Transcription factor USF2 is developmentally regulated in fetal lung and acts together with USF1 to induce SP-A gene expression

Erwei Gao, Ying Wang, Joseph L. Alcorn, and Carole R. Mendelson

Departments of 1Biochemistry and 2Obstetrics and Gynecology, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390-9038

Submitted 9 July 2002; accepted in final form 21 January 2003

Gao, Erwei, Ying Wang, Joseph L. Alcorn, and Carole R. Mendelson. Transcription factor USF2 is developmentally regulated in fetal lung and acts together with USF1 to induce SP-A gene expression. Am J Physiol Lung Cell Mol Physiol 284: L1027–L1036, 2003. First published February 7, 2003; 10.1152/ajplung.00219.2002.—Expression of the pulmonary surfactant protein A (SP-A) gene is lung specific, developmentally regulated, and enhanced by hormones and factors that increase cAMP. We previously identified two E-box-like enhancers termed distal binding element (DBE) and proximal binding element (PBE) in the 5'-flanking region of the rabbit (r) SP-A gene that are essential for cAMP induction of rSP-A promoter activity (Gao E, Alcorn JL, and Mendelson CR. J Biol Chem 268: 19697–19709, 1993). We also found that DBE and PBE serve as binding sites for the basic helix-loop-helix-leucine zipper transcription factor, upstream stimulatory factor-1 (USF1) (Gao E, Wang Y, Alcorn JL, and Mendelson CR. J Biol Chem 272: 23398–23406, 1997). In the present study, PBE was used to screen a rabbit fetal lung cDNA expression library; a cDNA insert encoding the structurally related rabbit upstream stimulatory factor-2 (rUSF2) was isolated. The levels of rUSF2 mRNA reach peak levels in fetal rabbit lung at 28 days of gestation, in concert with the time of maximal induction of SP-A gene transcription. In yeast two-hybrid analysis, rUSF2 was found to preferentially form heterodimers, compared with homodimers, with rUSF1. Binding complexes of nuclear proteins isolated from fetal rabbit lung type II cells with the DBE and PBE were supershifted by anti-rUSF2 antibodies. Binding activity (TBE; 5'-GGGTGGGG-3') (28, 38), an E-box transcription factor; upstream stimulatory factor; E-box; surfactant protein A; gene expression; development; type II cells; regulation

ADAPTATION OF THE FETUS to extraterine life is highly dependent on the maturity of the lung at birth and its capacity to produce surfactant. Surfactant, a developmentally regulated lipoprotein produced by pulmonary type II cells, acts to reduce alveolar surface tension and prevent atelectasis; its production is initiated in fetal lung only after ~75% of gestation is completed. Lung surfactant contains at least four associated proteins, surfactant protein (SP)-A, SP-B, SP-C, and SP-D, which appear to serve important roles in surface activity, phospholipid reutilization, and immune function within the alveolus (19). The surfactant protein genes are developmentally regulated and expressed in a lung-specific manner. The gene encoding SP-A is expressed primarily in type II cells and to a lesser extent in bronchioalveolar epithelial (Clara) cells (4, 37).

Transcription of the rabbit (r) SP-A gene is initiated in fetal lung tissue on day 24 and reaches maximal levels by day 28 (term = day 31) of gestation. In studies using transgenic mice carrying fusion genes comprising various amounts of 5'-flanking sequence from the rSP-A gene linked to the human growth hormone (hGH) structural gene, as reporter, our laboratory found that as little as ~378 bp of rSP-A 5'-flanking DNA were required to mediate appropriate lung tissue and cell-specific and development regulation of reporter gene expression (2).

SP-A gene expression in fetal lung is under multifactorial control; agents that increase cAMP (26) and cytokines (20) appear to play important roles in its regulation. In studies using cultured rabbit fetal lung explants, our laboratory found that cAMP causes a marked induction of rSP-A gene expression (8, 25); the stimulatory effect of cAMP appears to be mediated primarily at the transcriptional level (7). In type II cell transfection studies to functionally define the genomic regions that regulate basal and cAMP induction of rSP-A promoter activity, our laboratory found that rSP-A 5'-flanking sequences between −47 and −378 bp are essential for cAMP induction of SP-A promoter activity, whereas sequences between −378 and −991 bp enhance overall levels of basal and cAMP-induced expression (1).

Within the −378-bp SP-A 5'-flanking region, we have identified a number of conserved response elements, of which each is critical for cAMP induction of SP-A promoter activity. These include a putative nuclear receptor half-site [cAMP response element for the SP-A promoter (CRFSP-A); 5'-TGACCTCA-3'] (28, 38), a thyroid transcription factor-1 (TTF-1)-binding element (TBE; 5'-CTTCAAGG-3') (21), a GT box that binds Sp1 (5'-GCGGTGCGG-3') (39), and an E-box-
like sequence [proximal binding element (PBE); 5’-CTCGTG-3’]. By use of electrophoretic mobility shift assays (EMSA); we observed that the PBE (-87 to -70 bp) competed for binding to rabbit lung nuclear proteins with a structurally related element at -986 to -977 bp within the rSP-A 5’-flanking region, termed the distal binding element (DBE; CACGTG) (13). Binding activity for DBE and PBE was found to be greatly enriched in nuclear extracts of lung type II cells compared with those of whole lung tissue (13). In type II cell transfection studies to assess the functional roles of the DBE and PBE in cAMP regulation of SP-A promoter activity, we observed that rSP-A-990:hGH fusion genes containing both the DBE and PBE were induced ~30-fold by cAMP treatment (13). The finding that mutation of the DBE reduced basal and cAMP-induced expression to levels similar to those found for promoter constructs containing -378 bp of rSP-A 5’-flanking DNA (15-fold induction by cAMP) suggests that the DBE serves as a general, rather than as a specific, enhancer of cAMP-regulated expression. On the other hand, mutagenesis of the PBE [(rSP-A-990PBE-30:hGH) caused a marked reduction of basal expression and a loss of cAMP-stimulated expression to levels comparable to those of the basal promoter construct (13). It is apparent that the PBE serves a more critical role in basal and cAMP regulation of SP-A promoter activity than does the DBE. Whether this is due to its proximity to the promoter, or its interaction with other transcription factors bound to adjacent response elements, remains to be determined. Interestingly, the PBE lies near a DNase I hypersensitive site at approximately -100 bp, which was found to be present in nuclei from rabbit lung several days before the time of initiation of rSP-A gene transcription on day 24 but not in nuclei from liver or kidney tissues (10).

