Differences in the translation efficiency and mRNA stability mediated by 5′-UTR splice variants of human SP-A1 and SP-A2 genes

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Wang, Guirong, Xiaoxuan Guo, and Joanna Floros. Differences in the translation efficiency and mRNA stability mediated by 5′-UTR splice variants of human SP-A1 and SP-A2 genes. Am J Physiol Lung Cell Mol Physiol 289: L497–L508, 2005. First published May 13, 2005; doi:10.1152/ajplung.00100.2005.—Surfactant protein A (SP-A) plays an important role in host defense, modulation of inflammatory processes, and surfactant-related functions of the lung. The human SP-A (hSP-A) locus consists of two functional genes, SP-A1 and SP-A2. Several hSP-A 5′-untranslated region (UTR) splice variants for each gene have been characterized and shown to be translated in vitro and in vivo. In this report, we investigated the role of hSP-A 5′-UTR splice variants on SP-A production and molecular mechanisms involved. We used in vitro transient expression of hSP-A 5′-UTR constructs containing luciferase as the reporter gene and quantitative real-time PCR to study hSP-A 5′-UTR-mediated gene expression. We found that 1) the four (A′D′, ABD, AB′D′, and A′CD′) 5′-UTR splice variants under study enhanced gene expression, by increasing luciferase activity from 2.5- to 19.5-fold and luciferase mRNA from 4.3- to 8.8-fold compared with the control vector that lacked hSP-A 5′-UTR; 2) all four 5′-UTR splice variants studied regulated mRNA stability. The ABD variant exhibited the lowest rate of mRNA decay compared with the other three constructs (A′D′, ABD, and A′CD′). These three constructs also exhibited significantly lower rate of mRNA decay compared with the control vector; 3) based on the indexes of translational efficiency (luciferase activity/mRNA), ABD and AB′D′ exhibited higher translational efficiency compared with the control vector, whereas the translational efficiency of each A′D′ and A′CD′ was lower than that of the control vector. These findings indicate that the hSP-A 5′-UTR splice variants play an important role in both SP-A translation and mRNA stability.

surfactant protein A; 5′-untranslated region; regulation; NCI-H441 cell line; translational control

SURFACANT PROTEIN A (SP-A), the most abundant surfactant-associated protein in the alveolar lung space (12), plays an important role in host defense, the modulation of inflammatory processes, as well as in surfactant physiology including surfactant-related functions of the lung. The human SP-A (hSP-A) locus consists of two functional genes, SP-A1 and SP-A2, and a pseudogene (26). Genomic sequences for both SP-A1 and SP-A2 genes (38, 76) and their corresponding cDNAs (15) have been published. Several alleles for each hSP-A gene have been characterized based on the DNA sequence variation at the coding region (10, 11). Structural and functional differences between SP-A1 and SP-A2, as well as among some frequently observed alleles, have been observed (17, 65, 71, 73, 74).

The genomic structure of SP-A1 and SP-A2 consists of a 5′-untranslated region (UTR), four coding exons, and a 3′-UTR, which is part of the fourth coding exon (38, 76). The 5′-UTR of the SP-A genes is complex. Four exons or regions (A, B, C, D) for SP-A1 or three (A, B, D) for SP-A2 (36), shown in Fig. 1, splice in a number of configurations to form different 5′-UTR. The major splice patterns, as well as their relative frequency, vary between the two genes; the major pattern for SP-A1 is A*BD′ (81%), and the major patterns for SP-A2 are A*BD (44%) and A*BD′ (49%) based on our previous observation (36), where no distinction was made among the three transcription start sites of exon A (36). All three start sites (A, A′, A′′) were denoted here as exon A*. The major 5′-UTR splice variants of the mRNA transcripts of each SP-A gene are shown to be translated both in vitro (36) and in vivo (37).

SP-A expression is regulated at several levels, including at the tissue-specific level, by development stage, by a number of hormones, and other factors (52). Studies of hSP-A regulation by glucocorticoids (GC) in fetal lung explants (5, 32, 34, 43), of the 5′-flanking region (29), and of the 3′-UTR (27, 72) of SP-A have produced a complex picture of regulation. In fact, the two SP-A genes have been shown to be differentially regulated by certain agents including GC (29, 34, 43, 51, 64), and certain SP-A alleles may be differentially regulated by dexamethasone (Dex, a synthetic GC) (27, 72). Previous reports of the promoter of SP-A1 revealed that the −32/+63 region relative to the SP-A1 transcription start site was sufficient for both basal transcription and Dex repression (29). Removal of the +18/+63 region significantly reduced the ability of Dex to inhibit transcription. cis-elements similar to negative GR-binding sites were identified within the −32/+63 region (29).

Recent studies have shown that 5′-UTR plays an important role in the regulation of gene expression (see reviews, Refs. 19,

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human SP-A variants in front of a luciferase reporter gene using PCR cloning with the appropriate primers. First, the pcDNA3 vector was modified by removing one putative transcription start site in pcDNA3. Briefly, the pcDNA3 was digested with BamHI and EcoRI restriction enzymes to remove a 33-bp fragment of pcDNA3. The large fragment (~5.3 kb) of pcDNA3 was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Then PCR was performed with primer pair 1046/1047 (Table 1) using the large purified fragment of pcDNA3 as template. Primer 1046 contains HindIII and BamHI restriction sites in its 5'-ends, and primer 1047 contains XhoI and BamHI restriction sites in its 5'-ends. The PCR products consisted of 1× buffer 2, 0.125 mM of each dNTP, 1 ng/µl of each of the primers, and 3.5 units of Expand high-fidelity PCR system (Roche, Mannheim, Germany) in 50 µl of final volume. The cycling was at 94°C for 2 min, followed by 20 cycles at 94°C for 10 s, 68°C for 30 s, and 68°C for 7 min. The final extension step was at 68°C for 10 min. The PCR products were purified and digested with the BamHI restriction enzyme and then ligated with the T4 DNA ligase at 4°C overnight. These modifications removed a 133-bp fragment from nucleotide 836 to 970 of pDNA3, leaving only one BamHI restriction enzyme site in the vector. Transformation in Escherichia coli strain XL1 Blue was performed by standard protocol. Purification of plasmid was performed with QIAprep spin miniprep kit (Qiagen). The sequence of the modified pcDNA3 (rpDNA3) vector was verified by DNA sequencing (Molecular Genetics Core Facility at Pennsylvania State University College of Medicine).

