

## Brief Report

## A MUTATION IN THE SURFACTANT PROTEIN C GENE ASSOCIATED WITH FAMILIAL INTERSTITIAL LUNG DISEASE

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**I**NTERSTITIAL lung diseases are a heterogeneous group of disorders that are poorly understood at a molecular level.<sup>1,2</sup> The cause is often unknown, and the histologic diagnoses used in adults may represent different disease processes in children.<sup>3-5</sup> For example, cases of desquamative interstitial pneumonitis reported in infants are often more severe and refractory to treatment than those reported in adults.<sup>6,7</sup> Many of these cases probably represent chronic pneumonitis of infancy.<sup>8,9</sup> The lungs in patients with chronic pneumonitis of infancy are characterized by interstitial thickening with mesenchymal cells, rather than by an inflammatory infiltrate, and an alveolar infiltrate with variable amounts of proteinaceous material. A possible genetic basis for desquamative interstitial pneumonitis and chronic pneumonitis of infancy is suggested by reports of familial cases.<sup>6,8,10</sup> We tested the hypothesis that mutations in the gene (*SP-C*) encoding surfactant protein C, a hydrophobic, lung-specific protein, were associated with chronic lung disease in an infant with a family history of interstitial lung disease.

### CASE REPORT

A full-term baby girl was born to a woman who had been given a diagnosis of desquamative interstitial pneumonitis at 1 year of age and who had been treated with glucocorticoids until she was 15 years old. The infant's maternal grandfather had died from life-long lung disease of unknown cause. Respiratory symptoms of tachypnea and cyanosis while breathing room air developed in the infant at six weeks of age. Radiography of the chest showed hyperinflation with increased interstitial markings. Because of the family history, open-lung biopsy was performed. The histologic features were thought to resemble most closely cellular or nonspecific interstitial pneumonitis.<sup>11</sup> The infant was treated with supplemental oxygen and corticosteroids, and her respiratory symptoms im-

proved somewhat. The mother's lung disease worsened after delivery, and she died from respiratory failure.

### METHODS

Samples of lung tissue, blood, and bronchoalveolar-lavage fluid were obtained from the patient, and samples of lung tissue were obtained from her mother. These samples were received as part of a program to evaluate infants with lung disease of unknown cause for mutations in the surfactant protein genes. The institutional review boards of the participating institutions approved the protocols for these evaluations, and written informed consent for genetic testing was obtained from the infant's father.

The lung tissues used as controls came from donor lungs and from patients undergoing lung transplantation for end-stage pulmonary disease. This latter group of controls included a two-year-old child with bronchopulmonary dysplasia who was dependent on a ventilator and also adolescents with primary pulmonary hypertension.<sup>12</sup> The DNA samples used as controls came from adult subjects without a known history of lung disease.<sup>13</sup>

### Preparation and Analysis of DNA

Genomic DNA was prepared from blood leukocytes with use of a commercially available kit (PureGene, Gentra Systems, Minneapolis). Polymerase-chain-reaction (PCR) products spanning exons 1 and 2 (genomic positions, -143 to 996) and exons 3 to 6 (genomic positions, 1212 to 2522) of the *SP-C* gene were generated from genomic DNA by PCR and analyzed by direct sequencing of the PCR products with the use of previously described conditions.<sup>13</sup> The resulting *SP-C* sequences were compared with published *SP-C* sequences.<sup>14-16</sup> Restriction analyses were performed on PCR products with the use of reagents according to the manufacturer's specifications (New England Biolabs, Beverly, Mass.). DNA from formalin-fixed, paraffin-embedded tissue was extracted by means of a microwave-based method<sup>17</sup> and analyzed by nested PCR, in which 20 cycles were performed with primers spanning exon 4, and then 2  $\mu$ l of the product was amplified for another 20 cycles with primers that were internal to those used in the first reaction.