To characterize transcription factors that bind to these E-box motifs, radiolabeled PBE was used to screen a rabbit fetal lung cDNA expression library; cDNA inserts were isolated encoding two alternatively spliced forms of the basic-helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor upstream stimulatory factor-1 (rUSF1a and b) (14). We found that USF1 gene expression is developmentally regulated in fetal rabbit lung and reaches a maximum at 23 days of gestation, just before the time of initiation of rSP-A gene transcription (14). Overexpression of rUSF1 in lung adenocarcinoma cells stimulated rSP-A promoter activity, suggesting that rUSF1 may serve a key role in the regulation of SP-A gene expression in pulmonary type II cells (14).

In the present study, a cDNA insert for another PBE-binding protein was characterized and found to encode rabbit upstream stimulatory factor-2 (rUSF2). rUSF2, which also is enriched in type II cells, was found to heterodimerize with rUSF1 in vivo and to act synergistically with rUSF1 to increase SP-A promoter activity. rUSF2 mRNA levels also were found to be developmentally regulated in fetal rabbit lung in concert with rSP-A gene transcription.

**EXPERIMENTAL PROCEDURES**

Cloning of a cDNA insert encoding a PBE-binding protein. First-strand cDNA was synthesized from poly(A)+ RNA isolated from 24-day gestational age fetal rabbit lung tissues using random hexanucleotides and was used to generate second-strand cDNA using a cDNA synthesis kit (You-Prime cDNA synthesis kit, Pharmacia). The animal research protocols implemented in these studies were approved by the Institutional Animal Care and Use Advisory Committee of the University of Texas Southwestern Medical Center at Dallas. The double-stranded cDNAs with EcoRI/NotI linkers were inserted into λgt11 vector and packaged by use of Gigapack II gold packaging extract (Stratagene). A 5’-radiolabeled double-stranded oligonucleotide corresponding to the PBE (core sequence underlined) (5’-GAGGCCCTCCTGAGCACGG-3’) was used to screen the λgt11 cDNA expression employing standard techniques (32). Approximately 4 million recombinant phage clones were screened, and two specific cDNA clones encoding proteins that bound specifically to radiolabeled DBE (5’-GATCTCCAGTGGTGTGAAGCCAGG-3’) and PBE, but not to nonspecific 2 DNA (5’-TGCAAGGGCCAAAGCCTGGGCATC-3’) (13), were isolated. The cDNA inserts were subcloned into the pGEM-7Z plasmid vector (Promega) and sequenced using Sequenase 2.0 (USB). One of the cDNA inserts was found to be highly similar to that of human upstream stimulatory factor-1 (hUSF1), which has been reported (14). The nucleic acid sequence of the other 1,640-bp cDNA insert, termed pG-U2, was found to be highly similar to the sequence of human upstream stimulatory factor-2 (hUSF2), but it lacked a 5’-untranslated region and 87 bp of coding sequence for the amino terminus of the protein.

Construction of plasmids. GST-TU2, used for expression of the glutathione S-transferase (GST)-rUSF2 fusion protein in bacteria, was constructed by cloning the EcoRI fragment of pG-U2 into the EcoRI sites of pGEX-1AT (Pharmacia) (Table 1). pG-U2(90–228), which lacks the bHLH-LZ region, was constructed by cloning the PCR-generated cDNA fragment that encodes USF2(90–228) into pGEX-1AT. This was used for expression of the GST-rUSF2 fusion protein in bacteria and for raising antibodies.

For in vivo binding studies using a yeast system, a cDNA fragment termed U2hHLH-LZ, which encodes the region from the bHLH-LZ region to the stop codon (amino acid residues 229–346) of rUSF2, was linked either to the Gal4 DNA-binding domain (in the yeast expression vector pGBT9 from Clontech), referred to as pBD-U2, or to the Gal4 activation domain (in the yeast expression vector pGAD424 from Clontech), referred to as pAD-U2. To generate these fusion constructs, the cDNA fragment (U2hHLH-LZ) was amplified by PCR; EcoRI and BamHI sites were introduced at the 5’ and 3’ ends, respectively. The PCR-amplified fragment cut with EcoRI and BamHI was subcloned into pGBT9 and pGAD424 to produce fusion constructs with sequences encoding either the Gal4 DNA-binding domain or the transcription activation domains, pBD-U2, and pAD-U2, respectively. The yeast expression vectors that encode fusion proteins of the bHLH-LZ region of rUSF1 with DBD and AD were described previously (14).