Second, the firefly luciferase gene was cloned into rpDNA3 vector. The 1.7 kb of firefly luciferase gene fragment was generated by digestion of the pGEM-luc DNA (Promega, Madison, WI) with restriction enzymes Xhol and BamHI. This 1.7-kb fragment was then cloned into Xhol/BamHI-digested rpDNA3 vector (Fig. 2). The recombinant plasmid DNA (rpDNA3/LUC) was confirmed by sequencing.

Third, the hSP-A 5'-UTR variants were each inserted between the promoter and the luciferase reporter gene variant of the vector (rpDNA3/LUC) to generate the recombinant constructs under study. SP-A cDNA clones, each containing a different variant transcript of hSP-A, were used as templates to obtain the 5'-UTR variant sequence for cloning. PCR was performed with primer pair 1050/1502 or 1051/1052 (Table 1). Primer 1050 or 1051 contained a HindIII restriction enzyme site in its 5'-end, and 1052 contained a BamHI restriction site at its 5'-end. Primer 1050 was for exon A of 5'-UTR, and 1051 was for exon A' of 5'-UTR. Primer 1052 was for both D and D' regions of 5'-UTR. The PCR conditions consisted of 1× buffer 2, 0.125 mM of each dNTP, 1 ng/µl of each of the primers, and 7 units of Expand high-fidelity PCR system (Roche) in 100 µl of final volume. PCR was performed at 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The final extension step was at 72°C for 5 min. The PCR product was purified by electrophoresis with 1.5% BioGel of the MERmaid spin kit (Bio101, La Jolla, CA). The pure PCR products were digested with HindIII/BamHI and then were cloned into the HindIII/BamHI site of pcDNA3/LUC.

Fourth, because the cytomegalovirus (CMV) promoter is a very strong promoter, we speculated that it may not be appropriate for our study. With a strong promoter, we may run the risk of cofactor limiting reaction. Therefore, we replaced the CMV promoter of rpDNA3/5'-UTR/LUC construct with the SV40 promoter. The SV40 promoter DNA fragment was generated from the pSVL5V40CAT by PCR amplification with primer pair 1050/1502 and primer pair 1051/1052 (Table 1). Primer 1050 or 1051 contained a HindIII restriction enzyme site in its 5'-end, and 1052 contained a BamHI restriction site at its 5'-end. Primer 1050 was for exon A of 5'-UTR, and 1051 was for exon A' of 5'-UTR. Primer 1052 was for both D and D' regions of 5'-UTR. The PCR conditions consisted of 1× buffer 2, 0.125 mM of each dNTP, 1 ng/µl of each of the primers, and 7 units of Expand high-fidelity PCR system (Roche) in 100 µl of final volume. PCR was performed at 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The final extension step was at 72°C for 5 min. The PCR product was purified by electrophoresis with 1.5% BioGel of the MERmaid spin kit (Bio101, La Jolla, CA). The pure PCR products were digested with HindIII/BamHI and then were cloned into the HindIII/BamHI site of pcDNA3/LUC.

MATERIALS AND METHODS

Cell culture. The human lung adenocarcinoma cell line (H441) used in this study was purchased from the American Type Culture Collection (Manassas, VA). H441 cells were grown in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO), 1× antimycotic-antibiotic solution (Sigma, St. Louis, MO), and 1% L-glutamine (Sigma). The cells were subcultured weekly and maintained at 37°C in 5% CO₂ atmosphere.

Plasmid constructs. The pcDNA3 vector was purchased from Invitrogen and was modified to facilitate cloning of the 5'-UTRs of human SP-A variants in front of a luciferase reporter gene using PCR cloning with the appropriate primers. First, the pcDNA3 vector was modified by removing one putative transcription start site in pcDNA3. Briefly, the pcDNA3 was digested with BamHI and EcoRI restriction enzymes to remove a 33-bp fragment of pcDNA3. The large fragment (~5.3 kb) of pcDNA3 was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Then PCR was performed with primer pair 1046/1047 (Table 1) using the large purified fragment of pcDNA3 as template. Primer 1046 contains HindIII and BamHI restriction sites in its 5'-ends, and primer 1047 contains XhoI and BamHI restriction sites in its 5'-ends. The PCR products consisted of 1× buffer 2, 0.125 mM of each dNTP, 1 ng/µl of each of the primers, and 3.5 units of Expand high-fidelity PCR system (Roche, Mannheim, Germany) in 50 µl of final volume. The cycling was at 94°C for 2 min, followed by 20 cycles at 94°C for 10 s, 68°C for 30 s, and 68°C for 7 min. The final extension step was at 68°C for 10 min. The PCR products were purified and digested with the BamHI restriction enzyme and then ligated with the T4 DNA ligase at 4°C overnight. These modifications removed a 133-bp fragment from nucleotide 836 to 970 of pDNA3, leaving only one BamHI restriction enzyme site in the vector. Transformation in Escherichia coli strain XL1 Blue was performed by standard protocol. Purification of plasmid was performed with QIAprep spin miniprep kit (Qiagen). The sequence of the modified pcDNA3 (rpDNA3) vector was verified by DNA sequencing (Molecular Genetics Core Facility at Pennsylvania State University College of Medicine).

Second, the firefly luciferase gene was cloned into rpDNA3 vector. The 1.7 kb of firefly luciferase gene fragment was generated by digestion of the pGEM-luc DNA (Promega, Madison, WI) with restriction enzymes XhoI and BamHI. This 1.7-kb fragment was then cloned into XhoI/BamHI-digested rpDNA3 vector (Fig. 2). The recombinant plasmid DNA (rpDNA3/LUC) was confirmed by sequencing.

Third, the hSP-A 5'-UTR variants were each inserted between the promoter and the luciferase reporter gene variant of the vector (rpDNA3/LUC) to generate the recombinant constructs under study. SP-A cDNA clones, each containing a different variant transcript of hSP-A, were used as templates to obtain the 5'-UTR variant sequence for cloning. PCR was performed with primer pair 1050/1502 or 1051/1052 (Table 1). Primer 1050 or 1051 contained a HindIII restriction enzyme site in its 5'-end, and 1052 contained a BamHI restriction site at its 5'-end. Primer 1050 was for exon A of 5'-UTR, and 1051 was for exon A' of 5'-UTR. Primer 1052 was for both D and D' regions of 5'-UTR. The PCR conditions consisted of 1× buffer 2, 0.125 mM of each dNTP, 1 ng/µl of each of the primers, and 7 units of Expand high-fidelity PCR system (Roche) in 100 µl of final volume. PCR was performed at 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The final extension step was at 72°C for 5 min. The PCR product was purified by electrophoresis with 1.5% BioGel of the MERmaid spin kit (Bio101, La Jolla, CA). The pure PCR products were digested with HindIII/BamHI and then were cloned into the HindIII/BamHI site of pcDNA3/LUC.