### Protein Blotting, Immunohistochemical Analysis, and Electron Microscopy

Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis and protein blotting were performed on homogenates of lung tissue that had been frozen in liquid nitrogen at the time of biopsy, and immunohistochemical analyses were performed on formalin-fixed, paraffin-embedded tissue as previously described.<sup>12,13</sup> Antigen-retrieval methods were used for samples with no detectable staining or low levels of staining.<sup>18</sup> The production and characterization of polyclonal antiserum against surfactant protein A, surfactant protein B, surfactant protein B precursor protein, and surfactant protein C precursor protein have been described elsewhere.<sup>19-22</sup> Antibodies against mature surfactant protein C were generated with the use of recombinant human surfactant protein C (Byk-Gulden, Konstanz, Germany) and used as described elsewhere.<sup>23</sup> A commercial monoclonal antibody, CD68 (Dako, Carpinteria, Calif.), was used for the detection of human macrophages. Small pieces of snap-frozen lung tissue were thawed quickly in fixative at room temperature and prepared for electron microscopy as previously described.<sup>24</sup>

### RNA Analysis

RNA was prepared from frozen lung tissue as previously described,<sup>13</sup> and 5  $\mu$ g was reverse-transcribed with the use of a Superscript II reverse transcriptase kit (GIBCO BRL, Life Technologies, Gaithersburg, Md.), an oligo(dT) primer, and reagents, according to the manufacturer's instructions. *SP-C* complementary DNA (cDNA) was generated with the use of a forward primer corresponding to cDNA nucleotides 15 to 32 and a reverse primer corresponding to nucleotides 715 to 698. The PCR conditions were the same as those used for the amplification of cDNA for surfactant protein B.<sup>13</sup>

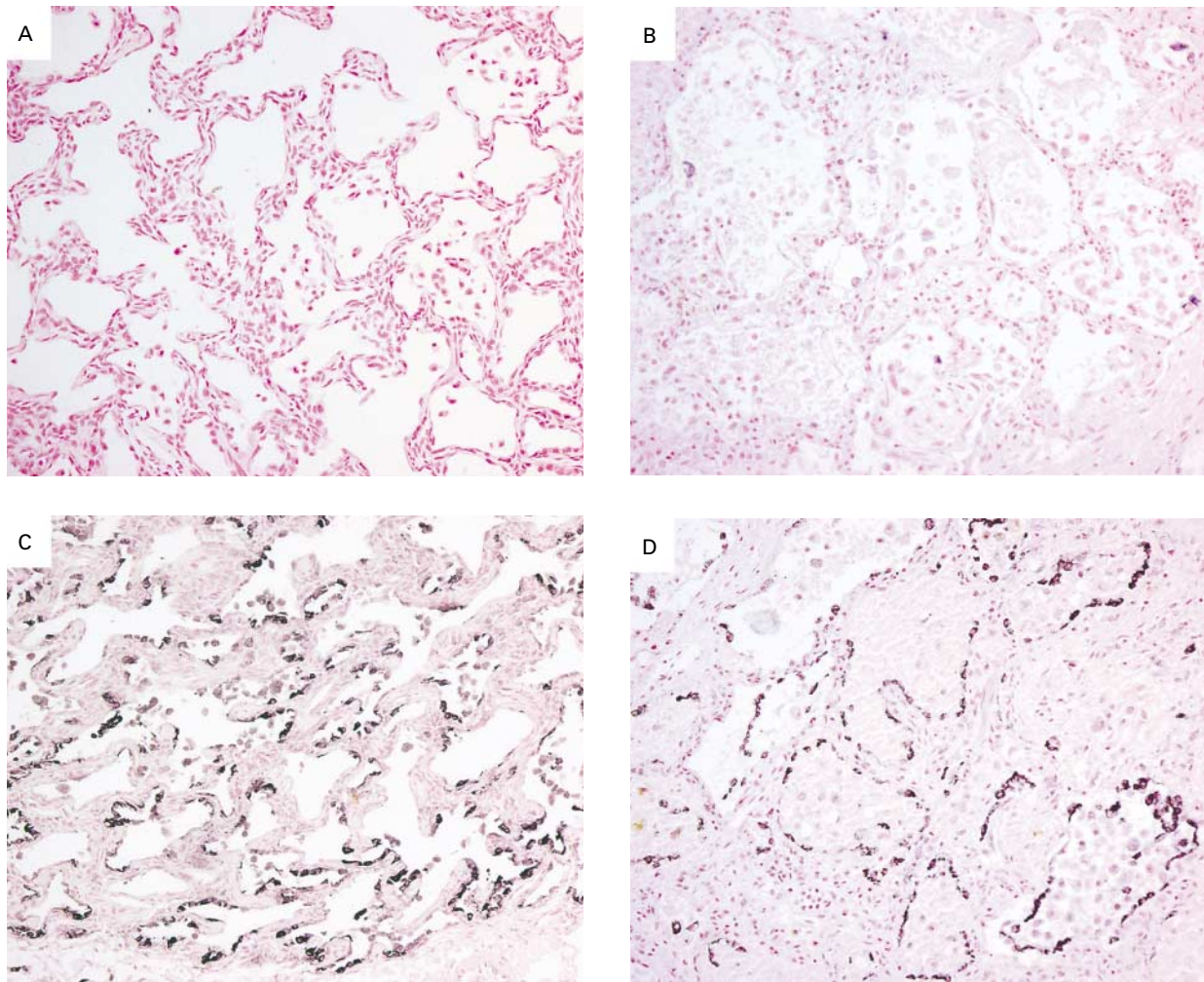
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## RESULTS

