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Hemoglobin Is Expressed by Alveolar Epithelial Cells*

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Hemoglobin gene expression in non-erythroid cells has been previously reported in activated macrophages from adult mice and lens cells, and recent studies indicate that alveolar epithelial cells can be derived from hematopoietic stem cells. Our laboratory has now produced strong evidence that hemoglobin is expressed by alveolar type II (ATII) cells and Clara cells, the primary producers of pulmonary surfactant. ATII cells are also closely involved in innate immunity within the lung and are stem cells that differentiate into alveolar type I cells. Reverse transcriptase-PCR was used to measure the expression of transcripts from the α- and β-globin gene clusters in several human and rodent pulmonary epithelial cells. Surprisingly, the two major globin mRNAs characteristic of adult erythroid precursor cells were clearly expressed in human A549 and H441 cell lines, mouse MLE-15 cells, and primary ATII cells isolated from normal rat and mouse lungs. DNA sequencing verified that these PCR products were indeed the result of specific amplification of globin gene cDNAs. These alveolar epithelial cells also expressed the corresponding hemoglobin protein subunits as determined by Western blotting, and tandem mass spectrometry sequencing was used to verify the presence of both α- and β-globin polypeptides in rat primary ATII cells. The function of hemoglobin expression by cells of the pulmonary epithelium will be determined by future studies, but this novel finding could potentially have important implications for the physiology and pathology of the lung.

The concept of hemoglobin as solely a carrier protein of O_2 and CO_2 in erythroid cells has undergone revision in recent years. Heme-binding proteins with the globin structural motif are found throughout the natural world, in both prokaryotes and eukaryotes (1, 2). Many of these proteins are involved in oxygen storage or transport, but all discovered thus far also function in binding products of nitric oxide (NO)[2] metabolism. Vertebrate hemoglobin itself has been clearly shown to not only scavenge NO and its metabolic derivatives, but also to transport and generate NO, releasing the gas in tissues to dilate blood vessels (3, 4). In fact, an emerging concept on the primordial function of hemoglobin-like proteins is that they protect cells against oxidative and nitrosative stress (5, 6).

Despite this widespread distribution of hemoglobin-like proteins (including myoglobin, neuroglobin, and cytoglobin), it has long been thought that hemoglobin itself is expressed only in cells of erythroid lineage in adult vertebrates. This notion may also be revised in the coming years, as the expression of non-erythroid hemoglobins has now been reported in activated macrophages from adult mice and cells of the lens (7, 8). Here we demonstrate that some types of respiratory epithelial cells of human, rat, or mouse origin, including alveolar type II (ATII) epithelial cells (also called type II pneumocytes) and Clara cells, also express hemoglobin. *In vivo*, these cells are the primary producers of pulmonary surfactant, essential for normal lung function as well as innate immunity (9–11). Also, ATII are stem cells that undergo significant phenotypic changes to terminally differentiate into alveolar type I (ATI) cells, which comprise much of the surface area of the alveolar epithelium and function in gas exchange (12, 13). Interestingly, although the exact details have yet to be elucidated, there is recent evidence that both ATI and ATII cells can be derived from hematopoietic stem cells, an intriguing discovery that may corroborate with our observation concerning hemoglobin expression (12–15).

The hemoglobin genes found in mammals arose by duplication before mammalian divergence and are similarly arranged in both primates and rodents (16, 17). α and β genetic loci are found on separate chromosomes and each includes numerous genes that are active at various stages of development. In erythroid cells, the expression of these genes is tightly coordinated to produce functional hemoglobin heterotetramers consisting of two α-globin-like and two β-globin-like chains. The genes of the α globin cluster in human (with rodent orthologs) include: α HBA-1 and -2 (Hba-1 and -2), ζ HBZ (Hba-x), and θ HBQ (Hbq). The β-globin cluster includes: β HBB (Hbb-b1 and b2 major/minor), δ HBD (rodent pseudogene), γ HBG-1 and -2 (Hbb-bh1/z), and ϵ HBE (Hbb-y). Each locus also includes several pseudogenes (17). Adult hemoglobin protein found in erythrocytes primarily consists of HbA, an $\alpha_2\beta_2$ tetramer with four bound heme prosthetic groups.

Here we demonstrate that adult forms of hemoglobin mRNAs and polypeptides are indeed expressed by human and rodent pulmonary epithelial cells. Our novel finding may have enormous implications in the physiology and pathology of the lung because of the many defined roles inherent to the structures of the hemoglobin molecule and its derived peptides, including gas exchange, NO metabolism, blood pressure regulation, and protection against oxidative and nitrosative stress.

MATERIALS AND METHODS

Cell Culture—The human ATII adenocarcinoma cell line A549 (18) and Clara-like adenocarcinoma cell line H441 (19) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Provo, UT). The mouse transformed ATII cell line MLE-15 (20) was cultured in HITES medium (RPMI 1640 with 5 μg/ml insulin, 10 μg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, and 10 mM HEPES) supplemented with 2% FBS. The human melanoma cell line FO-1 (21) and mouse fibroblast cell line NIH-3T3 (22) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The transformed murine erythroleukemia cell line MEL (23) was cultured in Iscove's modified Dulbecco's medium

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[2] The abbreviations used are: NO, nitric oxide; AE1, erythrocyte anion exchanger; ATI, alveolar type I cell; ATII, alveolar type II cell; FBS, fetal bovine serum; HbA, hemoglobin A heterotetramer; HBA, α-globin; HBB, β-globin; HBQ, θ-globin; MEL, mouse erythroleukemia cell line; qPCR, quantitative real-time PCR; RT, reverse transcription.

supplemented with 20% FBS and 55 μM 2-mercaptoethanol. MEL cells were induced to differentiate by 48 h culture with 6 units/ml epoetin (recombinant erythropoietin, Amgen, Thousand Oaks, CA) and 3 mM hexamethylene bisacetamide (Sigma). Primary ATII cells were obtained as previously described from Sprague-Dawley rats (24) or C57B/6 mice (25) and cultured on Matrigel (BD Biosciences) in Dulbecco's modified Eagle's medium, 10% FBS containing 10 ng/ml keratinocyte growth factor (Peprotech, Rocky Hill, NJ) to maintain the ATII phenotype. Except where noted, all culture media and additives were obtained from Invitrogen. Cell lines were obtained from the American Type Culture Collection (Manassas, VA), except FO-1, kindly provided by Dr. Sebastiano Gattoni-Celli, Medical University of South Carolina, and MEL, kindly provided by Dr. Clark Brown, Medical University of South Carolina. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air.