Fourth, because the cytomegalovirus (CMV) promoter is a very strong promoter, we speculated that it may not be appropriate for our study. With a strong promoter, we may run the risk of cofactor limiting reaction. Therefore, we replaced the CMV promoter of rpDNA3/5'-UTR/LUC construct with the SV40 promoter. The SV40 promoter DNA fragment was generated from the pSVL5V40CAT by PCR amplification with primer pair 1082/1083. Primer 1082 contains a XhoI restriction enzyme site in its 5'-end, and 1083 contains a BamHI restriction enzyme site at its 5'-end. Primer 1082 was for exon A' of 5'-UTR, and 1083 was for exon A of 5'-UTR. Primer 1084 was for both D and D' regions of 5'-UTR. The PCR conditions consisted of 1× buffer 2, 0.125 mM of each dNTP, 1 ng/µl of each of the primers, and 3.5 units of Expand high-fidelity PCR system (Roche) in 50 µl of final volume under the
following conditions: 94°C for 2 min, and 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The final extension step was at 72°C for 5 min. The PCR product was digested with NruI/HindIII and cloned into rpcDNA3/5'-UTR/LUC, and in rpcDNA/LUC control vector (not shown), to generate recombinant constructs that contained the SV40 promoter SP-A 5'-UTR and the firefly luciferase reporter gene (Fig. 2). The construct sequences were verified by DNA sequencing. To obtain plasmid DNA for transfection experiments, a large-scale purification of plasmids was performed using the Qiagen plasmid maxikit. Plasmid DNA was subsequently extracted with phenol-chloroform extraction kit. Plasmid DNA was subsequently extracted with phenol-chloroform extraction kit. Plasmid DNA was subsequently extracted with phenol-chloroform extraction kit.

Table 1. Primers used in this study

<table>
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<tr>
<th>Primer Name</th>
<th>Sequence, 5' to 3'</th>
<th>Comments</th>
<th>Position, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1046</td>
<td>GGGgatccACCTagctCTCTAGTTAAGCCGAGAG</td>
<td>Vector pcDNA3 remodeling, antisense</td>
<td>836–817</td>
</tr>
<tr>
<td>1047</td>
<td>GGggatccCTCCGAGGATCCCTAGAAG</td>
<td>Vector pcDNA3 remodeling, sense</td>
<td>973–990</td>
</tr>
<tr>
<td>1050</td>
<td>GGgagcttAACCTGAGGCAGGAGAACC</td>
<td>Human SP-A specific, sense</td>
<td>+1–+18</td>
</tr>
<tr>
<td>1051</td>
<td>GGgagcttGGGACCGGACGACCACCACG</td>
<td>Human SP-A specific, antisense</td>
<td>+6–+24</td>
</tr>
<tr>
<td>1052</td>
<td>GGggagcttGGCTCTGAGGAGGGCTGC</td>
<td>Vector pSVL for SV40 promoter, sense</td>
<td>+921–+904</td>
</tr>
<tr>
<td>1082</td>
<td>GGCTcgcgsCTCTGAGGAGGGCTGC</td>
<td>Vector pSVL for SV40 promoter, antisense</td>
<td>328–308</td>
</tr>
<tr>
<td>1152</td>
<td>GCCCGGGAAGGATTTA</td>
<td>Forward primer for pGL3</td>
<td></td>
</tr>
<tr>
<td>1153</td>
<td>TTTCGAAAGCTTGTGAGA</td>
<td>Reverse primer for pGL3</td>
<td></td>
</tr>
<tr>
<td>1156</td>
<td>GAGACATATGGAGGCCATGCAA</td>
<td>Forward primer for pRL-SV40</td>
<td></td>
</tr>
<tr>
<td>1157</td>
<td>GATACCTGAGCTGATAAGCATATATA</td>
<td>Reverse primer for pRL-SV40</td>
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<tr>
<td>Probe-pGL</td>
<td>VIC-TATCATGCGCCTGTAAGATCCGTTAGTAA-TAMRA</td>
<td>Probe for pGL3</td>
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<tr>
<td>Probe-pRL</td>
<td>VIC-TATCATGCGCCTGTAAGATCCGTTAGTAA-TAMRA</td>
<td>Probe for pRL-SV40</td>
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Nucleotide positions are relative to transcriptional start site (+1) of human surfactant protein (SP)-A except for primers 1046, 1047, 1082, 1083, and 1053. The latter are for the indicated vector sequences. Lower case indicates the additional sequences present in the given primers. These sequences are restriction enzyme recognition sites (see MATERIALS AND METHODS).

Transient transfection and luciferase activity assay. H441 cells were grown to 80–90% confluence in 10-cm dishes and subcultured into six-well culture plates with 1 × 10⁶ cells/well (~80% confluence) 24 h before transfection. Four hours before transfection, the RPMI-1640 plus 10% FBS was replaced with Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) containing neither FBS nor antibiotics.

The transfection procedure was performed with the Lipofectamine Plus reagent kit (Invitrogen). In brief, 1 μg of DNA experimental construct plus 0.05 μg of DNA of the transfection efficiency control pRL-SV40 plasmid (containing Renilla luciferase as the reporter gene) were diluted in 100 μl of DMEM without serum. PLUS reagent (6 μl) was added, and the mixture was incubated for 15 min at room temperature. In another tube, 4 μl of Lipofectamine reagent were diluted into 100 μl of DMEM without serum. Then, the components of the two tubes were combined, mixed, and incubated for another 15 min at room temperature. The above DNA-Lipofectamine complex was added to each well containing ~1 × 10⁶ H441 cells with 2.5 ml of fresh medium. Four hours after transfection, the DMEM with 10% FBS was added to normal culture volume (5 ml/well). Transfection was carried out for 36 h or for the indicated time for the time course of gene expression.

The dual-luciferase assay was performed with the Dual-luciferase reporter assay system (kit) (Promega). Transfected cells were harvested at 36 h after transfection or at other time points according to statements in the individual experiments. The cells were washed with 1× PBS and were dissolved in 500 μl of 1× passive lysis buffer. The culture plates were rocked at room temperature for 15 min, and the lysate was transferred to a tube and centrifuged for 1 min at 4°C to clear the cell lysate. Twenty microliters of cell extract were transferred into a luminometer tube containing 100 μl of luciferase assay reagent II. The tube was placed in the FB12 luminometer (Zylux, Maryville, TN) and Stop & Glo Reagent (100 μl) was placed into the tube to initiate the Renilla luciferase activity reading. The ratio of firefly luciferase activity to Renilla luciferase activity was calculated. In all the experiments, transfection and luciferase assays were performed in triplicate.

