Glucocorticoid Regulation of Human Pulmonary Surfactant Protein-B mRNA Stability Involves the 3'-Untranslated Region

Helen W. Huang¹, Weizhen Bi¹, Gaye N. Jenkins¹, and Joseph L. Alcorn¹

¹Department of Pediatrics, Division of Neonatal-Perinatal Medicine, The University of Texas Health Science Center at Houston, Houston, Texas

Expression of pulmonary surfactant, a complex mixture of lipids and proteins that acts to reduce alveolar surface tension, is developmentally regulated and restricted to lung alveolar type II cells. The hydrophobic protein surfactant protein-B (SP-B) is essential in surfactant function, and insufficient levels of SP-B result in severe respiratory dysfunction. Glucocorticoids accelerate fetal lung maturity and surfactant synthesis both experimentally and clinically. Glucocorticoids act transcriptionally and post-transcriptionally to increase steady-state levels of human SP-B mRNA; however, the mechanism(s) by which glucocorticoids act post-transcriptionally is unknown. We hypothesized that glucocorticoids act post-transcriptionally to increase SP-B mRNA stability via sequence-specific mRNA-protein interactions. We found that glucocorticoids increase SP-B mRNA stability in isolated human type II cells and in nonpulmonary cells, but do not alter mouse SP-B mRNA stability in a mouse type II cell line. Deletion analysis of an artificially-expressed SP-B mRNA indicates that the SP-B mRNA 3'-untranslated region (UTR) is necessary for stabilization, and the region involved can be restricted to a 126-nucleotide-long region near the SP-B coding sequence. RNA electrophoretic mobility shift assays indicate that cytosolic proteins bind to this region in the absence or presence of glucocorticoids. The formation of mRNA:protein complexes is not seen in other regions of the SP-B mRNA 3'-UTR. These results indicate that a specific 126-nucleotide region of human SP-B 3'-UTR is necessary for increased SP-B mRNA stability by glucocorticoids by a mechanism that is not lung cell specific and may involve mRNA-protein interactions.

Keywords: surfactant; SP-B; glucocorticoid; mRNA stability; 3'-untranslated region

Pulmonary surfactant is a unique complex of surface-active lipoprotein whose primary function is to reduce surface tension at the alveolar air-liquid interface, thereby preventing alveolar collapse upon expiration and allowing normal breathing (1). A developmental deficiency in the production of surfactant is recognized as the cause of respiratory distress syndrome (RDS) in prematurely born infants (2). While the phospholipid portion of surfactant, composed largely of dipalmitoylphosphatidylcholine, acts directly to reduce surface tension, one of the four major surfactant-associated proteins, surfactant protein-B (SP-B), is also essential in this function, and the lack of sufficient mature active SP-B leads to respiratory failure (3). In prematurely born infants, the levels of pulmonary surfactant and/or SP-B protein may not be sufficient for normal lung function. Infants that lack

CLINICAL RELEVANCE

The research begins to define the mechanism by which corticosteroids increase pulmonary surfactant protein B (SP-B) mRNA stability, which may lead to treatments specifically designed to augment SP-B protein levels in immature lungs.

sufficient SP-B must be maintained on respirators and high levels of oxygen, which predisposes them to develop bronchopulmonary dysplasia, a disorder that can result in chronic lung disease (4).

Expression of SP-B mRNA and protein is developmentally regulated and is restricted to alveolar type II epithelial cells and nonciliated bronchiolar epithelial (Clara) cells (5). Expression of SP-B mRNA is first detectable at 13 weeks of gestation in human lung, but SP-B protein is not detectable until 31 weeks of gestation, and protein levels increase exponentially thereafter (6). To augment expression of surfactant and SP-B, antenatal administration of glucocorticoids is commonly used to enhance lung maturation of prematurely born infants to prevent the onset and severity of RDS (7). Glucocorticoids have drastic and complex effects on human SP-B gene expression by increasing both SP-B gene transcription and SP-B mRNA stability. In explants from mid-trimester human fetal lung, dexamethasone (DEX) increased SP-B mRNA steady-state levels in a dose-dependent manner (8). Glucocorticoids not only increase the rate of transcription of the SP-B gene, but glucocorticoids increase SP-B mRNA stability 2.5-fold, from a half-life of 7 hours before treatment to a half-life of 19 hours after treatment (9). These studies suggest that glucocorticoids can act to stabilize SP-B mRNA; however, the mechanisms by which glucocorticoids increase human SP-B mRNA stability are still unknown.

While the study of gene expression has generally focused on transcriptional control mechanisms, the recognition of the importance of post-transcriptional mechanisms in gene expression has become more evident because the levels of any protein depend on the steady-state level of its mRNA. The mechanisms involved in the regulation of mRNA stability in mammalian cells are varied and complex, but the stability of mRNA appears to depend on specific mRNA-protein binding interactions (10). Regulation of mRNA stability is an important control point for the action of steroid hormones in a variety of biological systems, and there are several systems that are currently studied that suggest that hormonal control of mRNA stability is complex and the mechanisms and components involved vary for each mRNA species (11). For human SP-B mRNA, we hypothesize that regulation of SP-B mRNA stability by glucocorticoids is mediated via cytosolic proteins binding to a specific region(s) of the mRNA molecule. The aim of this study is to identify the mRNA regions that may be involved in the regulation of SP-B mRNA stability by glucocorticoids in alveolar type II epithelial cells.