For lung cell transfection studies, the following recombinant plasmids were used: rSP-A-990:hGH (containing 991 bp of 5’-flanking DNA from the rabbit SP-A gene fused to hGH, as reporter), rSP-A-976:hGH (lacking the DBE), and rSP-A-901PBE-30:hGH (containing a mutation in the PBE); and expression plasmids pCMV2 (empty expression vector) and pCMV:nlac (1, 13). An adapter containing NsiI and EcoRV
Table 1. Plasmid constructs

<table>
<thead>
<tr>
<th>Name</th>
<th>Vector</th>
<th>Insert</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg-U2</td>
<td>pGEM-7Z</td>
<td>rUSF2 cDNA*</td>
<td>Original cDNA</td>
</tr>
<tr>
<td>pg-GST-U2</td>
<td>pGEX-1A-T</td>
<td>cDNA encoding rUSF2-30–346†</td>
<td>EMSA</td>
</tr>
<tr>
<td>pg-GST-U230–228</td>
<td>pGEX-1A-T</td>
<td>cDNA encoding rUSF230–228‡</td>
<td>Raising antibodies</td>
</tr>
<tr>
<td>pGBT9</td>
<td></td>
<td>Gal4 DNA binding domain</td>
<td>Yeast system</td>
</tr>
<tr>
<td>pGAD424</td>
<td></td>
<td>Gal4 activation domain</td>
<td>Yeast system</td>
</tr>
<tr>
<td>pDBD-U2</td>
<td>pGBT9</td>
<td>cDNA encoding the bHLH-LZ domain of rUSF22 linked to Gal4 DNA binding domain</td>
<td>Yeast system</td>
</tr>
<tr>
<td>pAD-U2</td>
<td>pGAD424</td>
<td>cDNA encoding the bHLH-LZ domain of rUSF2 linked to Gal4 activation domain</td>
<td>Yeast system</td>
</tr>
<tr>
<td>pDBD-U1</td>
<td>pGBT9</td>
<td>cDNA encoding the bHLH-LZ domain of rUSF1 linked to Gal4 DNA binding domain</td>
<td>Yeast system</td>
</tr>
<tr>
<td>pAD-U1</td>
<td>pGAD424</td>
<td>cDNA encoding the bHLH-LZ domain of rUSF1 linked to Gal4 activation domain</td>
<td>Yeast system</td>
</tr>
<tr>
<td>SP-A -991PBE-→ hGH</td>
<td>pUC-12</td>
<td>Rabbit SP-A genomic DNA (−991 to +20 bp, with the PBE mutated) linked to hGH structural gene</td>
<td>Fusion reporter gene</td>
</tr>
<tr>
<td>pCMV</td>
<td>pBluescript</td>
<td>CMV promoter and translation initiation codon ATG</td>
<td>“Empty” expression vector</td>
</tr>
<tr>
<td>pCMV-nLac</td>
<td>pBluescript</td>
<td>cDNA encoding β-gal linked to CMV promoter</td>
<td>Transflection efficiency assay</td>
</tr>
<tr>
<td>pCMV-USF2</td>
<td>pBluescript</td>
<td>cDNA encoding rUSF2-30–346 linked to CMV promoter</td>
<td>Expression of rUSF2</td>
</tr>
</tbody>
</table>

USF-2, upstream stimulatory factor-2, rUSF-2, rabbit USF-2; rUSF1 rabbit upstream stimulatory; factor-1; EMSA, electrophoretic mobility shift assay; SP-A, surfactant protein A; hGH, human growth hormone; β-gal, β-galactosidase; bHLH-LZ, basic helix-loop-helix-leucine zipper. *Originally isolated from cDNA library using the proximal binding element as probe, lacking 5'-untranslated region and 29 amino acids at the NH2 terminus. †cDNA-encoding rUSF2 from amino acid 30 to the stop codon. ‡cDNA encoding rUSF2 from amino acids 30–228, lacking the bHLH-LZ region.

The orientations and the sequences of linking sites were confirmed by DNA sequencing. In all cases where inserts were generated by PCR, the nucleotide sequences of the resulting plasmid inserts were confirmed by DNA sequencing.

Expression and purification of GST fusion proteins. GST and GST fusion proteins were expressed and purified from Escherichia coli DH-5α (Life Technologies) as described by Smith and Johnson (35). After binding to glutathione-Sepharose 4B (Pharmacia), the proteins were washed and eluted with reduced glutathione (Sigma Chemical). The concentrations of the expressed proteins were determined by the method of Bradford (9) (Bio-Rad). The purity and sizes of the eluted proteins were then evaluated by Coomassie blue staining of SDS-polyacrylamide gels.

Preparation of USF2 antibodies. Polyclonal antibodies to rUSF2 were generated by immunizing guinea pigs with GST-rUSF230–228, using methods described previously (18). The IgGs against GST were removed by passing the antiseraum twice through a GST-agarose column. The antibodies produced recognized a protein band of 44 kDa, but they did not recognize GST or rUSF1 by Western blotting analysis. The immunoreactive band appears in some blots as a doublet, which may represent alternatively spliced forms or some type of posttranslational modification (data not shown).

EMSA. Nuclear proteins were prepared (16) from type II cells and fibroblasts isolated from cultured fetal rabbit lung explants (3). Binding reactions and gel electrophoresis were performed as described previously (13). Double-stranded oligonucleotides corresponding to the wild-type DBE and PBE and mutant forms of DBE (5'–GATCTCTTACGTTGGGTG-CAGGG-3') and PBE (5'–GAGGCTTTGCTGGACAGGG-3'), in which the 5' C in the E-box core sequence was mutated to T [underlined in core sequence (bold)], and the canonical CRE (24) were end-labeled using polynucleotide kinase and [γ-32P]ATP. The nuclear extracts (10 μg) or bacterially expressed GST (240 ng) or GST-rUSF2 fusion proteins (75 ng) were incubated at room temperature for 20 min in binding buffer (20 mM HEPES, pH 7.6, 150 mM KCl, 0.2 mM EDTA, 20% glycerol) with radiolabeled DNA probe and poly(dI-dC)-poly(dI-dC) (Pharmacia) as nonspecific competitor, followed by addition of guinea pig preimmune serum or the antibodies against rUSF2 followed by another 20-min incubation. The DNA-protein complexes were resolved on a 5% native polyacrylamide gel and visualized by autoradiography.