PCR and Quantitative PCR—RNA was purified from all cell types using a QuickPrep Total RNA kit, and cDNA was generated from 3 μg of purified total RNA and an oligo(dT) primer using a First Strand Synthesis kit (both kits from Amersham Biosciences). Conventional RT-PCR was performed using *Taq* DNA polymerase (New England BioLabs, Beverly, MA) for 35 cycles (60 °C annealing temperature). Reaction products were analyzed by electrophoresis on 4–12% polyacrylamide Tris borate-EDTA buffer gels (Novex, Invitrogen) followed by ethidium bromide staining. Results of experiments were verified by repetition of RT-PCR with RNA extracted from different aliquots of cells (at least three independent reactions performed per template/primer combination).

Quantitative real-time PCR (qPCR) and data analyses were performed using the MyiQ thermal cycler (Bio-Rad) (50 cycles, 62 °C annealing/extension). Sequence-specific internal probes dual-labeled with 6-carboxyfluorescein/carboxytetramethylrhodamine (TaqMan probes) were employed to measure fluorescence because of accumulating amplicons (Table 1). For relative quantification in qPCR, a mathematical model was used that incorporated the effects of the efficiency of amplification for each primer pair over a 10^4 range of template dilutions (26). qPCR were run in triplicate for each sample, and three independent experiments were performed. The results were expressed as mean ± S.D. To verify the synthesis of predicted amplicons, qPCR products were analyzed by electrophoresis on polyacrylamide gels followed by ethidium bromide staining.

Oligonucleotide primer pairs were designed for hemoglobin gene transcripts from the α- and β-globin clusters and are shown in Table 1. Some primer pairs were later redesigned for compatibility with qPCR analysis. All oligonucleotide primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA).

DNA Sequencing—Select PCR amplicons were gel purified and subjected to DNA sequencing by the Medical University of South Carolina Biotechnology Facility using the same primer pairs used for amplification. Resulting sequences were analyzed using the NCBI BLAST engine.

Western Blotting—Cells were washed four times in phosphate-buffered saline and lysed in 2% SDS. Whole cell lysates were subjected to SDS-PAGE (NuPAGE gels, Invitrogen), blotted onto nitrocellulose, and probed with polyclonal antibodies to α- or β-globin (Santa Cruz Biotechnology, Santa Cruz, CA) or pan hemoglobin (Bethyl Laboratories, Montgomery, TX) according to the manufacturers instructions. Horseradish peroxidase-conjugated secondary antibodies and SuperSignal West Dura chemiluminescence kit (both from Pierce Biotechnology) were used for visual detection with a FluorChem 8900 Imager (Alpha Innotech, San Leandro, CA). Purified human hemoglobin tetramer (HbA, Sigma) and α-globin purified from bovine pulmonary surfactant (27) (identity confirmed by N-terminal protein sequencing) were used as standards.

Protein Sequencing—Whole cell lysate from rat ATII primary cells was subjected to SDS-PAGE, silver-stained (28), and excised gel slices in the 16–20-kDa range were submitted to the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA). Peptides were generated by trypsin digestion before tandem mass spectrometry sequencing.

RESULTS

Hemoglobin mRNA Is Expressed by Pulmonary Epithelial Cells—Conventional RT-PCR was used to measure the expression of genes from the α- and β-globin clusters in several human and rodent cell lines and primary cell cultures. Oligonucleotide primer pairs specific for various globin gene transcripts were meticulously designed to amplify individual sequences among the closely homologous globin genes while distinguishing between cDNA and genomic DNA amplicons and avoiding common allelic variations (Table 1).

The expression of several globin genes was easily detectable by RT-PCR analyses of several types of pulmonary epithelial cells (Fig. 1). Specifically, PCR products corresponding to α-globin (HBA) and β-globin (HBB), the primary globin mRNAs expressed by erythroid precursor cells in adult mammals, were found in human cell lines A549 (ATII-like adenocarcinoma) and H441 (Clara cell-like adenocarcinoma), mouse cell line MLE-15 (transformed ATII cells), and ATII primary cells isolated from normal rat and mouse lungs (day 3 cultures). Interestingly, the human A549 and H441 cell lines also express the mRNA of θ-globin (HBQ), an α homolog that is the least-characterized globin gene (thought to be a pseudogene in rodents until recently) (29).

The usage of ATII primary cells from normal rat and mouse lungs, although difficult to maintain in culture, was important because it has been reported that transformation with herpes or Rous sarcoma viruses can activate α-globin transcription in some cell lines (30, 31), and certain patterns of aberrant gene expression are hallmarks of most tumor cell lines. As controls for cell phenotype and purity (especially for the primary cultures), Fig. 1 also shows that these cells expressed genes characteristic of pulmonary epithelial cells (*e.g.* surfactant protein B), but not other erythroid-specific genes, such as erythrocyte anion exchanger (AE1, band 3 protein) (32). In addition to microscopic examination of ATII preparations, the absence of a PCR product generated by using primers specific for the AE1 gene transcript (very abundant in erythroid precursors) strongly suggests that globin gene expression in these ATII primary cultures was not the result of erythroid cell contamination. All tested cell lines expressed the ubiquitous housekeeping gene ADP-ribosylation factor 1 (33).

Because of these findings, additional control reactions for RT-PCR analyses were also performed (Fig. 2). The human melanoma cell line FO-1 and mouse fibroblast cell line NIH-3T3 did not express transcripts from globin genes, surfactant protein B, or AE1. Amplification of cDNA prepared from human blood and the mouse erythroid cell line MEL (induced to differentiate in cell culture) clearly demonstrated the presence of HBA, HBB, HBQ, and AE1 transcripts. The unlikely contamination of cell culture medium (including 10% FBS) with globin gene nucleic acid template was analyzed by adding to the PCR both complete medium and medium "cDNA" (cDNA synthesis reaction was performed with undetectable product of RNA extraction of culture medium; no nucleic acid was actually detected spectrophotometrically). No PCR products were detected in cell culture medium (further confirmed by combining A549 template cDNA with the medium preparation to eliminate the possibility of false negatives because of PCR polymerase

TABLE 1

Oligonucleotide primers and probes used for PCR in these studies

Nomenclature for human globin genes is used for all globin orthologs. *HBA*, hemoglobin α ; *HBB*, hemoglobin β (rodent β major); *HBQ*, hemoglobin θ; *AE1*, erythrocyte anion-exchanger; *ARF*, ADP-ribosylation factor 1; *SPB*, surfactant protein B; *ACTB*, β -actin. For some amplifications in qPCR, alternate primer pairs were used as shown. Oligonucleotides were designed based on sequences found at the GenBank accession numbers listed in the right column.