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Correspondence and requests for reprints should be addressed to Joseph L. Alcorn, Jr., Ph.D., Department of Pediatrics, University of Texas-Houston Medical School, 6431 Fannin, suite 3.222, Houston, TX 77030. E-mail: Joseph.L.Alcorn@uth.tmc.edu

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MATERIALS AND METHODS

Plasmid Construction

Standard cloning, polymerase chain reaction (PCR) techniques, and bacterial manipulations were used to construct the plasmids shown in Figure 1. The human SP-B cDNA (GenBank #J02761) was obtained from the American Type Tissue Collection (#65984). The genomic DNA sequence immediately downstream of the consensus polyadenylation site was isolated by PCR of human genomic DNA derived from A549 cells using primers based on the complete SP-B gene sequence from Genbank (#M24461) (12); upstream primer (in SP-B gene intron 10) 5'-CCCGGGCAGTCGCTTTCCCC-3', downstream primer (3' of the polyadenylation addition site): 5'-GGATCCTCCTGCCTCGGCC-3'. The 3'-untranslated (UTR) encompassed entirely by exon 11 was subcloned into the SP-B cDNA sequence at the *XhoI* site at nucleotide 1774 as indicated in GenBank #J02761 to replace the very 3'-end of the cDNA and provide a *bona fide* polyadenylation cleavage site to the artificially expressed mRNA. The resulting sequence was placed under transcriptional control of the cytomegalovirus (CMV) E1 promoter in pShuttle (13) for ubiquitous expression (pSHcmvSPB). pSHcmvSPBSV40pA was constructed by replacing the SP-B 3'-UTR (all sequences downstream of the *BstEII* site at nucleotide 1225) with the SV40 3'-UTR and polyadenylation site from pShuttle-cmv (13). pSHcmvSPB Δ 7.6 and pSHcmvSPB Δ 7.6S were derived from pSHcmvSPB after digestion with appropriate restriction endonucleases and ligation of the modified DNA. In pSHcmvSPB Δ 7.6, the plasmid was digested with *BstEII* and *SacI*, the DNA region from nucleotide 1225 and 1475 removed, and the DNA ligated after repairing the ends of the DNA. In pSHcmvSPB Δ 7.6S, the plasmid was digested with *BstEII* and *ApaI*, the DNA region from nucleotide 1225 and 1347 removed, and the DNA ligated after repairing the ends of the DNA. pTET-hspB was constructed by placing the region containing the SP-B sequences in pSHcmvSPB and behind the tetracycline-repressed promoter of pTET-BBB (14). The absence or

presence of modifications was verified by DNA sequencing. Construction of plasmids shown in Figure 1B used to generate cRNA fragments were performed as follows: pT7-SPB7.6 includes the region of the SP-B mRNA 3'-UTR encompassed by *BstEII* (nucleotide 1225) and *SacI* (nucleotide 1475), pT7-SPB7.6S includes the region of the SP-B mRNA 3'-UTR encompassed by *BstEII* (nucleotide 1225) and *ApaI* (nucleotide 1347), pT7-SPB7.61 includes the region of the SP-B mRNA 3'-UTR encompassed by *BstEII* (nucleotide 1225) and *ApaI* (nucleotide 1347), pT7-SPB7.7 includes the region of the SP-B mRNA 3'-UTR encompassed by *SacI* (nucleotide 1475) and *XhoI* (nucleotide 1774), and pT7-SPB7.8 includes the region of the SP-B mRNA 3'-UTR encompassed by *XhoI* (nucleotide 1774) and nucleotide 2026.

Alveolar Type II Cell Isolation and Primary Culture

Alveolar type II epithelial cells were isolated from fetal lung explants as described previously (15). Lung explants from mid-trimester human abortuses obtained from Advanced Bioscience Resources, Inc. (Alameda, CA) in accordance with protocols approved by The Committee for the Protection of Human Subjects of the University of Texas-Houston Health Science Center. Tissues were maintained in organ culture for 5 days in serum-free Waymouth's MB 752/1 medium (#11220; Invitrogen Corp., Carlsbad, CA) in the presence of dibutyryl cAMP (Bt₂cAMP; 1 mM) (#D0627; Sigma Chemical Co., St. Louis, MO) to increase the number of type II cells. Type II epithelial cells were isolated from the tissue by digestion with collagenase type I (0.5 mg/ml, #C-0130; Sigma) and collagenase type IA (0.5 mg/ml, #C-9891; Sigma) for 15 minutes at 37°C with vigorous pipetting collagenase. The cell suspension was enriched for type II cells by incubation with DEAE-dextran (250 μ g/ml, #D9885; Sigma) and plated on 60-mm tissue culture dishes coated with extracellular matrix prepared from Madin-Darby canine kidney cells (ATCC CRL-6253). The resulting human type II epithelial cells were cultured in serum-free Waymouth's MB 752/1 medium in the presence of Bt₂cAMP in a humidified atmosphere of 95% air and 5% CO₂.

Figure 1. Diagrams of the surfactant protein B (SP-B) regions and chimeras used to investigate *in vivo* functional activity of the SP-B mRNA 3'-untranslated region (UTR), and to make *in vitro* transcribed SP-B cRNA probes. (A) Schematic diagram of the cytomegalovirus (CMV)-driven SP-B cDNA with the 3'-genomic region containing the *bona fide* site for polyadenylation of SP-B mRNA. pSHcmvSPB (wild-type), pSHcmvSPB Δ 7.6, mutation deleted in the 3'-UTR from *BstEII*-*SacI* (236 bp), pSHcmvSPB Δ 7.6S, mutation deleted in the 3'-UTR from *BstEII*-*ApaI* (126 bp), pSHcmvSPB-SV40pA, mutation in which the entire SP-B 3'-UTR is replaced with the SV40 3'-UTR from pShuttle-cmv (18). (B) Plasmids used to make *in vitro* T7-driven riboprobes. pT7-SPB7.6 results in a 254-base-long probe, pT7-SPB7.6S results in a 126-base-long probe, pT7-SPB7.61 results in a 110-base-long probe, pT7-SPB7.7 results in a 313-base-long probe, and pT7-SPB7.8 results in a 329-base-long probe.

Cell Culture

NCI-H441 (ATCC HTB-174), A549 (ATCC CCL-185), HeLa (ATCC CCL-2), and mouse MLE12 (ATCC CRL-2110) cells were cultured in Waymouth's MB 752/1 medium containing fetal bovine serum (FBS) (10% vol/vol), while HEK293(ATCC CCL-1573) (16) and 293 Tet-Off (#63098; Clontech Laboratories, Inc., Mountain View, CA) cells were cultured in high glucose Dulbecco's modified Eagle's medium containing FBS (10% vol/vol). When the cells were to be used for experiments, they were incubated in medium containing charcoal-stripped dextran-treated FBS (#SH30068; Hyclone, Inc., Logan, UT) 24 hours before treatment to eliminate possible effects from steroids in FBS. All cell culture incubations were performed in a humidified incubator at 37°C with 5% CO₂.