Total mRNA preparation and measurement by quantitative real-time PCR. Total mRNA was prepared from cells according to instructions of the RNA-Bee kit (TEL-TEST, Friendswood, TX). In brief, the culture medium in the wells was removed, and the cells were washed with PBS buffer. One milliliter of RNA-Bee solution was added, and the cells were homogenized after addition of 0.2 ml of chloroform. After centrifugation at 12,000 g for 10 min at 4°C, the aqueous phase was transferred to a clean tube, and total RNA was precipitated by adding 0.5 ml of isopropanol for 5–10 min at room temperature and

Fig. 2. Schematic representation of recombinant constructs of rpcDNA3/5'-UTR/LUC. The pcDNA3 vector was modified by introducing 2 restriction enzyme sites BamHI and XhoI in its multiple cloning site. A fragment of ~1.7 kb of the firefly luciferase gene was amplified from the pGEM-luc vector and cloned into the BamHI/XhoI site of the above modified pcDNA3 vector. The cytomegalovirus promoter of the modified pcDNA3 was replaced by the SV40 promoter as described in the MATERIALS AND METHODS. hSP-A 5'-UTR sequences varying from 62 to 137 bp were obtained from hSP-A cDNA clones and cloned into the HindIII/BamHI restriction enzyme sites of the modified vector. Thus the final recombinant construct (rpcDNA3/5'-UTR/LUC) contained the marker firefly luciferase gene and an hSP-A 5'-UTR splice variant located between the SV40 promoter and the firefly luciferase reporter gene. Four such constructs were generated, each containing a different 5'-UTR variant. The construct without hSP-A 5'-UTR fragment was used as an SP-A control and was referred to as LUC.
centrifuged at 12,000 g for 5 min at 4°C. The pellet was washed with 75% ethanol and dissolved in RNase-free double-distilled H2O. Any contaminating DNA was removed from the RNA preparations using a “DNA-free” kit (Ambion, Austin, TX).

Quantitative real-time PCR was performed using the ABI PRISM 7700 sequence Detection system and a kit of TaqMan one-step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA). In the present study, the real-time PCR probes and primers were designed to target the luciferase gene. With this design the influence of the endogenous SP-A gene expression of the host cells (H441) on the mRNA levels measured is eliminated. No luciferase gene activity was detected in the host cells. In brief, 100 ng of RNA were added into 50 μl of real-time-PCR mix buffer. The buffer contained a forward-reverse primer pair (each 50 nM), such as that of primer pair 1152/1153, and a probe, such as the probe-pGL (see Table 1), as well as other enzymes and reagents provided by the manufacturer (Applied Biosystems). The reaction was carried out via two steps) one cycle at 48°C for 30 min and 95°C for 10 min was performed to reverse transcribe the luciferase mRNA from each RNA preparation to cDNA, and the PCR reaction was carried out under the following conditions, 43 cycles, each at 95°C for 15 s and 60°C for 1 min. Then the threshold cycle or the number of cycles, where accumulation of PCR products can be detected, was determined (this occurs when fluorescence passes the baseline or fixed threshold). The threshold cycle number was used to determine the number of copies of the standard curve of the control plasmid (see below). The copy number of reporter luciferase cDNA in 100 ng of total RNA was determined and was used as a measurement for the mRNA content. To determine the copy number of the reporter luciferase cDNA, the control plasmid DNA (LUC) was used as standard. The concentrations of the control plasmid DNA varied from 10^{-6} to 1 μg in a 50-μl real-time PCR reaction. The copy number of the plasmid at each concentration and for different numbers of real-time PCR cycles was calculated with the following formula: i.e., copy number per gram of plasmid DNA is equivalent to 6.02 × 10^{23}/(bp number of plasmid × 660 Da/bp). According to the formula, 1 μg of 7-kb plasmid DNA has 1.3 × 10^{11} copies of plasmid molecules. Then the standard curve of the control plasmid DNA (number of copies vs. number of real-time PCR cycles) was used to calculate the number of copies in each experimental sample per 100 ng of total RNA in this study. To compare mRNA content of SP-A 5'-UTR variants at 30-h time point after transfection, the relative mRNA level of each SP-A 5'-UTR variants was calculated by normalizing each SP-A 5'-UTR variants to LUC control.

Prediction of RNA secondary structure. To study differences in mRNA secondary structure among hSP-A 5'-UTR variants, the sequences of SP-A 5'-UTR variants were analyzed with an online program (version 3.1) prepared by Dr. M. Zuker (78–80) (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/). Through this type of analysis we were able to predict the secondary structure of hSP-A 5'-UTR variants.

Analysis of mRNA stability following inhibition of transcription by actinomycin D. Actinomycin D, a polypeptide-containing antibiotic, can inhibit transcription through binding tightly and specifically to double-helical DNA. Actinomycin D has been extensively used as a transcription inhibitor (21, 70). In this experiment, H441 cells were transfected with recombinant hSP-A 5'-UTR constructs, and gene expression was inhibited using the optimal concentration of actinomycin D (5 μg/ml of the culture medium). The cells were sampled at several time points, 0, 0.5, 1, 2, 5, 10 h after the inhibitor actinomycin D was added, and mRNA levels of the transected gene (SP-A 5'-UTR/LUC) were determined by quantitative real-time PCR.

Statistical analysis. In the present study, at least three independent experiments were carried out. In addition, triplicate assays were performed in some experiments of reporter luciferase activity (n = 3–9). The data were analyzed by with the standard program software SigmaStat version 2.0 (SPSS). Differences between/among groups were assessed by the ANOVA test or multiple-comparison ANOVA (Tukey test). The results are expressed as means ± SE. Statistical significant differences were considered when the P < 0.05.