Primer pair or probe	Oligonucleotide sequence[a]	GenBank™
HBA, human	F: 5′-ATGTTCCTGTCCTTCCCCACCACCAAG-3′ R: 5′-GCTTAACGGTATTTGGAGGTCAGCACG-3′	BC050661
HBB, human	F: 5′-GTGAACGTGGATGAAGTTGGTGGTGAG-3′ R: 5′-TTGGACAGCAAGAAAGCGAGCTTAGTG-3′	NM_000518
HBQ, human	F: 5′-TACACGACAGAGGCCCTGGAAAGGACC-3′ R: 5′-AGTTCAGCGGTACTCGGAAACCAGCGC-3′	BC056686
HBA, mouse/rat	F: 5′-CTCTCTGGGGAAGACAAAAGCAAC-3′ R: 5′-GGTGGCTAGCCAAGGTCACCAGCA-3′	BC043020
HBB, mouse	F: 5′-CACAACCCCAGAAACAGACA-3′ R: 5′-CTGACAGATGCTCTCTTGGG-3′	NM_008220
HBB, rat	F: 5′-TGAACCCTGATGATGTTGGTGGCGAGG-3′ R: 5′-AAGACAAGAGCAGGAAAAGAGGTTTAG-3′	NM_033234
HBQ, mouse/rat	F: 5′-TACACGACCGAGGCCTTGGAGAGGAC-3′ R: 5′-AGTTCAGCGATCCTTGGAGACCAGTGC-3′	XM_220266
AE1, human	F: 5′-TGGACCTGCTGCTGGTAGTAG-3′ R: 5′-ATCTGGATGCCCGTGAATAAG-3′	NM_000342
AE1, mouse/rat	F: 5′-TGGCTGCTGTCATCTTCATCTAC-3′ R: 5′-TTTGGGCTTCATCACAACAGG-3′	NM_011403
SPB, human	F: 5′-GGACATCGTCCACATCCTTAACAAGATG-3′ R: 5′-ATTGCTGCTCGGAGAGATCCTGTGTGTG-3′	BC032785
SPB, mouse/rat	F: 5′-GTGCCAAGAGTGTGAGGATATTGTCCACCTCC-3′ R: 5′-GGACACAGCCACAGCCAGCACACCCTTG-3′	NM_147779
ARF, human/mouse/rat	F: 5′-GCCAGTGTCCTTCCACCTGTC-3′ R: 5′-GCCTCGTTCACACGCTCTCTG-3′	M36340
HBB, human qPCR	F: 5′-ACTCCTGAGGAGAAGTCTGCCGTTAC-3′ R: 5′-TTGTCACAGTGCAGCTCACTCAGTGTG-3′	NM_000518
HBA, mouse qPCR	F: 5′-ATGTTTGCTAGCTTCCCCACCACCAAG-3′ R: 5′-GGTGGCTAGCCAAGGTCACCAGCA-3′	BC043020
HBB, mouse qPCR	F: 5′-TGATGCTGAGAAGGCTGCTGTCTCTG-3′ R: 5′-GTGCCCTTGAGGCTGTCCAAGTGA-3′	NM_008220
ACTB, human qPCR	F: 5′-GGGAAATCGTGCGTGACATTAAG-3′ R: 5′-TGTGTTGGCGTACAGGTCTTTG-3′	X00351
ACTB, mouse qPCR	F: 5′-GGGAAATCGTGCGTGACATCAAAG-3′ R: 5′-TGTGTTGGCATAGAGGTCTTTAC-3′	NM_007393
HBA probe qPCR	5′-CAACTTCAAGCTCCTGAGCCACTGCCTGCTGGTG-3′	BC043020
HBB probe qPCR	5′-CCCTGGGCAGGCTGCTGGTTGTCTACCCTTG-3′	NM_008220
ACTB probe qPCR	5′-CCAGCCTTCCTTCTTGGGTATGGAATCCTGTGGCA-3′	NM_007393

[a] F, forward/sense; or R, reverse/antisense primers.

inhibition). All tested cells expressed ADP-ribosylation factor 1 transcript, demonstrating the integrity of the RNA preparations.

Hemoglobin Gene Expression Was Verified by DNA Sequencing—Select PCR products, including those from mouse ATII primary cells, were subjected to DNA sequencing to verify that they were indeed the result of specific amplification of globin gene transcripts. The sequences obtained were compared with those in GenBank using the NCBI BLAST engine and shown to be essentially perfect matches with the corresponding globin mRNA (Table 2).

Along with the absence of RT-PCR products obtained with tissue-specific primers like surfactant protein B and AE1, the species specificity of these globin amplicons confirmed by DNA sequencing further eliminates the unlikely possibility of PCR cross-contamination (*e.g.* from mouse MEL cells or bovine serum in culture medium). In addition, because of the design of the oligonucleotide primers, every PCR product shown in Figs. 1 and 2 and Table 2 would contain intron sequences if derived from amplification of contaminating genomic DNA. It is also worth noting that the results in Fig. 2 and Table 2 clearly demonstrate that the mouse erythroid cell line MEL expresses θ-globin, a poorly defined globin homolog, especially in rodents.