## Immunohistochemical Analysis

Histopathological findings in lung-tissue samples from the patient included well-preserved pulmonary architecture, hyperplasia of type II alveolar cells, and an interstitial infiltrate composed primarily of mature lymphocytes with scattered myofibroblasts. Some non-inflated alveoli were filled with desquamated cells, the majority of which were immunopositive for the macrophage-cell marker CD68. Normal-appearing lamellar bodies were observed in type II alveolar cells by means of electron microscopy. Lung tissue from the patient's mother had areas of diffuse fibrosis and honeycombing, with patchy areas of mild interstitial lymphocytic infiltration, accumulations of alveolar macrophages, and areas of superimposed alveolar damage.

Immunostaining for surfactant protein C precursor protein was absent in the lung tissue from the patient and was extremely weak or absent in most regions of lung tissue obtained at autopsy from her mother (Fig. 1). After antigen retrieval, however, immunostaining for surfactant protein C precursor protein was readily detected, indicating that the protein was present, although possibly in low levels. Staining for surfactant protein C precursor protein was restricted to type II alveolar cells and was not detected in luminal material. In lung tissue from both the patient and her mother, staining for surfactant protein A, mature surfactant protein B, and surfactant protein B



**Figure 1.** Immunohistochemical Staining for Surfactant Protein C Precursor Protein ( $\times 230$ ).

Before antigen retrieval, immunostaining for surfactant protein C precursor protein was undetectable in lung tissue from the patient (Panel A) and weak or absent in tissue from the patient's mother (Panel B). After antigen retrieval, strong staining for surfactant protein C precursor protein was observed in the alveolar epithelium in tissue from both the patient (Panel C) and her mother (Panel D). Strong staining was detected in tissue from controls without the need for antigen retrieval (not shown).

precursor protein was observed in type II alveolar cells, along the alveolar surface, in association with alveolar macrophages, and in intraalveolar exudates.

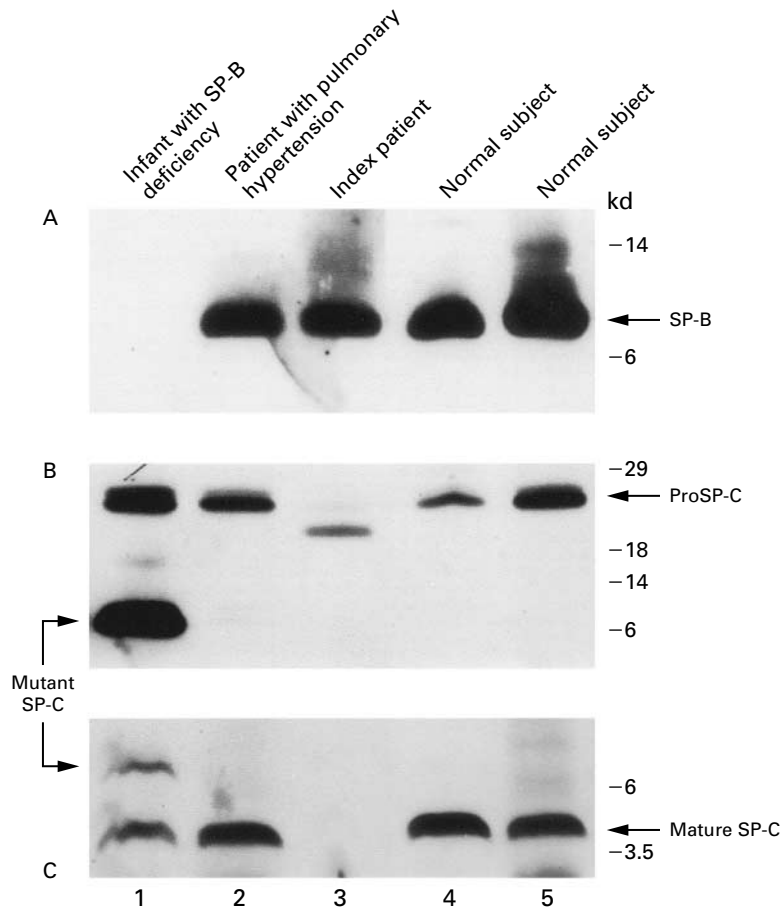
#### Immunoblot Analysis of Surfactant Proteins