RESULTS

Generation of the constructs with hSP-A 5'-UTR sequence variants. SP-A 5'-UTR variants used in the present study were obtained from SP-A cDNA clones by PCR and were cloned into the pcdNA3/LUC vector (Fig. 2), as described in materials and methods. The four constructs under study each included one of the following hSP-A 5'-UTR splice variants, A'D', AB'D', A'CD', and ABD (Fig. 1). A'D', AB'D', and A'CD' variants have been previously identified as transcripts of hSP-A1 and the ABD as hSP-A2 transcript (36). The sequence alignment of the four hSP-A 5'-UTR variants is shown in Table 2.

hSP-A 5'-UTR mediates expression of reporter luciferase gene activity: a time course. First, we determined optimal conditions for the luciferase activity assay. Each of the hSP-A 5'-UTR constructs (ABD and A'CD') along with the transfection efficiency control vector (pRL-SV40) were cotransfected into H441 cells. The transfection efficiency control vector contains the Renilla luciferase gene, and the Renilla luciferase activity serves as a denominator in our assays. The cells were harvested 36 h after transfection. Cell extracts were prepared and diluted from 0 to 100,000 times. Then, the firefly and Renilla luciferase activities were analyzed using the Dual-luciferase kit. The ratios of firefly-Renilla luciferase activity at different dilutions are shown in Table 3. The results indicated that the ratio of firefly-Renilla luciferase activity of cell extracts is relatively stable at a dilution range of 0–1,000 times (boldface in Table 3). In subsequent experiments we used undiluted cell extracts.

To determine the optimal time point of the luciferase activity we carried out time-course experiments with the ABD variant. The ABD construct was cotransfected into H441 cells along with the transfection efficiency control pRL-SV40 vector, and the cells were harvested after transfection at 12, 24, 30, 36, 42, and 48 h. The results indicated that the firefly luciferase activity increases from 12 to 24 h after transfection, reaching a plateau at about the 24-h time point (Fig. 3A). After that, no significant difference of the firefly luciferase activity was observed up to 48 h following transfection. A time course for the A'D' construct was also performed (data not shown), and although a plateau was reached by the 36-h time point, the slope of the increase seemed different from that of the ABD

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</table>
A time course of luciferase activity from 12 to 27 h after transfection indicated that the firefly luciferase activity of the cell extract after serial dilution increased throughout this period, as shown in the inset of Fig. 3A. In subsequent experimentation, a time point in the plateau (36 h) was chosen for luciferase activity measurement so that we could more accurately compare differences among hSP-A 5'-UTR splice variants.

Comparison of luciferase activity among hSP-A 5'-UTR splice variants. To compare 5'-UTR-mediated gene expression by each of the four hSP-A splice variants, each construct was cotransfected into H441 cells along with the pRL-SV40 vector. A construct without the hSP-A 5'-UTR variant sequence containing the SV40 promoter and the firefly luciferase gene served as an SP-A control (depicted as LUC in the figures). The cells were harvested 36 h after transfection, and the firefly luciferase activities (firefly, Renilla) were analyzed by the Dual-luciferase assay, and the ratio of firefly/Renilla luciferase activity for each construct was determined. The results (Fig. 3B) show that 1) all four hSP-A 5'-UTR splice variants (ABD, A'D', AB'D', and A'CD') exhibit significantly higher luciferase activity compared with the SP-A control, LUC (no hSP-A 5'-UTR) (P < 0.01). The level of luciferase activity increased from 2.5-fold (A'D') to 19.5-fold (ABD) of the LUC control; 2) the activity of the ABD splice variant is significantly higher than that of the other three variants (A'D', AB'D', or A'CD') (P < 0.01), and the ABD activity is 7.9-fold higher than A'D'; 3) the luciferase activity of the ABD' variant is significantly higher than that of A'D' or A'CD' (P < 0.01).

Role of hSP-A1 and hSP-A2 5'-UTR splice variants on mRNA levels. The luciferase activities measured above were taken to represent protein level. To further explore molecular mechanisms of the hSP-A 5'-UTR-regulated gene expression, we studied the impact of SP-A1 and SP-A2 5'-UTR on mRNA levels using quantitative real-time PCR. We performed a time-course experiment, by determining the mRNA level of luciferase of two 5'-UTR splice variants, ABD (SP-A2) and A'D' (SP-A1) at different time points (6, 15, 18, 24, 36, 48 h) after transfection. The results shown in Fig. 4A indicate that the mRNA level of the ABD variant increased up to 36 h after transfection and then showed a decrease as assessed by the 48-h time point measurement. A similar pattern was observed for the A'D'-mediated mRNA level, except that the absolute level of A'D' mRNA was lower than that of ABD.

Comparison of luciferase mRNA content among SP-A 5'-UTR splice variants. We compared hSP-A 5'-UTR-mediated luciferase mRNA level among the four 5'-UTR variants. Each of the four hSP-A 5'-UTR constructs (A'D', ABD, AB'D', or A'CD') and the control (LUC) were cotransfected into H441 cells along with the pRL-SV40 control vector. Total RNA from the cells was isolated at 30 h following transfection, and the copy number of luciferase mRNA per 100 ng of total RNA was determined by quantitative real-time PCR (see MATERIALS and METHODS). The relative luciferase mRNA content was determined following normalization of each SP-A 5'-UTR variant to LUC control. The results (Fig. 4B) indicated that 1) all hSP-A 5'-UTR variants have significantly higher (P < 0.01) mRNA levels compared with LUC control (no hSP-A 5'-UTR). The mRNA amount of the 5'-UTR variants varies from

Table 2. Sequence comparison of SP-A 5'-UTR splicing variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>nt 1</th>
<th>nt 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A'D'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB'D'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A'CD'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
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<tr>
<td>A'</td>
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<td></td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>AB'D'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A'CD'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nucleotide sequences of 5'-untranslated region (UTR) variant with underlining (_ _) represent exon A, with underline (____) represent exon A', with underline (_____ ) represent exon B, with underline (______) represent exon C, with underline (______ ) represent exon D, with underline (______ ) represent exon E. The nucleotide (nt) where each exon starts is noted (e.g., exons B, B', or C start at nt 45).

Table 3. Ratio of activity of luciferase of cell extract after serial dilution

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Firefly</th>
<th>Renilla</th>
<th>Ratio of Firefly/Renilla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A'CD'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undiluted</td>
<td>7606735</td>
<td>2929531</td>
<td>2.5966</td>
</tr>
<tr>
<td>10</td>
<td>987820</td>
<td>395834</td>
<td>2.4956</td>
</tr>
<tr>
<td>100</td>
<td>111691</td>
<td>46027</td>
<td>2.4243</td>
</tr>
<tr>
<td>1000</td>
<td>13028</td>
<td>5847</td>
<td>2.2283</td>
</tr>
<tr>
<td>10000</td>
<td>1560</td>
<td>1305</td>
<td>1.1946</td>
</tr>
<tr>
<td>100000</td>
<td>689</td>
<td>932</td>
<td>0.7394</td>
</tr>
</tbody>
</table>
4.3-fold (A’D’) to 8.8-fold (ABD) of the control (LUC); 2) the ABD mRNA level is significantly higher (P < 0.01) compared with that of the other three constructs (A’D’, AB’D’, or A’CD’); and 3) no significant differences are observed among AB’D’, A’D’, or A’CD’.