Relative Levels of Globin Gene Expression Are Different Among Cell Types—Using real time qPCR, the relative levels of HBA and HBB gene expression were compared between mouse pulmonary epithelial cells and induced MEL erythroid cells (Table 3). For this analysis, quality control was performed by determining amplification efficiency for each primer pair and by normalizing globin expression to β-actin expression to correct for differences in starting cDNA template concentrations (26). As expected, the erythroid cells clearly expressed much higher levels of both HBA and HBB mRNAs than did MLE-15 and ATII primary cells. In fact, HBA expression in MEL cells was slightly greater than β-actin expression, certainly making it one of the most abundant cellular mRNAs. Still, globin gene expression was easily detected in both mouse ATII cell types, and the amplification detection thresholds decreased in a dose-dependent manner over a 10^5-fold template dilution curve (data not shown). All three cells types expressed more HBA than HBB mRNA, with ratios of ~9:1, 10:1, and 27:1 for MEL, primary ATII, and MLE-15 cells, respectively.

qPCR was also performed with human A549 and H441 cells, and although not precisely compared with mouse cells because of the necessity for making even greater assumptions for reference gene normalization and internal probe efficiency, it was clear that globin gene expression in these human cell lines was closer to that of the mouse pulmonary epithelial cells rather than erythroid cells. Again, amplification detection thresholds for globin genes in A549 and H441 decreased in a dose-dependent manner over a template dilution curve, and more HBA than HBB was expressed in each cell line (data not shown).

Hemoglobin Protein Is Expressed by Pulmonary Epithelial Cells—Immunoblotting was performed to determine whether hemoglobin polypeptide subunits are expressed by lung epithelial cells (Fig. 3). Using polyclonal antibodies that demonstrated species cross-reactivity, α- and β-globin were detected in lysates from human A549 and H441 cells, mouse MLE-15 cells, and rat primary ATII cells (mouse primary ATII

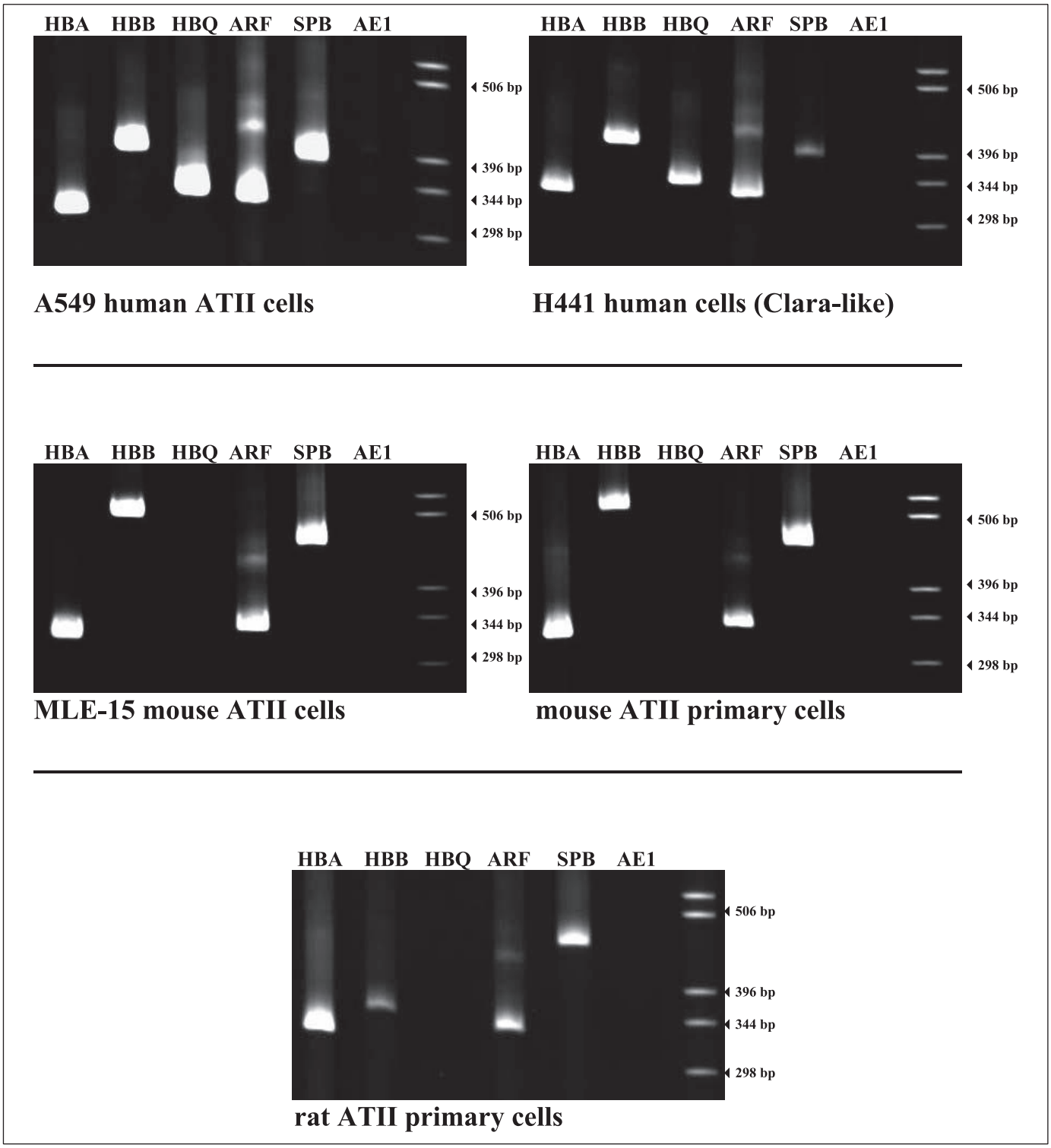

FIGURE 1. **Globin genes are expressed by pulmonary epithelial cells.** Total RNA was extracted from the indicated cell types, template cDNA prepared using an oligo(dT) primer, and PCR was performed using sequence-specific primer pairs. Amplified products were separated on polyacrylamide gels and visualized by ethidium bromide staining. All PCR products obtained were of the predicted size for cDNA amplification only (not genomic DNA contamination). Each panel represents the expression of various genes from a single cell type. Molecular weight markers are annotated in the *last lane* of each panel. Template cDNA: *A549*, human alveolar type II (ATII) cell line; *H441*, human Clara-like pulmonary cell line; *MLE-15*, mouse ATII cell line; *mouse primary ATII* cells (day 3 of culture); *rat primary ATII* cells (day 3 of culture). Sequence-specific oligonucleotide *primers*: *HBA*, human or mouse/rat α-globin (335 bp expected PCR products for each); *HBB*, human, mouse, or rat β-globin (411, 578, or 416 bp, respectively); *HBQ*, human or mouse/rat θ-globin (360 bp each); *ARF*, ADP-ribosylation factor 1 (human/mouse/rat) (336 bp); *SPB*, human or mouse/rat surfactant protein B (382 or 468 bp, respectively); *AE1*, human or mouse/rat erythrocyte anion exchanger (412 or 467 bp).