Transfection of Cells

Transfection of cell lines was performed using the protocol prescribed by the Lipofectamine Plus reagent (#11514-015; Invitrogen) with slight modifications; transfection of 60-mm plates took 4 µg of plasmid combined with 12 µl of the Lipofectamine reagent and 12 µl of the Plus reagent per plate was used. As a transfection efficiency control, β -galactosidase (*lacZ*) mRNA expression from pCH110 (17) (1 µg per plate) was used.

Isolation of RNA

RNA was isolated and purified from the cells using the Trizol reagent (#15596-026; Invitrogen). Trizol Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues that involves a single-step RNA isolation method. The concentration of the RNA was determined by measuring absorbance at 260 nm.

Northern Analysis of Surfactant mRNA

Total RNA (25 µg) was electrophoresed, transferred to nylon membrane (Zeta-Probe, #162-0165; Bio-Rad Laboratories, Inc., Hercules, CA), and probed using a radiolabeled human SP-B cDNA as described in detail previously (18). Analysis of β -galactosidase mRNA expression was performed using radiolabeled *lacZ* DNA. The relative levels of mRNA were assessed by autoradiography using BioMax MS imaging film (#829-4985; Eastman Kodak Co., New Haven, CT). Quantization of the resulting images was performed using the imaging and quantitating abilities of the ChemiGenius² imaging system (Syngene, Frederick, MD).

Real-Time Quantitative RT-PCR Analysis of SP-B mRNA

Quantitative real-time RT-PCR assays specific for the four human surfactant protein mRNAs were designed and performed by the Quantitative Genomics Core Laboratory in the Integrative Biology Department at The University of Texas Health Science Center–Houston using the 7700 Sequence Detector (Applied Biosystems, Foster City, CA) (19). Specific quantitative assays for human SP-B were developed using Primer Express software (Applied Biosystems) based on sequences from Genbank at the National Center for Biotechnology Information (NCBI, Bethesda, MD) (#NM000542). Forward primer, 216:5'-TGAGGACA TCGTCCACATCC-3'; reverse primer, 2892:5'-CCAGGAAGCTTC TCATCGTGT-3'. The fluorogenic primer 242:5'-AGATGGCCAA GGAGGCCATTTCC-3' was labeled with 6-carboxyfluorescein. Specific assays for mouse SP-B were developed based on sequences from Genbank (#NM14779). Forward primer, 567:5'-CAACAG CTCCCCATTCC-3'; reverse primer, 629:5'-TGAACCCGCTTGAT CAGAGT-3'; fluorogenic primer, 608:5'-CTGCAAGCCAGCAG AAGGGCAG-3'. SP-B cDNAs were synthesized in 10 µl total volume by the addition of 6 µl/well RT master mix consisting of: 400 nM assay-specific reverse primer, 500 µM deoxynucleotides, Stratascript buffer and 10 U Stratascript reverse transcriptase (Stratagene, San Diego, CA), to a 96-well plate (ISC Bioexpress, Kaysville, UT) followed by a 4-µl volume of sample (25 ng/µl). Each sample was measured in triplicate plus a control without reverse transcriptase. Each plate also contained an assay-specific sDNA (synthetic amplicon oligo) standard spanning a 5-log range and a no template control. Each plate was incubated in a thermocycler (MJR, Waltham, MA) for 30 minutes at 50°C followed by 72°C for 10 minutes. Subsequently, 40 µl of a PCR master mix (400 nM forward and reverse primers [IDT, Coralville, IA], 100 nM fluorogenic

probe [Biosource, Camarillo, CA], 5 mM MgCl₂, and 200 µM deoxy-nucleotides, PCR buffer and 1.25 U Taq polymerase [Invitrogen]) was added directly to each well of the cDNA plate. RT master mixes and all RNA samples were pipetted by a Tecan Genesis RSP 100 robotic workstation (Tecan US, Research Triangle Park, NC); PCR master mixes were pipetted using a Biomek 2000 robotic workstation (Beckman, Fullerton, CA). Each assembled plate was then covered with an optically clear film (Applied Biosystems) and run in the 7700 using the following cycling conditions: 95°C, 1 minute; 40 cycles of 95°C, 12 seconds and 60°C, 30 seconds. The resulting data were analyzed using SDS 1.9.1 software (Applied Biosystems). Synthetic DNA oligos used as standards (sDNA) encompassed the entire 5'-3' amplicon for the assay (Eurogentec via VWR, Sugarland, TX). Oligo standards were diluted in 100 ng/µl yeast tRNA-H₂O (Invitrogen) and span a 5-log range in 10-fold decrements starting at 0.8 pg/µl. Standards for housekeeping gene assays start 10-fold higher for abundant transcripts. It has been shown for several assays that *in vitro* transcribed sDNA standards have the same PCR efficiency when the reactions are performed as described above (G. L. Shipley, personal communication). The final data were normalized to the total amount of RNA in the samples. Linear regression was used to calculate the best fit of the data (SigmaPlot 2000 for windows 6.0; Systat Software, Inc., Point Richmond, CA).