Translation efficiency index of hSP-A 5’-UTR variants. Comparison of the findings, shown in Fig. 3B (luciferase activity, which is taken to reflect protein levels) and in Fig. 4B (mRNA levels), indicate that although no significant differences of the mRNA content were observed among A’D’, AB’D’, and A’CD’ (Fig. 4B), the luciferase activity of AB’D’ was significantly higher than that of A’D’ and A’CD’ (Fig. 3B). The translation efficiencies of the SP-A 5’-UTR variants were evaluated using the index of luciferase activity/relative mRNA content (Fig. 5). The larger the index, the higher the translation efficiency. The results indicate that both ABD and AB’D’ showed higher translation efficiency than the control (LUC), but the translational efficiencies of A’D’ and A’CD’ were lower than that of the control (LUC). This observation indicates that the ABD and AB’D’ variants exhibit increased translational efficiencies, and the A’D’ and A’CD’ show decreased translational efficiencies, although all four 5’-UTR variants enhanced the reporter gene luciferase activity and mRNA content compared with the control vector (LUC). These results demonstrate that the hSP-A 5’-UTR plays a role in the protein translation process and that alteration of translation efficiency may be one mechanism via which the hSP-A 5’-UTR variants regulate gene expression.

Comparison of predicted hSP-A 5’-UTR mRNA secondary structures. As a prelude to gain insight into potential factors contributing to differences observed among hSP-A 5’-UTR variants, we investigated the secondary structures of hSP-A 5’-UTR (ABD, A’D’, AB’D’, and A’CD’) using algorithms to identify the most energetically favored structure for each variant. The structure of each hSP-A 5’-UTR variant alone was predicted by Zuker algorithm of the mRNA fold online program (version 3.1) (78–80). The results showed that the ABD variant forms the most stable structure compared with the other 5’-UTRs. The secondary structure of the ABD, A’D’, and AB’D’ variants is shown in Fig. 6. Based on the principle of the lowest free energy and other relevant parameters of mRNA folding, the black-paired nucleotides in the predicted structure represent the highest stability. The next optimal folding is shown sequentially by colors red, purple, blue, green, light green, and yellow. The ABD secondary structure consists of a long stable stem shown by the black-paired nucleotides and a double hairpin-like head at each end of the stable stem (Fig. 6A). However, the A’D’, AB’D’, and A’CD’ variants (data of the A’CD’ not shown) appear to lack such a level of structural stability.

Each of the four 5’-UTR variants (when one considers only the 5’-UTR region, without including the luciferase gene and

![Fig. 3. hSP-A 5’-UTR mediates expression of reporter luciferase gene activity. A: a time course. After transient transfection of the ABD variant into H441 cells, activity measurements at various time points (12, 24, 36, 48, 50 h) were determined. The firefly luciferase activity exhibited a significant increase from 12 to ~24 h and then reached a plateau, which was maintained up to at least 50 h after transfection. The 36-h mid-time point in the plateau was used for further experimentation. The experiments were repeated 3 times (n = 3). Each point shows the mean ± SE. Inset: luciferase activity of a time course, focusing on a range from 12 to 27 h after transfection. The results indicate that the firefly luciferase activity kept increasing at this period of 12–24 h. The slope (r) of the ABD was calculated with a formula (r = a/b) (a = the increase in the amount of firefly luciferase activity from 12 to 24 h after transfection; b = the number of h from 12 to 24 h after transfection). B: luciferase activity. Recombinant constructs with ABD, A’D’, AB’D’, and A’CD’, or control vector (LUC) without hSP-A 5’-UTR variant, were cotransfected into H441 cells along with the transfection efficiency vector control (pRL-SV40). The cells were harvested 36 h after transfection, and the firefly and Renilla luciferase activities were measured using the Dual-luciferase assay, as described in MATERIALS AND METHODS. The ratio of firefly/Renilla luciferase activities was used to represent 5’-UTR-mediated gene expression. The ABD activity was significantly higher (**p < 0.01) than either of the constructs tested, A’D’, AB’D’, A’CD’, or control (LUC). The AB’D’ was significantly higher (##p < 0.01) than either A’D’, A’CD’, or control (LUC). The level of luciferase activity of the SP-A control (LUC) i.e., the puxDNA3 construct that lacked hSP-A 5’-UTR sequence, was significantly lower (##p < 0.01) than any of the constructs with hSP-A 5’-UTR variants. The experiments were repeated 3 times (n = 3). Bar shows ± SE.](http://ajplung.physiology.org/)
Comparison of luciferase mRNA stability among hSP-A 5′-UTR variants. To study whether hSP-A 5′-UTR variants influence mRNA stability and whether differences in mRNA stability are observed by different hSP-A 5′-UTR variants, we evaluated the rate of mRNA decay. The luciferase mRNA level was determined by quantitative real-time PCR assays following inhibition of the RNA transcription activity by actinomycin D.

To determine the optimal concentration of the transcription inhibitor actinomycin D, a dose-response experiment was carried out using a range of concentrations (0, 0.1, 1, 5, 10 μg/ml). The H441 cells were transiently transfected with the ABD construct, and 24 h after transfection the cells were treated with actinomycin D at the indicated concentrations. The cells were harvested at several time points, 0, 0.5, 1, 2, 5, and 10 h after actinomycin D treatment. The relative mRNA levels of luciferase are shown in Fig. 7A. The results show that gene transcription could not be inhibited completely when the cells were treated with 0.1 or 1 μg/ml of actinomycin D (Fig. 7A). However, the mRNA levels decreased significantly after treatment with 5 or 10 μg/ml of actinomycin D, with no significant difference detected between 5 and 10 μg/ml of actinomycin D. Therefore, a concentration of 5 μg of actinomycin D per ml was deemed optimal and was used in subsequent experiment.

Next, the rate of mRNA decay was compared between each of the four hSP-A 5′-UTR constructs (ABD, A′D′, AB′D′, and A′CD′) and the control (LUC) vector (without hSP-A 5′-UTR), and among the hSP-A 5′-UTR variants. These constructs were transfected into H441 cells as described in MATERIALS AND METHODS. Twenty-four hours after transfection, transcription was inhibited with actinomycin D (5 μg/ml), and the levels of luciferase mRNA transcripts at 0, 0.5, 1, 2, 5, and 10 h
were determined by real-time PCR. The results shown in Fig. 7B indicate that 1) after treatment with the inhibitor, all four 5′-UTR variants have higher mRNA levels compared with actinomycin D-treated control (LUC) vector; 2) the relative mRNA level of ABD is significantly higher than that of the other variants (A′D′, AB′D′, and A′CD′); and 3) no significant differences of the rate of mRNA decay were observed among A′D′, AB′D′, and A′CD′. These observations indicate that the hSP-A 5′-UTR variants increase mRNA stability compared with the control (LUC) and that the mRNA decay is differentially affected by the 5′-UTR splice variants.