cells not available for testing). Interestingly, the hemoglobin from pulmonary epithelial cells migrated more slowly than that from erythroid sources, including that from MEL cells. This observation is yet to be explained, but is consistent with that seen in previously reported non-erythroid hemoglobin expression (7). Not surprisingly, much higher levels of globin subunits were detected in induced MEL cells compared

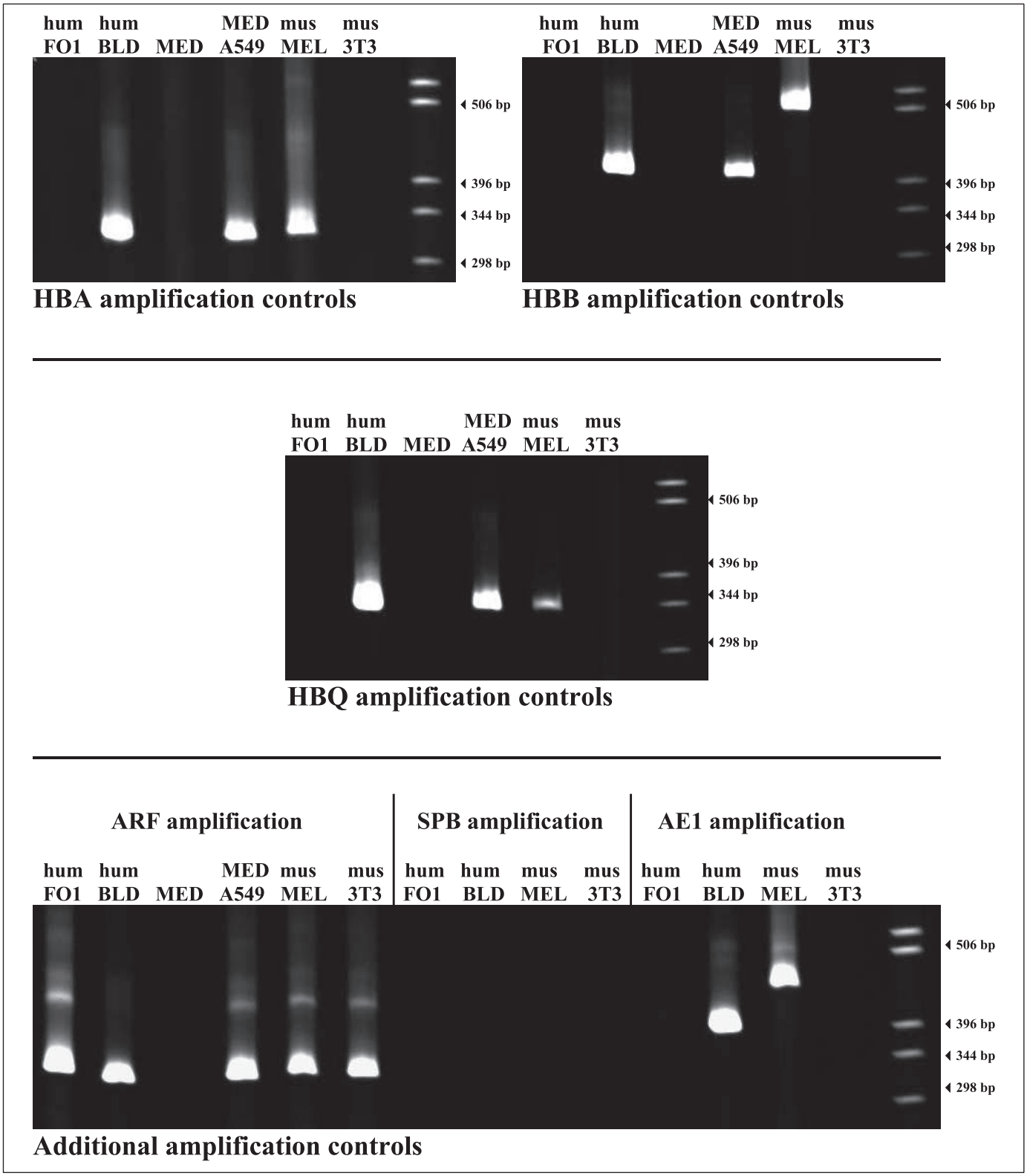

FIGURE 2. **RT-PCR analysis controls.** RT-PCR was performed as described in the legend to Fig. 1. All PCR products obtained were of the predicted size for cDNA amplification only (not genomic DNA contamination). Results obtained with various templates are grouped according to primer. Each *panel* represents the expression of individual genes among various cell types and other templates (*bottom panel* groups three different genes). Molecular weight markers are annotated in the *last lane* of each panel. Template cDNA: *FO-1*, human melanoma cell line; human blood (*BLD*); cell culture medium + 10% fetal bovine serum (*MED*, prepared as described in text); *MED + A549* cDNA (internal PCR control); *MEL*, mouse erythroid cell line (induced to differentiate); *NIH-3T3*, mouse fibroblast cell line. Sequence-specific oligonucleotide primers: same as described in the legend to Fig. 1.

with that in pulmonary epithelial cells (~25-fold less total MEL protein was loaded to obtain roughly comparable levels of detection). Most likely because of the extremely stable conformation of the hemoglobin molecule, multiple forms of the protein, including aggregates larger than the heterotetramer, were commonly detected by immunostaining even after detergent and heat denaturation. For example, the α-globin

TABLE 2

DNA sequencing of RT-PCR products

Selected PCR products were excised from polyacrylamide gels, purified, and subjected to DNA sequencing on both strands with the same sequence-specific primers used for original amplification. Sequences were compared to those in GenBank using the NCBI BLAST engine and examples of matches with numerous redundant data base files are shown.