Dimerization of rUSF2 in a yeast two-hybrid system. To study the interaction of rUSF2 in vivo, the Gal4 DBD-USF2bHLH-LZ fusion plasmid, pDBD-U2, Gal4 activation domain-USF2bHLH-LZ fusion plasmid, pAD-U2, and the Gal4 DBD-USF1bHLH-LZ fusion plasmid, pDBD-U1, Gal4 activation domain-USF1bHLH-LZ fusion plasmid, pAD-U1 (14), as well as control plasmids pGBT9 (containing Gal4 DBD) and pGAD424 (containing Gal4 AD) were transformed individually into yeast HF7c (provided in the Matchmaker Two-Hybrid System kit, Clontech) or were cotransformed in different combinations (29). The transformants were grown in appropriate medium and studied as described previously (14).

Northern blot analysis of type II pneumocyte and lung fibroblast mRNA. Total RNA was extracted from cells by homogenization in guanidinium isothiocyanate (4.0 M) using a Teflon-glass homogenizer. The cell extracts were centrifuged through a cesium chloride gradient (5.7 M), and the pelleted RNA was resuspended in water (11). Total RNA was electrophoresed, transferred to nitrocellulose, and probed using a 32P-labeled cDNA for rUSF2 using methods described in detail previously (8). Relative levels of mRNA were assessed by autoradiography.

RNase protection assay. RNase protection was used for analyzing developmental changes in USF2 mRNA levels because it is highly sensitive. Furthermore, 18S RNA levels were determined simultaneously for correction of loading and
transfer. A 107-bp 32P-labeled antisense RNA probe for rUSF2, corresponding to the region encoding amino acids 30–65 of rUSF2, was generated by in vitro transcription of the corresponding cDNA sequence. A 32P-labeled 18S probe was generated by in vitro transcription of pTRI RNA 18S (Ambion, Austin, TX). The radiolabeled probes were annealed with 2.5 μg of either yeast RNA or total RNA isolated from various gestational age fetal rabbit lung tissues and then were digested with RNase for 30 min. After ethanol precipitation, radiolabeled fragments were resolved on a 6% denaturing polyacrylamide gel and detected by autoradiography.

Transient transfections. A549 lung adenocarcinoma cells (ATCC CCL 185) (22) were plated at a density of 5–9 × 10^6 cells/60-mm dish 1 day before transfection. The cells were cultured overnight in Waymouth MB752/1 medium containing fetal calf serum (10%, vol/vol). The cells were then washed three times with Hanks’ balanced salt solution (Life Technologies) and incubated with 11 μg of each DNA fragment and 44 μg of Dotap (BMB) in Waymouth MB752/1 for 18 h. The medium was then aspirated and replaced with fresh Waymouth MB752/1 at 24-h intervals. Two days later, the culture media and cells were harvested. The concentrations of hGH in the media were analyzed by radioimmunoassay using an Allegro hGH kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). Variations in transfection efficiency were corrected by normalizing for β-galactosidase activity, which was assayed using a Galato-light kit (Tropix, Medford, MA).

RESULTS

Isolation of the rUSF2 cDNA clone. A cDNA isolated upon screening ~5 million recombinant phage clones from a kgt11 fetal rabbit lung cDNA expression library using 32P-labeled PBE as probe, encoded a protein that bound specifically to radiolabeled PBE and DBE but not to nonspecific C2 DNA (5’-TGCAGGGCCCAGAGCCTGGGCCCATC-3’) (13). The nucleic acid sequence of the 1,640-bp cDNA insert, termed pG-U2, was found to be highly similar to that of hUSF2 (34), but it lacked a 5’-untranslated region and 87 bp of sequence coding for the NH2 terminus of the protein (GenBank accession no. AY168774).

USF2 comprises one component of the complex of proteins bound to the DBE and PBE. To obtain expressed and purified rUSF2 protein for analysis of its properties, the rUSF2 cDNA insert was subcloned into the GST fusion vector pGEX-1X (Pharmacia). The GST-rUSF2 fusion proteins expressed in E. coli and purified using glutathione-agarose beads were used to analyze binding activity for the DBE and PBE by EMSA. As shown in Fig. 1, nuclear proteins from lung tissues of 28-day gestational age fetal rabbits, followed by in vitro translated rUSF2, which does not contain GST, was used in EMSA (14). The binding complexes of the DBE and PBE with fetal rabbit lung nuclear proteins were supershifted by addition of anti-rUSF2 antibodies (lane 12), indicating that the nuclear proteins that bound to the DBE and PBE comprise USF2 homodimers and/or USF2 heterodimers. The anti-rUSF2 antibodies also supershifted the complexes of expressed GST-rUSF2 bound to the DBE and PBE (lane 11).

Whereas the PBE core sequence (5’-CTCGTG-3’) differs by one nucleotide from that of the DBE (5’-CAGTG-3’), which contains a sequence known to bind

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Wild-type</th>
<th>Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GST-rUSF2</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 1. Upstream stimulatory factor-2 (USF2) comprises one component of the complex of rabbit lung nuclear proteins bound to the distal binding element (DBE) and proximal binding element (PBE). Radiolabeled (*) DBE (top) or PBE (bottom) was incubated for 20 min with bacterially expressed glutathione S-transferase (GST), GST-rabbit USF2 (rUSF2) fusion protein, or nuclear proteins isolated from lung tissues of 28-day gestational age fetal rabbits, followed by incubation with anti-rUSF2 antibodies or with preimmune guinea pig serum (pre-im), as control, for 20 min before electrophoresis. F, free probe; C, DNA-protein complexes; S, supershift of DNA-protein binding complexes with anti-rUSF2 antibodies.

play a more important role in mobility in native polyacrylamide gels than does molecular mass. Similar findings were obtained when GST-rUSF1 fusion proteins were used in EMSA (14). The binding complexes of the DBE and PBE with fetal rabbit lung nuclear proteins were supershifted by addition of anti-rUSF2 antibodies (lane 12), indicating that the nuclear proteins that bound to the DBE and PBE comprise USF2 homodimers and/or USF2 heterodimers. The anti-rUSF2 antibodies also supershifted the complexes of expressed GST-rUSF2 bound to the DBE and PBE (lane 11).
to hUSF2 (15), the PBE has binding activity similar to the DBE for expressed rUSF2 and for rabbit lung nuclear proteins. This may be explained by the finding that the nucleotide residues C and G are the most important nucleotides for binding (15). Accordingly, neither bacterially expressed GST-rUSF2 nor lung nuclear proteins bound to radiolabeled oligonucleotides containing mutant forms of DBE (5′-GATCTCCTAGCTGGGTGCAGGG-3′) and PBE (5′-GAGGCCTTCGTGACAGGG-3′) in which the 5′ C in the E-box core sequence, a nucleotide known to be critical for binding of USF1 (14), were mutated to T [underlined in core sequence (bold)] (lanes 14 and 15).