RNA Electrophoretic Mobility Shift Assays

RNA electrophoretic mobility shift assays (REMSAs) were performed as previously described (14). Cells were grown as described above in the absence or presence of dexamethasone (10⁻⁷ M). Cytosolic proteins were isolated from the cells in ice-cold lysis buffer (10 mM Tris, pH 7.6; 3 mM MgCl₂; 40 mM KCl; 2 mM DTT; 5% glycerol; 0.5% NP-40 in DEPC-treated H₂O). After a freeze/thaw cycle, the cells were centrifuged at 600 × *g* for 10 minutes to remove debris and the supernatant containing the cytosolic proteins were aliquoted, the protein concentration determined, and the aliquots stored at -80°C. Specific radiolabeled RNA probes were made from linearized plasmids described in Figure 1B with the Maxiscript T7 kit (#1314; Ambion, Inc., Austin, TX). Specific radiolabeled fragments were isolated after electrophoresis on a denaturing Tris-Borate-EDTA (TBE)-urea polyacrylamide gel as prescribed by the Maxiscript T7 kit. For the assay, 10 to 25 µg cytosolic proteins were combined with 10⁵ CPM specific RNA fragment in reaction buffer (10 mM Tris, pH 7.6; 3 mM MgCl₂; 70 mM KCl; 2 mM dithiothreitol; 5% glycerol, 2 µg/ml heparin, 2 µg/ml tRNA). These reactions were performed in 15-µl volumes. After 15 minutes at room temperature, 1 U RNase T1 was added, and incubation continued for 10 minutes. If nonradioactive RNA probe is needed as a specific competitor, 100× molar excess was used in the assays. The samples were then electrophoresed on a nondenaturing 3.5% TBE polyacrylamide gel. The resulting gel was dried and exposed to BioMax MS imaging film (#829-4985; Eastman Kodak). Quantization of the resulting images was performed using the imaging and quantitating abilities of the ChemiGenius² imaging system (Syngene).

RESULTS

Glucocorticoids Increase Human SP-B mRNA Stability in Human Type II Cells in Primary Culture, but Do Not Increase Murine SP-B mRNA Stability in a Mouse Type II Cell Line (MLE-12)

Previous studies indicate that glucocorticoids increase SP-B mRNA stability in human fetal lung tissue in organ culture (9, 20), but the effect of glucocorticoids in isolated human fetal type II cells has never been investigated. To determine if glucocorticoids have the same effect on SP-B stability in isolated type II cells in primary culture, type II cells were isolated and grown in serum-free medium in the absence or presence of DEX (10⁻⁷ M) for 72 hours. To stop nascent transcription, the transcription inhibitor actinomycin D (10 µg/ml, #A9415; Sigma) (21) was used. RNA was isolated at various times after addition of actinomycin D and subjected to real-time quantitative RT-PCR analysis to determine the levels of SP-B mRNA in the samples. Shown in Figure 2A are the results of the determinations. As can be seen,

Figure 2. QRT-PCR of SP-B mRNA stability in human alveolar type II epithelial cells in primary culture and MLE12 mouse lung epithelial cell line. Human type II cells in primary culture and mouse lung epithelial cells (MLE12) were incubated in the absence or presence of dexamethasone (DEX) (10^{-7} M) for 24 hours. RNA was isolated various times after addition of actinomycin D ($10 \mu\text{g/ml}$) to the medium. Quantitative analysis of SP-B mRNA transcripts relative to total input RNA (100 ng) was determined by real-time quantitative PCR. Shown is a plot of SP-B mRNA remaining as a function of time ($n = 3$ mean $\pm$ SEM). Linear regression was used to calculate the best fit of the data. (A) Analysis of SP-B mRNA in human type II cells in primary culture. (B) Analysis of murine SP-B mRNA in the MLE12 cell line.

SP-B mRNA half-life is 7 hours when the cells are incubated in the absence of DEX, while SP-B mRNA half-life increases 2.3-fold (16 h) in the presence of DEX. This magnitude of change is very similar to that reported for SP-B in fetal lung tissue in organ culture (7.5 h to 18.8 h) (9), indicating that isolated type II epithelial cell possess the capacity for DEX regulation of SP-B mRNA stability.

In addition to the determination of DEX regulation of SP-B mRNA stability in isolated human type II epithelial cells in primary culture, we also investigated the ability of DEX to regulate mouse SP-B mRNA stability in a well-characterized mouse cell line, MLE12 (22), since these immortalized type II cells possess the ability to express SP-B and SP-C mRNA. The cells were grown in serum-free media in the absence or presence of DEX (10^{-7} M) for 48 hours, at which time actinomycin D was added to inhibit transcription initiation. RNA was isolated at various times after addition and the amount of mouse SP-B mRNA was determined by real-time quantitative RT-PCR analysis using primers specific for mouse SP-B sequences. As can be seen in Figure 2B, mouse SP-B mRNA half-life is 5 hours in cells incubated in the absence or presence of DEX. These results indicate that either murine SP-B mRNA stability is not regulated by DEX or the cells no longer possess this capacity. To date, there are no published reports of the possible effects of DEX on murine SP-B mRNA stability, although there is a report that the addition of DEX plus keratinocyte growth factor (KGF) somewhat increases the stability of murine SP-B mRNA stability (23). Comparison of the sequences of the human and mouse SP-B 3'UTR using the capabilities of the Blast alignment engine available from the National Center for Biotechnology Information (NCBI) indicates the absence of any significant similarity other than a 34-bp region surrounding the polyadenylation sequences at the very 3'-end of the mRNA, suggesting that the

mechanism by which SP-B mRNA stability is increased by DEX may involve sequences unique to the human region.

DEX Increases SP-B mRNA Stability in Transfected Cells of Pulmonary and Nonpulmonary Origin