Sequenced PCR product	GenBank™ match
A549, HBA primers	*Homo sapiens* hemoglobin, α 1 and 2 (HBA), mRNA[a] (example: 100% nucleotide match to bases 120–449 of GenBank BC032122)
A549, HBB primers	*H. sapiens* hemoglobin, β (HBB), mRNA (example: 100% nucleotide match to bases 109–515 of GenBank NM_000518)
A549, HBQ primers	*H. sapiens* hemoglobin, θ 1 (HBQ), mRNA (example: 100% nucleotide match to bases 95–454 of GenBank BC056686)
H441, HBA primers	*H. sapiens* hemoglobin, α 1 and 2 (HBA), mRNA[a] (example: 100% nucleotide match to bases 120–454 of GenBank BC032122)
H441, HBB primers	*H. sapiens* hemoglobin, β (HBB), mRNA (example: 100% nucleotide match to bases 109–510 of GenBank NM_000518)
H441, HBQ primers	*H. sapiens* hemoglobin, θ 1 (HBQ), mRNA (example: 100% nucleotide match to bases 95–454 of GenBank BC056686)
MLE, HBA primers	*Mus musculus* hemoglobin α, adult chain 1, mRNA (example: 100% nucleotide match to bases 22–356 of GenBank BC043020)
MLE, HBB primers	*M. musculus* hemoglobin, β major chain (Hbb-b1), mRNA (example: 99% nucleotide match to bases 36–551 of GenBank XM_489729)
MEL, HBQ primers	*M. musculus* hemoglobin, θ 1 (Hbq1), mRNA (example: 100% nucleotide match to bases 123–460 of GenBank NM_175000)
Mouse ATII,[b] HBA primers	*M. musculus* hemoglobin α, adult chain 1, mRNA (example: 100% nucleotide match to bases 22–352 of GenBank BC043020)
Mouse ATII, HBB primers	*M. musculus* hemoglobin, β major chain (Hbb-b1), mRNA (example: 100% nucleotide match to bases 35–551 of GenBank XM_489729)

[a] Human HBA1 and HBA2 mRNAs share identical coding region sequences as flanked by these primers.
[b] Mouse primary ATII cells.

TABLE 3

Quantitative analysis of globin gene expression

Expression of α- (HBA) and β- (HBB) globin mRNAs was analyzed in mouse primary ATII cells (ATII), MLE-15 ATII-like cell line, and induced MEL erythroid cells. Globin gene expression was determined using real-time PCR and normalized to β-actin expression after determining amplification efficiency of each template/primer mixture. Values shown are mean ± S.D. All differences in relative levels of expression were statistically significant (defined as $p < 0.05$).

Ratios	Normalized globin mRNA expression
	-fold
Comparison of globin gene expression in different mouse cells	
MEL HBA:ATII HBA	35.8 ± 4.4
MEL HBA:MLE-15 HBA	147 ± 9.3
MEL HBB:ATII HBB	40 ± 4.7
MEL HBB:MLE-15 HBB	440 ± 16.3
ATII HBA:MLE-15 HBA	4.1 ± 1.1
ATII HBB:MLE-15 HBB	11.0 ± 2.2
Relative expression of different globin genes	
MEL HBA:HBB	9.0 ± 2.8
ATII HBA:HBB	10.1 ± 2.6
MLE-15 HBA:HBB	26.9 ± 3.9

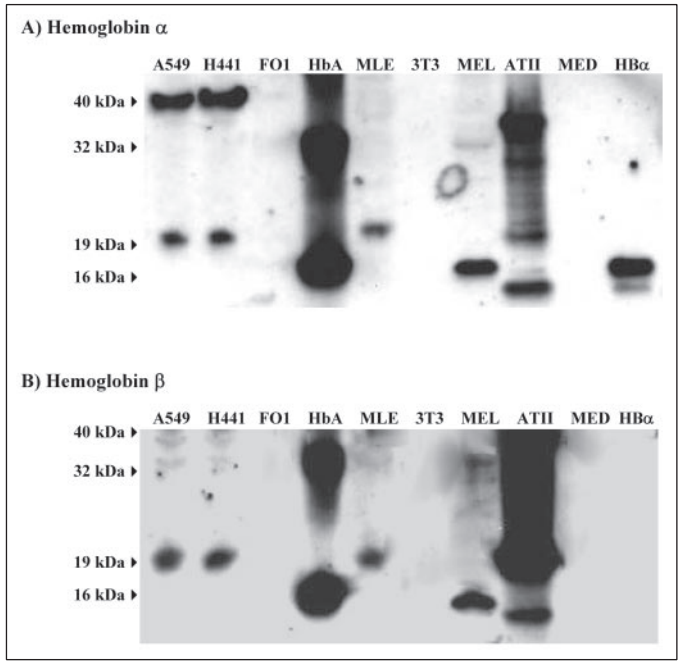

FIGURE 3. **Pulmonary epithelial cells express hemoglobin proteins.** Western blots of duplicate SDS-polyacrylamide gels were probed with anti-hemoglobin α (*A*) or β (*B*) polyclonal antibodies (anti-human, mouse, rat, or bovine), followed by horseradish peroxidase-conjugated secondary antibody, and visualized by chemiluminescence. Molecular weights are approximations based on the migration of stained protein markers. *A549*, lysate from A549 human ATII cell line (50 μg of total protein); *H441*, lysate from H441 human Clara-like cell line (50 μg of total protein); *FO-1*, lysate from FO1 human melanoma cell line (50 μg of total protein); *HbA*, purified human HbA (adult α/β heterotetramer; 200 ng); *MLE*, lysate from MLE-15 mouse ATII cell line (50 μg of total protein); *3T3*, lysate from NIH 3T3 mouse fibroblast cell line (50 μg of total protein); *MEL*, lysate from induced MEL mouse erythroid cell line (2 μg of total protein); *ATII*, lysate from rat ATII primary cells (day 3 culture; 50 μg of total protein); *MED*, cell culture medium + 10% fetal bovine serum (10 μl); *HBα*, purified bovine α-globin subunit (300 ng).

dimers shown in Fig. 3 were consistently the most abundant forms seen in SDS-PAGE analyses of A549 and H441 cell lysates (in fact, monomers were occasionally difficult to detect even with MEL lysates). Because of this variability, the use of purified hemoglobin tetramer for comparison in immunoblotting greatly assisted in the interpretation of the protein expression data.

Although all cells were thoroughly washed before lysis, culture medium containing 10% FBS was also tested to eliminate the possibility of contamination with bovine hemoglobin. No immunostaining was detected with these antibodies, which clearly would recognize bovine α-globin as demonstrated with a purified standard.