Only a small amount of surfactant protein C precursor protein was present in lung tissue from the patient, and the predominant band migrated at a lower molecular weight than did those of the controls (Fig. 2). Mature surfactant protein C was undetectable in lung tissue and bronchoalveolar-lavage fluid from the patient, but it was readily detected in bronchoalveolar-lavage fluid from age-matched controls. Mature surfactant protein B, surfactant protein A, and

surfactant protein B precursor protein were present in amounts similar to those in controls.

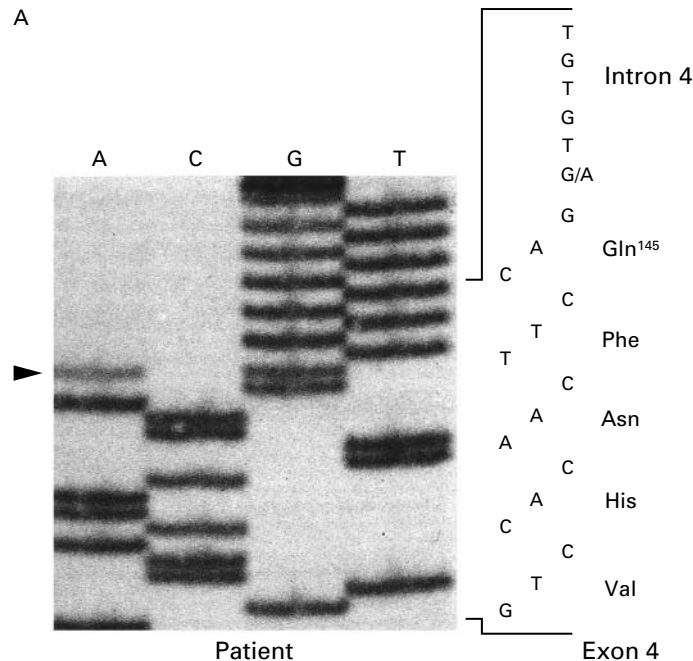
#### DNA-Sequence Analysis

A heterozygous substitution of A for G was identified at the first base of intron 4 (genomic DNA base 1728; cDNA base 460 + 1 [c.460 + 1 G→A]) of the patient's *SP-C* gene. This mutation would abolish the normal donor splice site (Fig. 3A). No other deviations from the published *SP-C* sequences or intron-exon boundaries were observed.<sup>14-16</sup> This mutation eliminated a recognition site for the restriction enzyme *Bst*NI. Restriction analysis confirmed the presence of the mutation in the patient and her mother (Fig. 3B),



**Figure 2.** Immunoblotting for Surfactant Proteins in Lung Tissue.

Mature surfactant protein B (SP-B) was detected in lung tissue from all the controls except one infant with a known hereditary deficiency of surfactant protein B (Panel A). The amount of surfactant protein C precursor protein (ProSP-C) in tissue from the patient was small and was of a lower molecular weight than the surfactant protein C precursor protein in tissues from the controls (Panel B). Mature surfactant protein C (SP-C) was detected in lung tissue from the controls but not in tissue from the patient (Panel C). Aberrantly processed surfactant protein C precursor protein peptides, characteristically found in the lung tissue of infants with hereditary mutations causing deficiencies of surfactant protein B,<sup>13,22</sup> were not observed in lung tissue from the patient with an *SP-C* mutation or in tissue from the controls. The results shown are representative of at least three separate experiments.