**DISCUSSION**

Regulation of gene expression is achieved through a series of complex mechanisms including control of processes involved in gene transcription, posttranscriptional modifications, and translation. The hSP-A locus consists of two functional genes, SP-A1 and SP-A2, and a pseudogene. SP-A1 and SP-A2 gene expression is regulated at several different levels, including developmental, tissue/cell-specific gene expression, allelic-specific expression (13, 46, 52). Several regions of the hSP-A gene, including 5′-flanking and promoter regions (29) as well as the 3′-UTR (27, 72), are involved in these regulatory processes. Although the coding sequences of both SP-A1 and SP-A2 genes share a high degree of similarity, structural and functional differences between the two gene products and even among the alleles have been observed in recent studies (17, 31, 65, 71, 73, 74). Both SP-A1 and SP-A2 genes are expressed in the lung tissue and generate at least three and two 5′-UTR splice variants, respectively (36). However, it is not clear what role(s) the hSP-A 5′-UTR variants play in the regulation of SP-A expression and whether the 5′-UTR variants differentially affect SP-A expression. In the present study, we studied the role of hSP-A 5′-UTR variants in gene expression, by evaluating the role of 5′-UTR on protein, mRNA content, and mRNA stability. Our findings indicate that the 5′-UTR splice variants exhibit differential impact on several regulatory steps. The SP-A2 ABD 5′-UTR splice variant was found with higher activity, higher mRNA stability, and higher translation efficiency compared with SP-A1 variants. Of the SP-A1 variants the AB′D′ showed higher translation efficiency index, and based on theoretical considerations, a higher secondary structure stability was observed compared with A′D′ and A′CD′ variants; the mRNA stability of all three SP-A1 variants was virtually identical. We speculate that the hSP-A 5′-UTR modulates expression via several mechanisms and that 5′-UTR-mediated differences contribute to differences observed in SP-A levels among individuals.

Published work has indicated an important role for 5′-UTR in posttranscriptional modification and/or translation (19, 68). Initiation of translation is one of the most important steps that could influence the level of gene expression, and 5′-UTR sequences may greatly contribute to this step. Five structural elements near the 5′-end of the mRNA determine initiation of translational efficiency (19, 68). These are 1) the m7G cap, 2) the primary sequence or context surrounding the AUG codon, 3) the presence of the AUG codons in the 5′-UTR, 4) the secondary structure of mRNA, and 5) the 5′-UT leader length. It is likely that the composition and/or the length of the leader sequence plays an important role in the translation efficiency and may account for differences among hSP-A 5′-UTR splice variants. Between the most frequently observed SP-A2 (ABD and AB′D′) (AB′D′ was not studied in this work) and SP-A1 (A′D′) variants, the length of the SP-A2 leader sequence is clearly longer (ABD and AB′D′ are 100 and 97 nt, respectively) than the SP-A1 (A′D′ is 62 nt). Although the SP-A2 ABD variant with the longer leader sequence (compared with A′D′) exhibited higher translation efficiency and higher mRNA stability, other less frequently found variants with even longer leader sequence (AB′D′ = 137 nt, A′CD′ = 122 nt) than either A′D′ or ABD exhibited translation efficiency (A′CD′) equivalent to A′D′ or lower (AB′D′) than the ABD. These
Fig. 7. Effect of 5'-UTR-mediated mRNA stability. A: dose-response inhibition of transcription by actinomycin D (act. D). H441 cells were transfected with the ABD construct. The cells (24 h after transfection) were treated with the transcription inhibitor act. D at various concentrations (0, 0.1, 1, 5, 10 µg/ml) and harvested at various time points (0, 0.5, 1, 2, 5, and 10 h) after treatment with the inhibitor. The luciferase mRNA level was determined by real-time PCR. The relative mRNA levels of luciferase (mRNA amount at the 0-h time point is shown as 100%) are shown. Concentrations of 0.1 µg/ml of act. D did not inhibit transcription, but concentrations of 1 µg/ml of act. D/ml could significantly decrease transcription compared with control without act. D treatment (**P < 0.01). When a concentration of 5 or 10 µg/ml of act. D/ml was used, the transcriptional activity of luciferase was completely inhibited. The experiments were repeated 3 times (n = 3). Each point shows the mean ± SE. B: comparison of luciferase mRNA stability among hSP-A 5'-UTR variants. H441 cells were transfected with each of the constructs, ABD, A'D', ABD', A'CD', and the control (LUC) vector. The cells were treated with the transcription inhibitor act. D (5 µg/ml) 24 h after transfection, and harvested, at time points 0, 0.5, 1, 2, 5, and 10 h after inhibitor treatment. RNA was extracted at each time point, and the amount of luciferase mRNA was measured by real-time PCR. The relative mRNA levels of luciferase are shown. The mRNA content is set to 100% at the time point of 0 h (before inhibitor treatment). All 4 5'-UTR variants exhibited higher relative mRNA levels compared with act. D-treated control (LUC) vector without hSP-A 5'-UTR (***P < 0.05, **P < 0.01). Of the 4 5'-UTR variants, the ABD variant had significantly higher relative mRNA level than the other constructs (A'D', AB'D', or A'CD') (##P < 0.01). No significant differences were observed among A'D', AB'D', and A'CD'. The experiments were repeated 3 times (n = 3). Each point shows the mean ± SE.

Fig. 8. Potential recognition binding sites for regulatory factors located at hSP-A 5'-UTR variants. Each exon or region of each hSP-A 5'-UTR variant is denoted with a rectangle box, and the number of base pairs of each is shown within the box. The total base pairs of each 5'-UTR variant are listed on the right. Several DNA binding factors are shown under the box for each 5'-UTR variant, where potential recognition binding sites for these factors were detected, i.e., ADR1, alcohol dehydrogenase gene regulator 1; GATA-1, GATA-binding factor 1; HSF, heat shock transcription factor; p300, a major E1A-associated cellular protein 300-kDa product; AP-1, activator protein 1; c-Myb, a hematopoietic lineage-restricted transcription factor; Sn, Snail factor.