USF2 DNA-binding activity is enriched in type II pneumocytes compared with lung fibroblasts. The SP-A gene is expressed predominantly in type II pneumocytes and to a lesser extent in Clara cells of rabbit lung tissue (4, 37). Binding activity of rabbit fetal lung nuclear proteins (13) and of rUSF1 (14) for the DBE and PBE is enriched in type II cells compared with whole lung tissue and with lung fibroblasts isolated from the same collagenase-digested cell suspension of cultured fetal rabbit lung tissue. To evaluate the possible role of rUSF2 in the type II cell-specific regulation of SP-A gene expression, EMSA was used to assess the binding activity of rUSF2 in nuclear proteins from an enriched population of the type II cells compared with nuclear proteins from an enriched population of fibroblasts isolated from the same collagenase-digested cell suspension of cultured fetal rabbit lung tissue. As can be seen in Fig. 2A, binding activity for radiolabeled DBE and PBE was greatly enriched in type II cells (lane TII); essentially no binding activity was detected in lung fibroblasts (lane Fb). The binding complexes of the DBE and PBE with fetal rabbit lung type II cell nuclear proteins were supershifted by addition of anti-rUSF2 antibodies, indicating that one of the type II cell nuclear proteins that bound to the DBE and PBE was rUSF2. To evaluate the integrity of the fibroblast and type II cell nuclear extracts, we compared their abilities to bind a radiolabeled double-stranded oligonucleotide containing the CRE sequence, TCTAGCTGGGTGCAGGG, derived from rat somatostatin gene, which binds to the ubiquitous transcription factor CRE binding protein (27). Binding activities of nuclear proteins from fibroblast and type II cells for the CRE were similar (data not shown), indicating that these two preparations contained equivalent amounts of protein binding activity. The absence of rUSF2 expression in lung fibroblasts was further supported by Northern blot analysis, in which we obtained a clear signal for rUSF2 mRNA in rabbit lung type II cells but were unable to detect rUSF2 mRNA transcripts in lung fibroblasts isolated in the same cell preparation (Fig. 2B).

Previously, we reported that the PBE is required for cAMP induction of SP-A promoter activity (13). In preliminary studies, our laboratory observed that cAMP treatment of type II cells has little or no effect on PBE DNA-binding activity in nuclear extracts obtained from the cells (K. N. Islam and C. R. Mendelson, unpublished observations).

rUSF2 preferentially forms heterodimers with rUSF1 in vivo. It has been reported that hUSF2 binds to an E-box motif as either a homodimer or a heterodimer with USF1 (15, 34, 36). By studying the interaction of 35S-labeled rUSF2 and rUSF1, as well as bacterially expressed GST-rUSF2 and GST-rUSF1 fusion proteins in the absence or presence of the DBE and PBE in vitro, we found that rabbit USF2 also can form homodimers and heterodimers with rUSF1 in solution in the absence of binding to the DBE and PBE and that binding of rUSF2 to these
E-boxes does not influence dimerization of rUSF2 (data not shown). A yeast two-hybrid system (5) was used to determine whether homodimerization of rUSF2 and heterodimerization of rUSF2 with rUSF1 occurs in vivo. As shown in Fig. 3, the bHLH-LZ region of rUSF2 homodimerized and, in turn, enhanced expression of the His3 gene in yeast HF7c (pDBD-U2/pAD-U2). The apparent efficiency of rUSF2 bHLH-LZ domains to form homodimers was only one third of that of the bHLH-LZ domains of rUSF1. On the other hand, the efficiency of rUSF2 and rUSF1 bHLH-LZ domains to form heterodimers and consequently transactivate expression of the His3 gene in yeast HF7c was fivefold greater than that of the bHLH-LZ regions of rUSF2 to form homodimers and nearly twice as efficient as rUSF1 homodimerization. These findings suggest that heterodimerization of rUSF2 and rUSF1 occurs with greater efficiency than does homodimerization and are in support of findings of others using alternative methodologies (36).

rUSF2 mRNA levels are developmentally regulated in rabbit lung tissue during development. SP-A gene expression is developmentally regulated in fetal lung tissue (26). In rabbits, SP-A gene transcriptional activity is first detectable on day 24 and reaches maximal levels by day 28 of the 31-day gestation period (7). To analyze developmental changes in the levels of rUSF2 mRNAs, aliquots of total RNA isolated from lung tissues of 21- to 28-day gestational age fetal rabbits and from neonates and adults were analyzed by RNase protection assays. As shown in the autoradiogram (Fig. 4A) and the accompanying graph of the scanned and corrected data (Fig. 4B), rUSF2 mRNA was present at comparable levels in fetal rabbit lung tissue on days 21 and 23 of gestation. rUSF2 mRNA levels increased greater than twofold on day 25 of gestation, reached maximal levels on day 28 and declined markedly after birth. Similar findings were obtained using Northern blot analysis of total RNA isolated from fetal rabbit lung tissue at these different developmental stages. Again, relatively low levels of rUSF2 mRNA transcripts were evident on days 21 and 23 of gestation with a marked induction on day 25 and further increase on day 28 (data not shown). This developmental pattern is similar to that for SP-A gene transcription in rabbit lung tissue (7, 8).