While verification of the ability of DEX to regulate SP-B mRNA stability in isolated human type II cells suggests an *in vitro* cell model that can be used in these studies, the difficulty of using these cells in primary culture for transfection is a huge impediment to investigation. Therefore, the ability of other cells to mediate DEX-regulated stability of SP-B mRNA produced from an artificial expression system was determined; any cells that possess the ability could serve as a possible *in vivo* cell model. The plasmid pSHcmvSPB was constructed in which the coding sequence of the human SP-B mRNA was placed under transcriptional control of the ubiquitously expressed CMV E1 promoter (shown in Figure 1A). In this construct, the *bona fide* genomic DNA that flanks the SP-B mRNA polyadenylation addition site was introduced into the SP-B cDNA sequence to circumvent any problems arising from sequence-specific alterations in polyadenylation and mRNA maturation (24). Various cells of pulmonary and nonpulmonary origin were transfected with pSHcmvSPB and pCH110 (to normalize transfection efficiency) as described in the MATERIALS AND METHODS. After 24 hours, the cells were then incubated in the absence or presence of DEX for 48 hours, treated with actinomycin D, and RNA isolated both immediately and various times after addition. After Northern analysis to determine levels of SP-B mRNA in the cells, the half-life of the plasmid-expressed SP-B mRNA was determined for each cell type. Shown in Table 1 are the determinations of the half-life of SP-B mRNA in the cells. As demonstrated earlier, the SP-B mRNA half-life in isolated human fetal type II cells in primary culture is exceptionally similar to that seen in human fetal lung tissue in organ culture. Stability of SP-B mRNA in transformed cells derived from kidney (HEK-293) is greater than in isolated human fetal type II cells in primary culture, but DEX still increases SP-B mRNA stability 1.6-fold, indicating that the cellular components that comprise the mechanism by which DEX increases SP-B mRNA stability is not specific for lung cells. However, SP-B mRNA stability in a pSHcmvSPB-transfected uterine cell line (HeLa) does not increase in the presence of DEX, yet SP-B mRNA half-life is similar to that found in type II

TABLE 1. SP-B mRNA HALF-LIFE DETERMINATION IN VARIOUS CELLS

Tissue/Cell	$t_{1/2}$ (hours)		Fold-Change
	-Dexamethasone	+Dexamethasone	
Human Fetal Lung Tissue (37)	7.5 ± 0.4	18.8 ± 2.9	2.5
Human type II cells (primary lung epithelial)	7.0 ± 1.8	$16 \pm 4.0^\dagger$	2.3
A549 (Lung epithelial cell line)*	32 ± 3.5	$70 \pm 12.5^\dagger$	2.2
HEK293 (Kidney epithelial cell line)*	10.3 ± 3.2	$17.3 \pm 1.2^\dagger$	1.6
HeLa (Uterine epithelial cell line)*	16.0 ± 2.9	16.5 ± 1.7	1.05
MLE12 (Mouse lung epithelial cell line)	5.0 ± 0.6	5.0 ± 0.2	1.0

SP-B mRNA was expressed endogenously or comes from a transfected vector. The determinations of half-life were performed three times, values are mean $\pm$ SEM.

* Cells transfected with the pSHcmvSPB plasmid containing the CMV promoter driving expression of the human SP-B mRNA.

$^\dagger P < 0.05$, determined using the student's t-test.

epithelial cells incubated in the presence of DEX. As expected, the half-life of SP-B mRNA in transfected A549 cells (human type II cell in origin but no longer expresses any surfactant protein mRNA) is increased greater than 2-fold after DEX treatment. But unexpectedly, the intrinsic stability of the SP-B mRNA also increased nearly 5-fold. Importantly, the experiment suggests that the mechanism(s) responsible for the increase in SP-B mRNA stability by DEX can act on mRNA that has been artificially expressed. Despite the cell-specific differences in SP-B mRNA stability found in these analyses, the results indicate that transfected A459 and HEK-293 cells can be used in subsequent analyses.

The Stabilization of Human SP-B mRNA by DEX Can Be Demonstrated Using an Alternative Strategy that Eliminates Potential Adverse Effects of Actinomycin D

While the use of transcription inhibitors (such as actinomycin D) to determine molecular components and sequences involved in regulating mRNA stability has generated important information regarding these processes, these compounds have a profound effect to alter mRNA stability (25). To demonstrate the absence of any confounding effects of actinomycin D on the results of this study, we chose to employ a well-described system that uses a tetracycline-regulated promoter as a means to provide a pulse of transcription of a specific mRNA (14). In this investigation, the human SP-B sequences of pSHcmvhsbP were placed under transcriptional control of the tetracycline-repressed promoter of pTET-BBB (14), resulting in the plasmid pTET-hsbP. 293 Tet-Off are cells that stably express the protein that prevents transcription of a specific promoter when incubated in the presence of the tetracycline analog doxycycline. These cells were transfected with the pTET-hsbP (and co-transfected with pCH110 as a transfection control) and incubated in the presence of doxycycline (10 μ g/ml) and in the absence or presence of DEX for 48 hours. Doxycycline was removed for 4 hours to allow a burst of transcription in the cells from the de-repressed CMV promoter, then doxycycline was added to the cells to prevent continued transcription of SP-B mRNA. Samples were taken at 0 and 24 hours after addition of doxycycline. RNA was isolated from the samples, Northern analysis performed to determine the levels of SP-B and *lacZ* mRNAs, and the levels quantitated by densitometry. Shown in Figure 3 are the results of the assay in which the values at $t = 0$ hours are normalized as 1. As can be seen, the level of SP-B mRNA remaining after 24 hours is reduced when compared with levels at $t = 0$ hours (when transcription is repressed by the presence of doxycycline, and the amount remaining depends on the stability of the SP-B mRNA). However, the relative level of SP-B mRNA remaining after 24 hours in the presence of DEX is significantly increased as compared to the relative levels in cells incubated in the absence of DEX, and the approximate 2-fold increase is similar to that seen in Table 1 with transfected HEK293 cells after treatment with actinomycin D. Since the relative change in stability is similar when assayed by the disparate methods (2-fold versus 1.6-fold), these results suggest that actinomycin D has no effect on the ability of DEX to increase human SP-B mRNA stability. In addition, it can be surmised that whatever the mechanism, regulation is resistant to the possible effects of actinomycin D.