Observations support the notion that the leader sequence composition is more important than simply the length of this sequence. We speculate that the sequence composition of exon B contributes to the enhanced activity of the ABD variant, via binding of positive regulatory factors to exon B recognition binding sites. We further speculate that exon B lacks binding factors that negatively affect the translation efficiency as it may be in the case of the AB'D' variant (see below). This putative differential regulation of translation efficiency among 5'-UTR variants may help explain the varying mRNA (14, 35) and protein (24) levels of SP-A observed among individuals. However, future experiments are warranted to determine elements that may bind to exon B and the impact of these on translation.

Cis-acting elements in 5'-UTR have been implicated in the regulation of gene expression of a number of genes. For instance, the 5'-UTR of GLUT1 glucose transporter increased the expression of the luciferase gene, and deletion of 5'/3'-UTRs markedly reduced the expression of luciferase, suggesting that cis-acting elements of 5'-UTR play important roles in the translation of GLUT1 (2–4). The effect of regulation may involve different mechanisms, such RNA-protein interactions. A well-known cis-acting element is iron-responsive element (IRE) (23). IRE had been identified and characterized in several genes, in which cytoplasmic RNA binding proteins, iron regulatory protein-1 (IRP-1) and IRP-2, specifically bind the mRNA stem-loop structure of IRE in the 5'-UTR and regulate translation (23, 40). In addition, a number of DNA binding sites of regulatory factors were identified on the SP-A 5'-UTR splice variants in the present study (Fig. 8). For example, the ABD variant and specifically exon B contains recognition sites for alcohol dehydrogenase gene regulator 1, GATA-1, and heat shock transcription factor. These sites, although present in the 5'-UTR play important roles in the regulation of GLUT1 (2–4). The effect of regulation may involve different mechanisms, such RNA-protein interactions. A well-known cis-acting element is iron-responsive element (IRE) (23). IRE had been identified and characterized in several genes, in which cytoplasmic RNA binding proteins, iron regulatory protein-1 (IRP-1) and IRP-2, specifically bind the mRNA stem-loop structure of IRE in the 5'-UTR and regulate translation (23, 40).
recognition sites present in B, B', or C exon are functionally active, either in transcriptional regulation or other mechanism.

Our previous study has implicated an AP-1-like binding sequence within intron 1 (or between exons A and B of human SP-A) (28) in the regulation of SP-A in response to phorbol ester (28, 53). Whether the AP-1 site in exon B' plays a role in SP-A regulation remains to be determined. It is also of interest to note that GATA-6, a member of the zinc finger protein family, has been shown to be required for maturation of the lung in late gestation (47). One study showed that transforming growth factor-β1 inhibits vascular endothelial growth factor receptor-2 gene expression and this inhibition was mediated by a palindromic GATA site located in the 5'-UTR (54). The levels of gene expression and translation efficiency have been shown to be differentially regulated through the 5'-UTR splice variants in the proinsulin gene (66), estrogen receptor-α gene (41), and heat shock protein 70 gene (62). Therefore, the potential recognition binding sites in exons B, B', and C may contribute to changes in SP-A content via a variety of mechanisms.

The 5'-UTR has also been shown to affect translation efficiency through cap-independent internal ribosome entry site (IRES)-mediated mechanism (for reviews see Refs. 22, 67). Although this novel mechanism of the translation initiation was first discovered in picornaviral RNAs (33, 55), growing evidence indicates that some eukaryotic mRNAs also possess a similar mechanism (reviewed in Refs. 22, 67). The IRES-mediated translation initiation requires specific sequences that can form a Y-shaped secondary structure and a short stem loop near the start of the AUG codon (42, 62). About 6.4% of the human 5'-UTR sequences contain IRES, and these leader sequences are usually >200 bp in length (7, 44, 56). However, it is currently unknown whether shorter leader sequences (with <200 bp), such as the hSP-A 5'-UTR variants under study, can involve IRES-mediated translational mechanism.

Several studies have suggested that the 5'-UTR regulates gene expression by altering mRNA stability in both prokaryotic and eukaryotic cells (1, 21, 30, 77). Hambraeus et al. (21) found that a 5'-UTR stem-loop and ribosome binding are important for the stability of Bacillus subtilis aprE mRNA; the rate of mRNA decay of the mutated 5'-UTR of aprE was significantly increased compared with that of the wild-type sequence. Recently, a similar mechanism was found in eukaryotic cells (1, 30, 77). A stem-loop region of the tobacco psbA 5'-UTR was shown to play an important role in determining mRNA stability and translation efficiency (77), and the first 92 bp of the At-P5R 5'-UTR of Arabidopsis were sufficient in mediating mRNA stability and translation inhibition (30). These published findings indicate that 5'-UTR sequences and relevant secondary structures of transcripts from both prokaryotic and eukaryotic cells could influence both mRNA stability and/or translation efficiency. In the present study, we found that the hSP-A 5'-UTR variants appear to also modulate mRNA stability, following inhibition of transcription by actinomycin D. All four hSP-A 5'-UTR exhibited higher mRNA stability compared with the control vector (LUC), with the ABD variant showing a considerably lower rate of mRNA decay compared with the other three constructs. The present data identify differential mRNA stability as yet another mechanism of SP-A regulation. It is possible that the stability of 5'-UTR ABD secondary structure (compared to other variants) is key to higher mRNA stability observed for the ABD variant. Whether this contributes to the differential regulation of SP-A1 and SP-A2 mRNA in basal levels, in response to various agents, or during fetal lung development (32, 34, 43, 51) remains to be determined.

In summary, the hSP-A 5'-UTR variants regulate SP-A gene expression, at least through mechanisms involving mRNA stability and translation efficiency. 1) All four hSP-A 5'-UTR variants have higher mRNA level and luciferase activity compared with the control vector without hSP-A 5'-UTR. 2) Differences in the rate of mRNA decay and translation efficiency are observed among four 5'-UTR variants. 3) The ABD variant has not only a lower rate of mRNA decay and possibly the most stable mRNA secondary structure, but also the highest translation efficiency among the four 5'-UTR variants studied. 4) The AB'D' variant has higher translation efficiency than A'D' and A'CD', but all three have the same rate of mRNA decay stability. 5) Although the A'D' and A'CD' had a higher mRNA content and exhibited a lower rate of mRNA decay compared with the control vector, their translation efficiency indexes were lower than that of the control vector. These findings indicate that the 5'-UTR in hSP-A plays an important regulatory role in SP-A expression by modulating several levels of regulatory control.

ACKNOWLEDGMENTS

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GRANTS

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