rUSF2 acts cooperatively with rUSF1 to increase SP-A:hGH fusion gene expression in A549 cells. In previous studies, we found that rSP-A:hGH fusion genes containing −991 bp of rSP-A 5′-flanking sequence linked to the hGH structural gene, as reporter (rSP-A−991:hGH), were expressed in primary cultures of type II cells and that their expression was markedly stimulated by cAMP (1, 13). Mutagenesis of the DBE or PBE in the context of the 991 bp of 5′-flanking region resulted in a pronounced reduction of basal and cAMP-induced fusion gene expression (13). To determine the capacity of rUSF2 to regulate expression of the rSP-A gene via the DBE and PBE and to act together with rUSF1, A549 cells were cotransfected with either SP-A−991:hGH, SP-A−976:hGH (which lacks the DBE), or SP-A−991PBE−hGH (containing a mutation in the PBE) alone or in combination with pCMV:USF2 and pCMV:USF1 expression vectors, added alone or in combination. A549 is a lung adenocarcinoma-derived cell line of presumed type II cell origin (22); however, these cells do not produce SP-A mRNA or protein at detectable levels. Although the expressed rUSF2 lacked 29
amino acids from the NH2 terminus, we assumed that this protein would have transcriptional activity comparable to the full-length protein. In studies by Luo and Sawadogo (23), it was found that a truncated form of USF2 lacking 40 amino acids from the NH2 terminus had essentially full transcriptional activity compared with the full-length protein. The transcriptional activation and DNA binding and dimerization domains are located within the COOH terminal two-thirds of the USF2 protein and are contained within our truncated rUSF2 construct. As shown in Fig. 5 (open bars), when A549 cells were cotransfected with the intact rSP-A-991:hGH fusion gene and with pCMV-USF2, only a modest induction of hGH expression was found compared with that observed with cotransfection of the rSP-A-991:hGH fusion gene with “empty” expression vector plasmid pCMV as control. As we observed previously (14), when A549 cells were cotransfected with rSP-A-991:hGH and with pCMV-USF1a, an approximately fourfold induction of hGH expression was found. Interestingly, when A549 cells were cotransfected with rSP-A-991:hGH and with pCMV-USF2 and pCMV-USF1a in combination, a synergistic eightfold induction of rSP-A promoter activity was observed. By contrast, when A549 cells were cotransfected with rSP-A-976:hGH (lacking the DBE; hatched bars), little or no induction of fusion gene expression was found on cotransfection of rUSF2 and rUSF1 expression vectors, alone or in combination. When the cells were transfected with rSP-A-991PBE(−):hGH (containing a mutation in the PBE; solid bars) fusion gene expression was essentially undetectable and was unchanged on cotransfection of pCMV-USF2 or pCMV-USF1a, alone or in combination. These findings suggest that rUSF1/ rUSF2 synergistically increase rSP-A promoter activity by binding to the DBE and PBE. Furthermore, synergistic activation of the rSP-A promoter is dependent on the integrity of both E-boxes.

Fig. 4. rUSF2 mRNA levels are developmentally regulated in rabbit lung tissue. Total RNA isolated from lung tissues of fetal rabbits of 21–28 days (D21–D28) of gestational age and from neonates (N) and adults (A) were analyzed for USF2 mRNA using RNase protection. A 32P-labeled (*) 107-nucleotide rUSF2 cRNA probe and/or a 32P-labeled 18S probe was annealed with 2.5 μg of either yeast RNA (lane 11) or total RNA isolated from the rabbit lung tissues and then was digested with RNase for 30 min (lanes 3–11). After ethanol precipitation, radiolabeled fragments were resolved on 6% denaturing polyacrylamide gel. The gel was dried, and an autoradiogram (A) was generated. The autoradiogram was scanned using computing laser densitometry, and the scanned data were corrected for 18S RNA (B). This experiment is representative of data obtained in 5 different series of RNase protection analyses using RNA isolated from pooled fetal lung tissues from 2 different litters of fetuses at each gestational age.
TRANSCRIPTIONAL REGULATION OF SP-A BY USF2 AND USF1

The 5′-flanking region of the rabbit SP-A gene contains two structurally similar E-box-like sequences, termed DBE and PBE that compete for binding to rabbit lung nuclear proteins (13). Deletion or mutation of the DBE or PBE in rSP-A hGH fusion genes reduced basal and cAMP induction of rSP-A promoter activity in transfected type II cells in primary culture. The DBE contains the E-box core sequence CACGTG, which is known to interact with a subset of gene regulatory proteins containing a bHLH structure. The PBE contains the sequence CTGGTG, which differs by one nucleotide from the DBE (13). Because the PBE and the DBE compete for lung nuclear protein binding (13), we considered it likely that similar or identical proteins bind to these elements.

A fetal rabbit lung cDNA expression library was screened using the radiolabeled PBE as probe; two cDNA inserts were isolated that encode proteins that negatively regulate USF2 expression, whereas USF2 expression decreases in USF2-null mice. This suggests USF1/USF2 heterodimers comprise a principal component of the complex of type II nuclear proteins that bind to the DBE and PBE.

The results of lung cell transfection studies also provide evidence for the functional importance of binding of USF1/USF2 heterodimers to the DBE and PBE in activation of the rSP-A promoter. Cotransfection of A549 cells with pCMV:USF2 only modestly increased SP-A hGH expression over basal levels. Whereas cotransfection of pCMVUSF1a caused an approximately fourfold induction of reporter gene expression, cotransfection of both USF1a- and USF2-containing expression vectors caused an approximately eightfold increase in rSP-A promoter activity. These findings correlate with those obtained using the yeast two-hybrid system, in which the efficiency of USF1 and USF2 heterodimerization was approximately twice that for USF1 homodimerization and approximately fourfold greater than that for USF2 homodimerization. It should also be noted that the transactivation domains in USF1 have been reported to be relatively weak (12); therefore, heterodimerization of USF1 and USF2 may be critical in the regulation of SP-A gene expression. Our findings are in accord with those of previous studies suggesting that USF1/USF2 heterodimers comprise the predominant DNA-binding form in a variety of cells and tissues, and that USF1 and USF2 homodimers constitute only a minor component of the USF binding activity (33, 36).