Regulation of SP-B mRNA Stability by DEX Requires the Presence of a Specific Region of the SP-B mRNA 3'-UTR

After demonstrating that cells transfected with pSHcmvhsbP can recapitulate the effect of DEX to increase SP-B mRNA stability seen in fetal human alveolar epithelial type II cells in primary culture, we used transfected cells as an alternative

Figure 3. Determination of SP-B mRNA stability regulation by DEX using a tetracycline-regulated pulse strategy. 293Tet-Off cells were transfected with pTET-hsbP and incubated in the absence or presence of DEX (10^{-7} M) for 48 hours and with tetracycline (10 μ g/ml) to prevent transcription of the SP-B sequence. Tetracycline was removed for 4 hours to allow a pulse of transcription of the SP-B DNA and then reintroduced to stop transcription ($t = 0$ h). Samples of RNA were isolated at $t = 0$ hours and $t = 24$ hours, and the SP-B mRNA transcript levels were determined by Northern analysis. (A) Shown is a representative Northern analysis of RNA isolated from the cells and probed for the presence of SP-B transcripts and *lacZ* transcripts (as normalizer). (B) Shown is a plot of SP-B mRNA signal at $t = 0$ hours and $t = 24$ hours with the levels normalized to the signal at $t = 0$ hours in the absence of DEX ($n = 3$, mean $\pm$ SEM). Statistical significance was determined using the Student's t test.

model of cellular regulation of SP-B mRNA stability. Expression plasmids were constructed in which various regions of the mature SP-B mRNA were deleted to functionally determine mRNA sequences necessary and/or responsible for DEX regulation of SP-B mRNA stability. Deletions were restricted to the 3'-UTR of the SP-B mRNA, since many, but not all, post-transcriptional mechanisms that regulate mRNA stability reside in the 3'-UTR (26). Figure 1A shows the schematic for the plasmid pSHcmvhsbPSPSV40pA in which most of the SP-B mRNA

3'-UTR was replaced with the region containing the SV40 late region polyadenylation signals to direct proper processing of the 3'-end of the chimeric mRNA (27). A549 cells were transfected with either pSHcmvhSPB or pSHcmvhSPBSV40pA (and the normalizing plasmid, pCH110), incubated for 24 hours in cell culture, then incubated for 48 hours in serum-free medium in the absence or presence of DEX (10^{-7} M). Cells were then treated with actinomycin D (10 μ g/ml), samples taken for $t = 0$, then samples taken 24 hours later. RNA was isolated from the samples, and the levels of SP-B mRNA was determined by Northern analysis, transfection normalized by *lacZ* mRNA levels from pCH110 in cells at $t = 0$ hours, and levels of SP-B mRNA remaining after 24 hours relative to $t = 0$ was determined. The results are shown in Figure 4. As can be seen, the amount of SP-B mRNA remaining after 24 hours in the absence of DEX is 31% of $t = 0$ hours, while the amount left after 24 hours in the presence of DEX is 71%. The greater level of SP-B mRNA in the presence of DEX can be attributed to the expected increase in the half-life of the SP-B mRNA. On the other hand, levels of SP-B mRNA expressed from pSHcmvhSPBSV40pA remaining after 24 hours in the absence of DEX is 62% and in the presence of DEX is 73%, indicating that the human SP-B mRNA 3'-UTR is necessary for DEX regulation of SP-B mRNA stability. It is important to note that the presence of actinomycin D affected the levels of *lacZ* mRNA remaining in the samples after 24 hours in such a way that resulting in a net increase in SP-B mRNA as compared to levels at 0 hours when normalized to *lacZ* mRNA. We found similar results using β -actin as a normalizer in the results described in Figure 2: a net increase in SP-B mRNA levels after addition of actinomycin D when levels were normalized. In this instance, transfection efficiency was determined using levels at 0 hours, and that value was used for the 24-hour samples.

Two other plasmids were designed based on evidence described later in Figures 5 and 6 in which we found that proteins bind the region of the SP-B 3'-UTR designated SP-B7.6. To determine the *in vivo* relevance of this region in DEX regulation of SP-B mRNA stability, plasmids were constructed in which this region is deleted, pSHcmvhSPB Δ 7.6 and pSHcmvhSPB Δ 7.6S (Figure 1A). The stability of the mRNA expressed from these plasmids in the absence or presence of DEX was determined in transfected A549 cells. As seen in Figure 4, the remaining SP-B mRNA expressed from pSHcmvhSPB Δ 7.6 24 hours after addition of actinomycin D is 28% of $t = 0$ hour levels and is 32% of $t = 0$ hours in the presence of DEX, indicating that this 245-nucleotide (nt)-long region is necessary for DEX regulation of human SP-B mRNA stability. Similar results are seen with pSHcmvhSPB Δ 7.6S in which a 125-nt long region is deleted; DEX does not significantly alter the amount of remaining SP-B mRNA. These results imply that the sequences of the human SP-B 3'-UTR is necessary for DEX regulation of SP-B mRNA stability, and that these sequences may reside in a region near the SP-B termination codon (57 nt to 182 nt downstream TGA).

Cytosolic Proteins from Isolated Type II Cells and Other Cells Bind to a Specific 236-nt Fragment of the SP-B mRNA 3'-UTR in the Absence or Presence of DEX

After functionally identifying the SP-B mRNA 3'-UTR as necessary for DEX-mediated stabilization of the mRNA, the possible formation of mRNA:protein complexes to this region in the absence or presence of DEX was investigated. The 878-nt-long human SP-B 3'-UTR can be divided into three nearly equal size fragments by digesting the cDNA with several unique restriction endonuclease enzyme sites, *Bst*EII, *Sac*I, *Xho*I, and

Figure 4. Functional activity of the SP-B 3'-UTR in glucocorticoid regulation of SP-B mRNA stability. A549 cells were transfected with various CMV-driven SP-B expression plasmids altered in the region transcribed into the human SP-B mRNA 3'-UTR. Forty-eight hours after transfection and growth in the absence or presence of DEX (10^{-7} M), the cells were treated with actinomycin D (10 μ g/ml). RNA was isolated and the SP-B mRNA transcript levels were determined by northern analysis using SP-B cDNA as a probe. (A) Shown is a representative Northern analysis of RNA isolated from the cells and probed for the presence of SP-B transcripts and *lacZ* transcripts (as normalizer). Only *lacZ* transcripts are shown from 0 hours; actinomycin D restricts the use of these transcripts as a normalizer. (B) Shown is a plot of SP-B mRNA signal at $t = 0$ hours and $t = 24$ hours with the levels normalized to the signal at $t = 0$ hours in the absence of DEX ($n = 3$ mean $\pm$ SEM). Solid bars represent cells grown in the absence of DEX, while hatched bars represent cells grown in the presence of DEX. Statistical significance was determined using the Student's *t* test.