**Figure 3.** DNA Analyses.

In Panel A, a heterozygous substitution immediately after the last base of codon 145 (the first base in intron 4) in the patient's *SP-C* DNA sequence (arrowhead) eliminated the invariant G in the normal splice-donor consensus sequence. Restriction analysis (Panel B) showed that the c.460+1 G→A mutation eliminated a restriction site for the enzyme *Bst*NI. Arrows indicate the locations of the inner primers (genomic positions 1564 to 1582, forward, and genomic positions 1778 to 1757, reverse) used in the nested PCR reactions. After PCR amplification of the region containing the mutation and digestion of the PCR products with *Bst*NI, the presence of a 126-bp band in lanes 4 and 5 of Panel C indicate that both the patient and her mother carried the mutation on one allele.

but it was not found on 100 chromosomes from controls, indicating that it is not a common polymorphism.

#### RNA Analysis

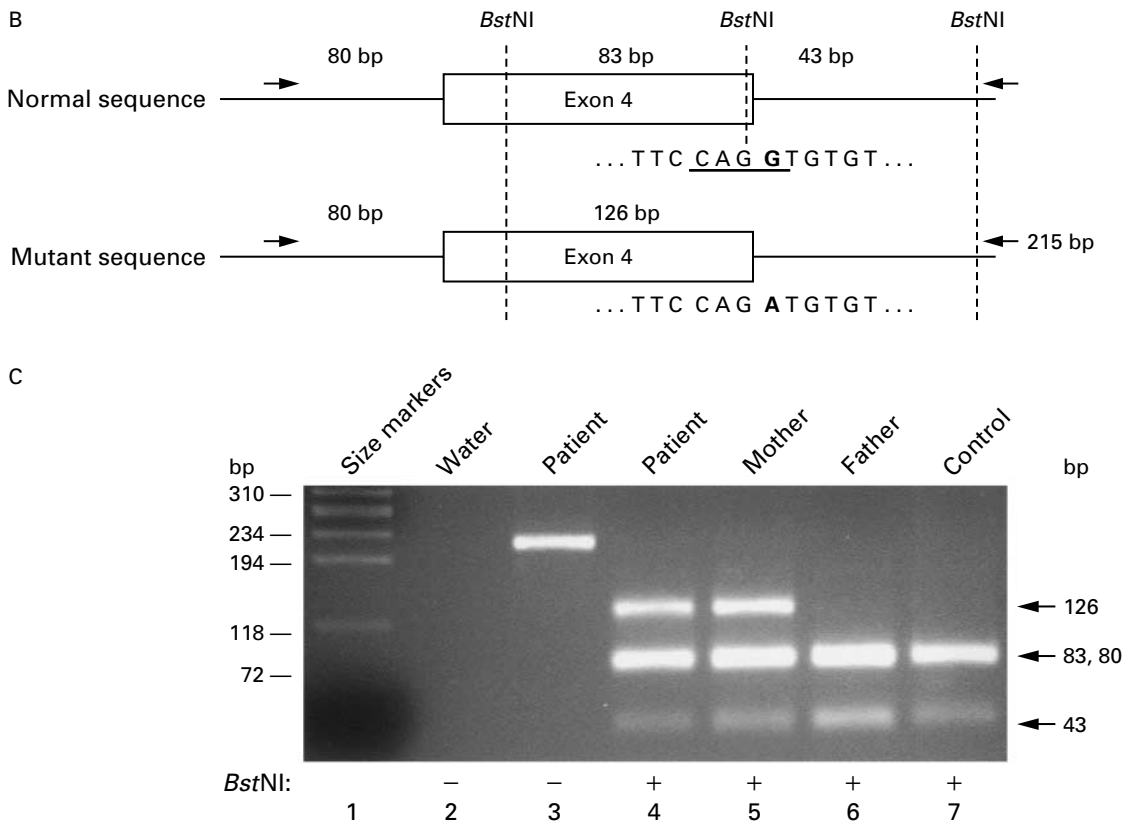
*SP-C* products of reverse-transcriptase PCR that were of the expected size and one that was shorter by approximately 110 bp were amplified from RNA prepared from the patient's lung tissue (Fig. 4). Sequence analysis indicated that the shorter product lacked the sequence corresponding to exon 4. Analysis of single-nucleotide polymorphisms in the *SP-C* gene indicated that the shorter transcripts were derived from the allele with the c.460+1 G→A substitution. No other deviations from the published *SP-C* sequences were observed.<sup>14,16</sup>