A critical functional role of USF1/USF2 heterodimers is also suggested from studies of mice carrying targeted deletions in the Usf1 and Usf2 genes (33). Whereas, Usf1−/− mice were viable and fertile, Usf2−/− mice manifested a pronounced growth defect. Interestingly, USF2 expression was increased in USF1-null mice, whereas USF1 expression was markedly decreased in USF2-null mice. This suggests USF1 negatively regulates USF2 expression, whereas USF2 positively regulates USF1 expression and that the apparently normal growth in the Usf1−/− mice may be due to a compensatory upregulation of USF2 (33).

In contrast to the single knockouts, an embryonic lethal phenotype was observed in mice that were homozygous for the Usf2 mutation and either homozygous or heterozygous for the mutation in Usf1 (33). There was no

**Fig. 5.** rUSF2 and rUSF1 act synergistically to increase surfactant protein A (SP-A):human growth hormone (hGH) fusion expression in transfected A549 cells. SP-A−976:hGH, SP-A−991:hGH (DBE deleted), and SP-A−991PBE−:hGH (PBE mutated) fusion genes were individually cotransfected into A549 cells with either pCMV, pCMV-USF2, or pCMV-USF2 plus pCMV-USF1a. Shown are the levels of immunoreactive hGH that accumulated in the culture medium over a 24-h period, 48 h after transfection. When A549 cells were cotransfected with the SP-A−991PBE−:hGH fusion gene in the absence or presence of USF expression vectors, hGH expression was below detectable levels. Values are means ± SE of 3 determinations from a representative of 3 experiments.

**DISCUSSION**

The 5′-flanking region of the rabbit SP-A gene contains two structurally similar E-box-like sequences, termed DBE and PBE that compete for binding to rabbit lung nuclear proteins (13). Deletion or mutation of the DBE or PBE in rSP-A hGH fusion genes reduced basal and cAMP induction of rSP-A promoter activity in transfected type II cells in primary culture. The DBE contains the E-box core sequence CACGTG, which is known to interact with a subset of gene regulatory proteins containing a bHLH structure. The PBE contains the sequence CTGGTG, which differs by one nucleotide from the DBE (13). Because the PBE and the DBE compete for lung nuclear protein binding (13), we considered it likely that similar or identical proteins bind to these elements.

A fetal rabbit lung cDNA expression library was screened using the radiolabeled PBE as probe; two cDNA inserts were isolated that encode proteins that specifically bind to the DBE and PBE elements. We previously reported (14) that one of these cDNAs encodes the rabbit homolog of hUSF1 (17, 30, 31). In the present study, we found that the second cDNA encodes the rabbit homolog of USF2/Pos-interacting protein (6, 34, 36). USF1 (43 kDa) and USF2 (44 kDa) are heat-stable, ubiquitously expressed proteins that bind as a dimer to the core E-box motif CACGTG (30, 31). Previously, we observed binding to the DBE and PBE of heat-stable type II cell nuclear proteins of molecular mass of 69,000, 45,000 and 22,000; the 45-kDa protein(s) appeared to be the predominant species and to bind as a dimer (13). In EMSA of rabbit type II cell nuclear protein binding to the PBE and DBE, we found that antibodies raised against rUSF1 supershifted a major portion of the binding complexes (14). In the present study, we observed a similar phenomenon using antibodies that are specific for rUSF2. These findings, together with those from yeast two-hybrid studies, indicating preferential interaction of USF1/USF2 heterodimers compared with homodimers, suggest that USF1/USF2 heterodimers comprise a principal component of the complex of type II nuclear proteins that bind to the DBE and PBE.

In the present study, we found that the second cDNA encodes the rabbit homolog of USF2/Pos-interacting protein (6, 34, 36). USF1 (43 kDa) and USF2 (44 kDa) are heat-stable, ubiquitously expressed proteins that bind as a dimer to the core E-box motif CACGTG (30, 31). Previously, we observed binding to the DBE and PBE of heat-stable type II cell nuclear proteins of molecular mass of 69,000, 45,000 and 22,000; the 45-kDa protein(s) appeared to be the predominant species and to bind as a dimer (13). In EMSA of rabbit type II cell nuclear protein binding to the PBE and DBE, we found that antibodies raised against rUSF1 supershifted a major portion of the binding complexes (14). In the present study, we observed a similar phenomenon using antibodies that are specific for rUSF2. These findings, together with those from yeast two-hybrid studies, indicating preferential interaction of USF1/USF2 heterodimers compared with homodimers, suggest that USF1/USF2 heterodimers comprise a principal component of the complex of type II nuclear proteins that bind to the DBE and PBE.

The results of lung cell transfection studies also provide evidence for the functional importance of binding of USF1/USF2 heterodimers to the DBE and PBE in activation of the rSP-A promoter. Cotransfection of A549 cells with pCMV:USF2 only modestly increased SP-A hGH expression over basal levels. Whereas cotransfection of pCMVUSF1a caused an approximately fourfold induction of reporter gene expression, cotransfection of both USF1a- and USF2-containing expression vectors caused an approximately eightfold increase in rSP-A promoter activity. These findings correlate with those obtained using the yeast two-hybrid system, in which the efficiency of USF1 and USF2 heterodimerization was approximately twice that for USF1 homodimerization and approximately fourfold greater than that for USF2 homodimerization. It should also be noted that the transactivation domains in USF1 have been reported to be relatively weak (12); therefore, heterodimerization of USF1 and USF2 may be critical in the regulation of SP-A gene expression. Our findings are in accord with those of previous studies suggesting that USF1/USF2 heterodimers comprise the predominant DNA-binding form in a variety of cells and tissues, and that USF1 and USF2 homodimers constitute only a minor component of the USF binding activity (33, 36).