Figure 5. RNA electrophoretic mobility shift assays (REMSAs) of various regions of the SP-B mRNA 3'-UTR in human alveolar type II cells. Cytosolic proteins were isolated from human type II cells in primary culture for 4 days incubated in the absence or presence of DEX (10^{-7} M). Radiolabeled *in vitro* transcribed RNA fragments encompassing the SP-B mRNA 3'-UTR were incubated in the absence or presence of the proteins and subjected to REMSA. Shown is an autoradiograph of the resulting complexes. Arrows denote the positions of mRNA-protein complexes.

*Bam*HI (Figure 1A). Each of these regions was subcloned behind the promoter that binds T7 polymerase. Radiolabeled *in vitro* transcribed cRNA could be produced by digesting plasmids with the appropriate restriction endonucleases (Figure 1B) and incubated with T7 polymerase and radiolabeled nucleotides. The resulting purified radiolabeled fragments (236 nt long [SPB7.6], 313 nt long [SPB7.7], and 329 nt long [SPB7.8]) were incubated with cytoplasmic proteins isolated from human fetal alveolar epithelial type II cells incubated in the absence or presence of DEX (10^{-7} M) for 2 or 4 days. REMSAs were performed as described in MATERIALS AND METHODS. After formation of presumed mRNA-protein complexes, they were separated by native PAGE, and subjected to autoradiography. As can be seen in the REMSA shown in Figure 5, stable complexes are formed only with radiolabeled SPB7.6 fragment, while no stable complexes are formed with SPB7.7 or SPB7.8 fragment. In addition, neither the number nor the intensity of the complexes formed with SPB7.6 is altered whether the cells are grown in the absence or presence of DEX. These results suggest that while cytoplasmic proteins in lung epithelial type II cells form stable complexes only with sequences in the 7.6 region of the SP-B mRNA 3'-UTR, formation of these complexes are not altered by the presence of DEX in the culture medium.

As indicated in Table 1, DEX regulation of human SP-B mRNA stability can occur in cells types other than human fetal alveolar epithelial type II cells in primary culture. A REMSA using cytoplasmic proteins from H441, A549, and HEK293 cells grown in the absence or presence of DEX was performed using the same radiolabeled cRNA fragments of the human SP-B 3'-UTR used in Figure 5. As seen in Figure 6, stable mRNA-protein complexes were formed only with the SPB7.6 RNA fragment and cytoplasmic proteins from the various cell lines. As was demonstrated with the binding of cytosolic proteins derived from primary type II alveolar epithelial cells to RNA fragments derived from SP-B7.6, the number and intensity of the complexes did not consistently change regardless whether the cells were grown in the absence or presence of DEX.

Figure 6. REMSAs of various regions of the SP-B mRNA 3'-UTR in various cell lines. Cytosolic proteins were isolated from A549, H441, and HEK293 cells incubated in the absence or presence of DEX (10^{-7} M). Radiolabeled *in vitro* transcribed RNA fragments encompassing the SP-B mRNA 3'-UTR were incubated in the absence or presence of the proteins and subjected to REMSA. Shown is an autoradiograph of the resulting complexes. Arrows denote the positions of mRNA-protein complexes. FP indicates free probe with no cytosolic protein added to the sample.

Binding of Cytosolic Proteins to the SP-B7.6 Region Is Sequence Specific

The specificity of binding of the cytoplasmic proteins to the SPB7.6 fragment was tested by performing competition assays with excess cold cRNA derived from various regions of the SP-B mRNA 3'-UTR. In these experiments, radiolabeled SPB7.6, SPB7.6S, and SPB7.61 cRNA fragments (Figure 1B) were incubated with cytoplasmic proteins isolated from A549 cells. Incubations took place in the absence or presence of 100× molar excess of cold competitor cRNA, and the REMSA is shown in Figure 7. In competition experiments using radiolabeled SPB7.6 fragment, self-competition could be seen using excess cold SPB7.6, SPB7.6S, and SPB7.61 fragments (Figure 7, arrow). These results demonstrate that binding is specific for the 7.6 region. Identical results are observed using radiolabeled SPB7.6S and SPB7.61 fragments. In both assays, self-competition is evident using excess cold self and SPB7.6 fragments, and SPB7.61 can compete with SPB7.6S binding and vice versa. This suggests that the mRNA:protein interactions are similar throughout the SPB7.6 region. More importantly, the presence of excess cold cRNA derived from other regions of the SP-B mRNA 3'-UTR does not compete for binding of proteins to any of the SPB7.6 regions, implying specific mRNA-protein interactions.

DISCUSSION

The importance of post-transcriptional mechanisms has become recognized as a major point of regulation since the levels of expression of a particular protein depend on the levels of its mRNA. Differential regulation of mRNA stability and mRNA turnover is primarily determined by interactions between specific sequences within mRNA (*cis*-acting elements) and cellular RNA-binding proteins (*trans*-acting factors) modulating nuclear export, stabilization, and translation of transcripts, as well as ribonuclease degradation of mRNA (28). The mechanisms involved in these interactions are varied and complex (10, 29), but ultimately the cellular steady-state levels of any mRNA depend on its rate of degradation. In the present study, we have begun to identify the molecular components of mechanism involved in

Figure 7. Competition assays of the 7.6 region of the SP-B mRNA 3'-UTR to demonstrate specific binding. Radiolabeled *in vitro* transcribed RNA fragments were incubated with cytosolic proteins isolated from A549 cells incubated in the absence or presence of 100× molar excess of the indicated nonradiolabeled *in vitro* transcribed RNA as competitor and subjected to EMSA. Shown is an autoradiograph of the resulting complexes. Arrows denote the positions of mRNA:protein complexes. FP indicates free probe with no cytosolic protein added to the sample.