#### DISCUSSION

Pulmonary surfactant is the mixture of lipids and proteins needed to reduce surface tension and prevent end-expiratory atelectasis. Deficiency of pulmonary surfactant is the principal cause of respiratory distress syndrome in premature infants.<sup>25</sup> Surfactant protein B and surfactant protein C are hydrophobic

proteins that enhance the surface-tension-lowering properties of surfactant lipids, and both are present in the preparations of lung-derived surfactant that are used to treat infants with respiratory distress syndrome.<sup>26</sup> The inability to produce surfactant protein B causes lethal neonatal lung disease both in genetically engineered mice and in infants who are homozygous for mutations in the *SP-B* gene.<sup>12,13,27</sup> We identified a mutation in the *SP-C* gene in two members of the same family who did not have respiratory symptoms at birth but in whom interstitial lung disease subsequently developed. These observations suggest that although surfactant protein C may not be critical for respiratory adaptation at birth, it is important for normal postnatal lung function, and that mutations in the gene may be associated with interstitial lung disease.

An *SP-C* mutation was identified on only one allele in the patient and her mother, as is consistent with the autosomal dominant pattern of inheritance, although occult mutations may have been present on the other alleles. The c.460+1 G→A mutation resulted in the production of an abnormal proprotein, and the levels of transcripts encoding normal surfactant



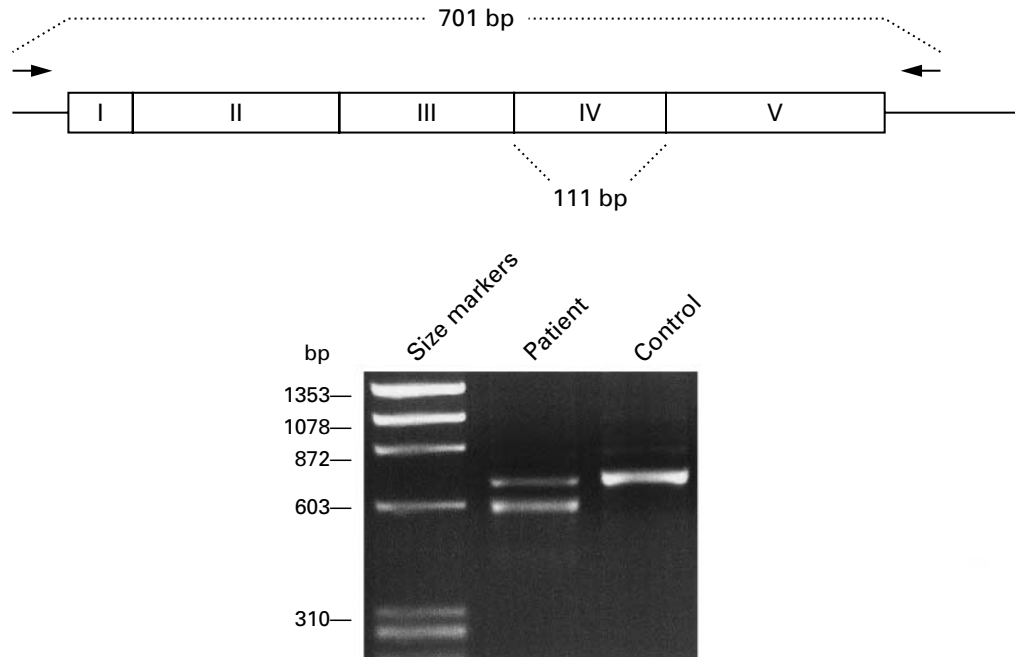
protein C precursor protein were similar to those of transcripts encoding the abnormal protein. These observations suggest that the abnormal protein had a dominant negative effect on the function or metabolism of surfactant protein C. Mature surfactant protein C is derived through the proteolytic processing of a 197-amino-acid proprotein (or a 191-amino-acid proprotein with alternative splicing).<sup>14-16</sup> Surfactant protein C precursor protein is an integral membrane protein that is anchored in the membrane by the hydrophobic core of mature surfactant protein C.<sup>28</sup>