A critical functional role of USF1/USF2 heterodimers is also suggested from studies of mice carrying targeted deletions in the Usf1 and Usf2 genes (33). Whereas, Usf1−/− mice were viable and fertile, Usf2−/− mice manifested a pronounced growth defect. Interestingly, USF2 expression was increased in USF1-null mice, whereas USF1 expression was markedly decreased in USF2-null mice. This suggests USF1 negatively regulates USF2 expression, whereas USF2 positively regulates USF1 expression and that the apparently normal growth in the Usf1−/− mice may be due to a compensatory upregulation of USF2 (33). In contrast to the single knockouts, an embryonic lethal phenotype was observed in mice that were homozygous for the Usf2 mutation and either homozygous or heterozygous for the mutation in Usf1 (33). There was no
indication in that report as to the stage of development that embryonic demise occurred.

SP-A gene transcription is developmentally regulated in fetal rabbit lung and is first detectable in lung nuclei on day 24 of gestation (term = day 31), reaching maximal levels by day 28 (7). By use of quantitative RT-PCR, we previously observed that rUSF1 mRNA is readily detectable on day 21 of gestation, reaches peak levels on day 23, just before the time of activation of rSP-A gene transcription, and subsequently declines to relatively low levels on day 28 (14). By contrast, in the present study, we found that rUSF2 mRNA levels are relatively low in fetal rabbit lung on day 21 of gestation, increase approximately twofold on day 24 and reach maximal levels on day 28, in concert with the time of maximal induction of rSP-A gene expression. These findings suggest that the temporal induction of SP-A gene transcription in fetal rabbit lung may be regulated, in part, by the relatively high levels of USF1 and USF2 on day 24 of gestation, facilitating their heterodimerization and binding to enhancers in the 5′-flanking region of the rSP-A gene. Because the findings in gene-targeted mice suggest that USF1 negatively regulates Usf2 gene expression (33), it is possible that the developmental increase in rUSF2 expression in rabbit fetal lung occurs only after the levels of rUSF1 begin to decline.

In studies using transgenic mice carrying rSP-A: hGH fusion genes containing various amounts of 5′-flanking DNA from the rabbit SP-A gene, our laboratory has found that fusion genes containing as little as ~378 bp of rSP-A 5′-flanking DNA are expressed in a lung-specific manner, specifically in type II cells and in bronchoalveolar epithelial cells (2). The rSP-A_{-378}:hGH transgenes are also developmentally regulated in concert with the endogenous mouse SP-A gene (2). These findings indicate that response elements within the 378 bp 5′-flanking region are most critical for tissue/cell-specific and developmental regulation of rSP-A expression and suggest that the PBE may play a more crucial role than the DBE in this regulation. Previously, we observed that a fusion gene containing both the DBE and PBE (rSP-A_{-99}:hGH) was induced ~25-fold by cAMP treatment of transfected type II cells. Whereas mutation of the PBE within this construct markedly reduced basal and essentially eliminated cAMP induction of rSP-A promoter activity, removal of the DBE reduced both basal and cAMP-induced expression to levels similar to that observed using an rSP-A_{-378}:hGH fusion gene so that a 15-fold stimulation by cAMP of rSP-A promoter activity was still observed (13). We therefore suggest that the DBE serves as an enhancer required for high levels of basal and cAMP stimulated SP-A expression in cultured type II cells, whereas the PBE, together with other response elements within the ~378-bp 5′-flanking sequence, plays a more essential role. It should be noted that we have identified another E-box sequence (median binding element; MBE) (CACGTG) at ~340 bp within the rSP-A 5′-flanking region that we have found to also be important for cAMP regulation of rSP-A promoter activity in transfected type II cells (E. Gao and C. R. Mendelson, unpublished observations). It is possible that within chromatin, USF1/USF2 proteins bound to the PBE act cooperatively with those bound to the MBE and to other critical response elements to mediate appropriate temporal and spatial regulation of rSP-A gene expression. Although USF1 and USF2 are ubiquitously expressed proteins, their patterns of developmental regulation and the enhanced levels of USF1/USF2 DNA-binding activity in type II cells, compared with lung fibroblasts, may serve an important role in developmental and cell-specific expression of the SP-A gene in fetal lung.

USF2 and USF1 contain two protein interacting domains, HLH and LZ. It should be noted that USF2 was initially isolated by its interaction with the LZ domain of c-Fos, and was found to cooperatively interact with c-Fos in activation of an activator protein-1-responsive promoter (6). As mentioned previously, we have observed that cAMP stimulation of SP-A promoter activity in transfected type II cells is critically dependent on the integrity of a number of response elements, including a putative nuclear receptor binding site (CRESP-A) (28, 38), a TTF-1-binding element (21), the PBE (13), and a GT-box that binds Sp1; mutagenesis of any one of these sites severely reduces basal and cAMP stimulation of SP-A promoter activity (39). Cooperative interaction of USF2/USF1 with other transcription factors bound to the proximal SP-A promoter is, therefore, likely to play a critical role in developmental, hormonal, and tissue-specific regulation of SP-A gene expression.

The authors gratefully acknowledge the expert assistance of Margaret Smith in the isolation and culture of lung type II cells and fibroblasts.

This research was supported by National Heart, Lung, and Blood Institute Grant R37 HL-50022 (to C. R. Mendelson) and by Grant-in-Aid 94R-084 from the American Heart Association, Texas Affiliate (to E. Gao).

Present address for J. L. Alcorn: Dept. of Pediatrics, Univ. of Texas Medical School at Houston, 6431 Fannin, Ste. 3-222, Houston, TX 77030.

REFERENCES