DEX regulation of SP-B mRNA stability. We hypothesized that regulation of SP-B mRNA stability by DEX is mediated via cytosolic proteins binding to a specific region(s) of the mRNA molecule. We have shown that DEX regulation of SP-B mRNA stability occurs in isolated human alveolar epithelial type II cells in primary culture. DEX regulation can also occur in nonpulmonary cells, indicating a mechanism that is not lung cell specific, but that may be species specific. The mechanism for DEX regulation of SP-B mRNA stability requires the SP-B mRNA 3'-UTR. Deletion of a 126-nt sequence in the SP-B 3'-UTR near the SP-B coding region does abolish DEX regulation of mRNA stability, but it is unclear if the effect is due to the deletion of the sequences, or if the deletion alters the structure or spatial relationships of putative regions of the SP-B 3'-UTR that is involved in the regulatory mechanism. This same region specifically and stably binds cytosolic proteins in the absence or presence of DEX derived from a variety of cells. Interestingly, other regions of the SP-B 3'-UTR do not form stable mRNA-protein complexes with cytosolic proteins derived from type II epithelial cells or other cells. These results suggest that protein binding to a specific region of the SP-B mRNA 3'-UTR necessary for DEX regulation of SP-B mRNA stability may be a component in the mechanism of regulation.

Nucleotide patterns or motifs located in 3'-UTRs can interact with specific RNA-binding proteins, but the biological activity of regulatory motifs at the RNA level relies on a combination of primary and secondary structure (30), leading to mRNA stabilization or degradation. In addition, many RNA-binding proteins involved in the cytoplasmic post-transcriptional regulation of gene expression also participate in other regulatory processes within the nucleus (31), and the connection between post-transcriptional events in the nucleus and in the cytoplasm can affect its cytoplasmic fate. Several examples of stabilization of mRNA are given here. The Elav/Hu family of RNA-binding proteins is involved in the stabilization of several mRNAs. Rapid degradation of mRNAs is signaled by AU-rich elements (AREs) in their 3'-UTR regions (32). Ubiquitously expressed HuR binds AREs and stabilizes the mRNAs (33). While there are no ARE sequences found in the human SP-B mRNA 3'-UTR, the region designated as 7.6 in this study does contain extensive cytosine

tracts. The poly(C)-binding proteins (PCBPs) are important in mRNA stabilization, and can be divided into two groups: hnRNPs K/J and the α -CPs. These proteins bind poly(C)-rich regions of the 3'-UTR (a CCCUCCC motif) of various mRNAs and are involved in regulation of mRNA stability (34, 35).

Regulation of mRNA stability is an important control point for the action of steroid hormones in a variety of biological schemes, and there are several mechanisms that are currently studied to understand this emerging area (11). Perhaps the best studied is the estrogen-mediated stabilization of vitellogenin mRNA in *Xenopus* liver. Activation of the estrogen receptor by estrogen induces a protein that binds to a particular segment of the 3'-UTR of the vitellogenin mRNA containing the sequence ACUGUA, increasing its stability 30-fold (36). This protein, vigilin prevents site-specific endonucleolytic cleavage of the mRNA. A 5-fold increase in estrogen receptor- α (ER) mRNA levels in the endometrium of ewes treated with estradiol is entirely due to an increase in ER mRNA stability. The 3'-UTR of ER mRNA contains discrete sequences required for stability; two of these estradiol-modulated stability sequences contain a common 10-base, uridine-rich sequence that is predicted to reside in a loop structure (37). In osteoblasts, cortisol has been shown to increase collagenase 3 mRNA stability by increasing the binding of two proteins to AU-rich sequences of the 3'-UTR of the mRNA (38). These proteins are believed to be involved in cellular sub-localization and protection of the mRNA from site-specific endonucleolytic cleavage. On the other hand, glucocorticoids regulate the expression of cyclin D3 mRNA by destabilizing the mRNA through sequences within the 3'-UTR. Three elements have been mapped that include three (25, 26, and 37 bp) pyrimidine-rich domains and two proteins bind to these elements. The polypyrimidine tracts are predicted to form a stable stem-loop structure, and the proteins bind the regions in either the absence or presence of dexamethasone (39). A recent review concerning the role of steroid hormones and post-transcriptional regulation of mRNA stability by Ing (40) indicates that in general, glucocorticoids destabilize mRNAs, except for the mRNAs for fatty acid synthase and growth hormone. It seems that the role of glucocorticoids is more closely associated with the destabilization of the mRNAs for inflammatory response proteins (41).

It is significant to note that during REMSA analysis of proteins that bind to the 7.6 region of the human SP-B mRNA 3'-UTR, the presence of DEX does not alter, impede or induce binding as compared the complexes formed in the absence of DEX. The most obvious interpretation is that a DEX-induced change in SP-B mRNA stability does not involve the binding or release of an RNA-binding protein. There still remains the possibility that post-translational modifications of the binding proteins may be affected by the absence or presence of DEX. While such modifications have not been reported in specific cases of RNA-binding proteins directly involved in regulating mRNA stability, it is known that some classes of RNA-binding proteins involved in translation and localization of mRNA are post-translationally modified. Dephosphorylation of the eIF4E protein decreases its affinity for mRNA 5'-caps, allowing formation of the translational apparatus (42). On the other hand, phosphorylation of mex64 to dissociate from the nuclear complex is a key step in the translocation of mature mRNA from the nucleus to the cytoplasm (43). It has also been shown that arginine methylation of Sam68 and SLM proteins markedly reduced their poly(U) binding ability *in vitro* (44). In all cases, however, modification altered the capacity of the protein to bind to bind mRNA, unlike the results described here.

In summary, the mechanism of regulation of surfactant protein gene expression in the lung occurs by a variety of mechanisms, but the mechanisms by which mRNA stability is changed remains unclear. Here we have defined a cell culture system that will allow investigation of the molecular components of the mechanism by which human SP-B mRNA stability is regulated by DEX. We have also shown that the mechanism requires the presence of a 126-nt region in the SP-B mRNA 3'-UTR. Cytosolic proteins specifically bind this region in the absence or presence of DEX, suggesting the involvement of specific mRNA-protein interactions. Future studies are underway to identify the specific sequences involved in DEX regulation of SP-B mRNA stability and to characterize the proteins that may be involved.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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