The c.460+1 G→A mutation resulted in the skipping of exon 4 and the deletion of 37 amino acids in the carboxy-terminal domain of surfactant protein C precursor protein. Deletions in this domain have been shown to disrupt the intracellular transport of surfactant protein C precursor protein.<sup>29,30</sup> Surfactant protein C can form oligomers and interacts with surfactant phospholipids and surfactant protein B.<sup>31</sup> Interactions between normal and abnormal surfactant protein C precursor protein could hinder the transit of normal surfactant protein C precursor protein through the processing pathway or enhance its degradation. Competitive inhibition by the abnormal proprotein could also interfere with the processing of normal surfactant protein C precursor protein. The lack of ma-

ture surfactant protein C in lung tissue and bronchoalveolar-lavage fluid from the patient supports the notion that surfactant protein C precursor protein was not being processed and secreted properly.

Several mechanisms may relate the observed abnormalities in the metabolism of surfactant protein C to the development of lung disease, although it is also possible that they were not causally related. The abnormal proprotein is unlikely to have folded properly. Since surfactant protein C is extremely hydrophobic, improperly folded surfactant protein C precursor protein may have resulted in the formation of protein aggregates, secondary cellular injury, and subsequent inflammation.<sup>32</sup> Since the expression and processing of surfactant protein C are developmentally regulated,<sup>33</sup> the postnatal onset of lung disease could be related to the increased expression or accumulation of abnormal surfactant protein C precursor protein. Accumulation and misrouting of improperly folded proteins have been increasingly recognized as causes of disease, including  $\alpha_1$ -antitrypsin deficiency and cystic fibrosis.<sup>34</sup> Agents that enhance the intracellular processing and transport of misfolded proteins may thus have a role in therapy for interstitial lung disease.<sup>35,36</sup>

Lung disease may also have resulted from a defi-



**Figure 4.** Reverse-Transcriptase PCR Analysis for *SP-C* cDNA.

In the top portion of the figure, the translated sequences of the *SP-C* mRNA are represented by boxes corresponding to the exons (I through V) in the *SP-C* gene, and the untranslated sequences are represented by lines, with arrows indicating the locations of the primers used for amplification. As shown in the lower portion of the figure, two *SP-C* cDNAs of different sizes were amplified from the patient, the smaller one corresponding to the size of the deletion of the exon IV sequence.

ciency of mature surfactant protein C. Genetically engineered mice that are incapable of producing surfactant protein C survive to adulthood but have abnormal surfactant that is unstable at low lung volumes (Glasser S: unpublished data). Deficiency of surfactant protein C could thus predispose persons to recurrent atelectasis, lung injury, and inflammation. The lack of surfactant protein C may have secondary effects on the metabolism and function of other surfactant components, or surfactant protein C may have an as yet unknown but essential function. In vitro studies examining the effect of *SP-C* mutations on the metabolism and function of surfactant protein C precursor protein and surfactant protein C and the creation of an animal model expressing this mutation will be necessary to prove that this mutation causes lung disease and to clarify its pathogenesis.

Further study is required to determine how frequently interstitial lung disease is associated with this particular mutation and with *SP-C* gene mutations in general. The natural history of interstitial lung disease of childhood and its response to different therapeutic agents, such as glucocorticoids and chloroquine, are variable and may depend in part on the cause of the disease. In cases with genetic causes, the disease may be less likely to respond to these thera-

pies. The histologic diagnoses in the patient and her mother in this report illustrate the diversity and the lack of specificity of the pathological findings in interstitial lung diseases. The identification of the *SP-C* gene mutation associated with interstitial lung disease in these cases may lead to a more accurate classification of these diseases.

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