When the staining intensities of these antibodies were normalized (assuming the HbA heterotetramer standard contained equimolar amounts of α- and β-globin), only MEL cells appeared to express roughly equal levels of α- and β-globin subunits. Similar to the qPCR analysis of steady-state mRNA expression, more α- than β-globin subunit was detected in A549, H441, and MLE-15 cells; however, β-globin stained very intensely in the rat ATII primary cells. It is unknown if this staining phenomenon accurately reflects relative levels of the globin subunits or is because of differences in species cross-reactive binding or epitope recognition (*e.g.* because of heme presence/absence) of these two antibodies used. For comparison, an additional polyclonal antibody recognizing all hemoglobin tetramers (and apparently, α-globin-containing dimers and monomers) was also used to probe these immunoblots. Although staining intensities with this antibody were considerably lower (even for standards and MEL lysates), globin polypeptides were detected in the same pulmonary epithelial cells at similar molecular weights as those recognized by the other antibodies tested (data not shown).

To verify the results of immunoblotting, a tryptic digest of proteins in the ~16-kDa PAGE gel band from rat ATII primary cells was subjected to sequencing by tandem mass spectrometry (Fig. 4). Peptides mapping

HBA_RAT Rattus norvegicus (rat), hemoglobin alpha-1 and alpha-2 chains
Swiss-Prot Accession Number: P01946

VLSADDKTNI KNCWGKIGGH GGEYGEEALQ RMFAAFPTTK TYFSHIDVSP
GSAQVKAHGK KVADALAKAA DHVEDLPGAL STLSDLHAHK LRVDPVNFKF
LSHCLLVTLA CHHPGDFTPA MHASLDKFLA SVSTVLTSKY R [MASS=15197 kDa]

6 Peptides Sequenced	VLSADDKTNIK (pos 1-11)
	IGGHGGEYGEEALQR (pos 17-31)
	MFAAFPTTK (pos 32-40)
	TYFSHIDVSPGSAQVK (pos 41-56)
	LRVDPVNFK (pos 91-99)
	FLASVSTVLTSK (pos 128-139)

HBB1_RAT Rattus norvegicus (rat), hemoglobin beta chain, major-form
Swiss-Prot Accession Number: P02091

VHLTDAEKAA VNGLWGKVNP DDVGGEALGR LLVVYPWTQR YFDSFGDLSS
ASAIMGNPKV KAHGKKVINA FNDGLKHLDN LKGTFAHLSE LHCDKLHVDP
ENFRLLGNMI VIVLGHHLGK EFTPCAQAAF QKVVAGVASA LAHKYH
[MASS=15848 kDa]

9 Peptides Sequenced	VHLTDAEK (position 1-8)
	AAVNGLWGK (pos 9-17)
	VNPDDVGGEALGR (pos 18-30)
	LLVVYPWTQR (pos 31-40)
	YFDSFGDLSSASAIMGNPK (pos 41-59)
	KVINAFNDGLK (pos 66-76)
	VINAFNDGLK (pos 67-76)
	LHVDPENFR (pos 96-104)
	VVAGVASALAHK (pos 133-144)

Some Other Proteins Identified:

H4_RAT	Histone H4; Swiss-Prot Accession: P62804; 8 peptides sequenced
PROF1_RAT	Profilin-1; P62963; 7 peptides sequenced
MYL6_RAT	Myosin light polypeptide 6; Q64119; 3 peptides sequenced
NLTP_RAT	Nonspecific lipid-transfer protein, mitochondrial; P11915; 3 peptides

FIGURE 4. **Protein sequencing data from rat primary ATII cells.** Proteins of ~16–17 kDa in an excised silver-stained gel slice from SDS-PAGE of whole cell lysate from rat ATII primary cells (day 3 culture) were subjected to sequencing by tandem mass spectrometry (peptides were generated by trypsin digestion before sequencing). Peptides mapping to 24 different protein species were identified, including rat HBA (6 peptides sequenced, *top panel*) and HBB (9 peptides sequenced, *middle panel*). All other identified proteins with 3 or more sequenced peptides are listed in the *bottom panel*. SWISS-PROT accession numbers are shown for each matched protein. Complete amino acid sequences and molecular weights were also taken from this data base.

to 24 different protein species were identified, including rat HBA (6 peptides sequenced) and HBB (9 peptides sequenced).

DISCUSSION

For the first time, the results reported herein clearly demonstrate that hemoglobin is expressed by alveolar epithelial cells in human, mouse, and rat. Specifically, mRNA transcripts and polypeptides were detected for both globin subunits of adult hemoglobin in ATII cell lines, a Clara-like cell line, and rodent ATII primary cells. Perhaps it is not surprising that this discovery has been elusive. Of course, any hemoglobin transcript or protein found in lung tissue samples would likely be attributed to the enormous levels found in contaminating erythroid cells. Also, there are only a few cell lines in existence that can be definitively classified as retaining the ATII phenotype, and primary cells are difficult to maintain in culture. The main reason for this is that ATII are the stem cells for the type I pneumocyte (ATI cell) of the alveolar epithelium and can rapidly change their pattern of gene expression *in vitro* (12, 13, 34, 35). For example, the expression of the characteristic surfactant protein genes in ATII can be greatly influenced by cell culture conditions, including cytokine and hormonal stimulation. It is likely that ATII *in vivo* have a phenotype that may never be exactly duplicated *in vitro* because of the wide variety of environmental factors known to affect gene expression in these cells, including cell-cell interaction, extracellular matrix contact, hormone/cytokine stimulation, influences of innate and adaptive immune responses, and the unique nature of the highly oxidative lung environment itself (12, 34–38). Hopefully, further studies will elucidate the patterns and mechanisms of hemoglobin expression in ATII cells during cellular differentiation and under various environmental conditions, a somewhat complicated task because ATI cells are more difficult to isolate and maintain in culture than ATII, and truly characteristic cell lines are essentially nonexistent because of the terminally differentiated ATI phenotype.

The easily detectable levels of HBQ transcript in pulmonary epithelial cells is particularly unexpected. HBQ is a poorly understood hemoglobin that may actually represent a pseudogene that is transcribed in primates as a result of insertion of a mobile genetic element. The promoter of this globin is more characteristic to that of a housekeeping gene, and computer models suggest that translation of the θ-globin coding sequence may result in a polypeptide that would be hypothetically unstable. Interestingly, low levels of θ-globin transcript can be found in erythroid cells at most developmental stages, including adult, although no polypeptide has been found (29, 39).

Interestingly, the hemoglobin from pulmonary epithelial cells migrated more slowly than that from erythroid sources, including that from MEL cells. This observation is yet to be explained, but may be because of post-translational modifications (*e.g.* glycosylation), heme-binding differences, or tight linkage to other proteins/peptides among many intriguing possibilities, and is consistent with that seen in previously reported non-erythroid hemoglobin expression (7). Immunostaining with an alternate antibody detected globin polypeptides in the same pulmonary epithelial cells at similar molecular weights as those recognized by the other antibodies tested.

It has not been determined if the heme prosthetic group is present on the globin polypeptides found in these lung epithelial cells. Unlike MEL

erythroid cells and the purified HbA heterotetramer standard, A549, H441, MLE-15, and rat ATII primary cells do not appear red, although this may be because of the different levels of hemoglobin expression. Also, it is not known if heme binding contributes to epitopes recognized by the polyclonal antibodies used here, although for anti-HBA at least, the detection of the colorless bovine α-globin subunit standard suggests that heme presence is not necessary for antibody binding *per se*. Although globin subunits were qualitatively detected with all antibodies tested, possible differences in antibody binding because of species cross-reactivity and heme presence made it difficult to accurately compare relative levels of protein expression.

Our initial results suggest that globin protein production is not as tightly coordinated in pulmonary epithelial cells as in erythroid cells. Despite higher ratios of HBA to HBB mRNA transcript levels detected in all cell types, only induced MEL erythroid cells demonstrated approximately equal amounts of α- and β-globin protein subunits as detected by immunostaining. Again, this may be simply because of antibody binding differences (*e.g.* heme contributing to epitope), but could also result from the absence of a similar translational control system in pulmonary epithelial cells that in developing erythroid cells links the synthesis of globin subunits to heme availability (40). Also, it has yet to be determined whether the pulmonary epithelial cells produce the well characterized hemoglobin heterotetramer that functions in oxygen transport and exchange. Globin gene expression by these cells may result in the production of individual polypeptide subunits that may have unique functions. It is interesting to note that, despite the tight coordination of expression displayed by erythroid cells, the α- and β-globin clusters are on different chromosomes, are characterized by very different genomic arrangements, and individual globin genes contain elements that result in their temporal expression during vertebrate development (16, 41, 42). In addition, the amounts of globin polypeptides produced by erythroid cells are much higher than those found in our studies. Taken together, this complexity suggests that it is entirely plausible that globin genes may not be similarly regulated in ATII and erythroid cells.

Although the actual significance has yet to be determined, this novel and surprising finding that hemoglobins are expressed by pulmonary epithelial cells may have enormous implications in the physiology and pathology of the lung because of the many defined roles inherent to the structures of the hemoglobin molecule and its derived peptides, including gas exchange, NO metabolism, and blood pressure regulation, and protection against oxidative and nitrosative stress. Also, because it is traditional dogma that globin genes are normally expressed solely by erythroid tissue in most vertebrates, these results raise many important questions in hematology, genetics, and development. Those questions are further complicated by the wide variety of hematologic disorders associated with aberrant forms or production of hemoglobin, which potentially could affect alveolar physiology as well.

A few bioactive peptides derived from the proteolytic degradation of both α- and β-globin have been described. These so-called "hemorphins" are ligands for opioid and possibly G-protein-coupled receptors and have roles in processes as diverse as blood pressure regulation, neurotransmission, and neutrophil activity (43–48). The best characterized hemorphin, LVVH-7 (derived from β-globin), is an inhibitor of angiotensin-converting enzyme (a protein found at the highest concentration in the mammalian lung), and can help regulate blood pressure through the renin-angiotensin system (45). Interestingly, although hemorphins were previously thought to be produced solely by erythrocyte-scavenging macrophages (44), the cathepsin proteases involved in their synthesis are also expressed by ATII cells, where they are involved in post-translational processing of surfactant proteins (43, 49, 50). In addition, LVVH-7 has been found in the brochoalveolar lavage fluid of patients with certain types of lung cancer (51). Although speculative, further studies may determine that ATII cells produce hemorphins that could possibly affect cellular activity in the pulmonary microenvironment.

Hemoglobin expression by cells of the pulmonary epithelium could potentially have important consequences for gas exchange and NO metabolism. The protein may function in oxygen or carbon dioxide transport across the blood-airway interface, although this activity may depend on the production of the heterotetramer as discussed above. Alternatively, the protein may have a simpler, myoglobin-like role in oxygen storage or even trap toxins like carbon monoxide. Hemoglobin can also help regulate cellular pH by binding H^+, which may be important considering the large amount of CO_2 that passes through these cells. Of course, the presence of hemoglobin in pulmonary epithelial cells could have a major role in protection against the oxidative stress of this cellular environment. The genetic and functional integrity of ATII cells are imperative to the generation and maintenance of the pulmonary epithelium and production of surfactant, processes that may be severely hindered by high levels of reactive oxygen intermediates.

In addition to its roles in respiratory gas exchange, hemoglobin has been shown to be intimately involved in nitric oxide metabolism. In fact, some types of primordial hemoglobins function primarily to relieve nitrosative stress (5, 6). In vertebrates, the scavenging activity of hemoglobin can have a tremendous effect on the bioavailability of free NO, reducing its half-life in the cellular environment to a few seconds, which in turn has important implications on NO function, including its role in regulating smooth muscle relaxation and blood pressure. Also, hemoglobin has recently been shown to generate NO by reduction of nitrite, and NO attachment to hemoglobin is involved in the normal binding and release of oxygen by the protein (3, 4, 52–54). The levels of NO can be greatly increased in lung epithelial cells after exposure to proinflammatory cytokines, oxidative stress, and viral infections, and by binding NO, hemoglobin could potentially reduce nitrosative stress by reducing the production of reactive intermediates like peroxynitrite (53, 55–58). In addition to their central role in the development and regeneration of the alveolar epithelium, ATII cells also function in surfactant production and innate immunity of the lung, processes known to be greatly affected by NO, and consequently, could also be affected by hemoglobin (59–62).

Taken together, this discovery may have significant impact not only in the basic biology of hemoglobin and the lung, but in the understanding of pathologies associated with acute respiratory distress syndrome, sickle cell anemia (acute chest syndrome), thalassemias, asthma, bronchial pulmonary disease, and microbial infections, among others.

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Hemoglobin Is Expressed by Alveolar Epithelial Cells

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