Surfactant Protein D Gene Regulation

INTERACTIONS AMONG THE CONSERVED CCAAT/ENHANCER-BINDING PROTEIN ELEMENTS*

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Surfactant protein D (SP-D) plays roles in pulmonary host defense and surfactant homeostasis and is increased following acute lung injury. Given the importance of CCAAT/enhancer-binding protein (C/EBP)-binding elements in the systemic acute-phase response and lung development and the expression of C/EBP isoforms by lung epithelial cells, we hypothesized that conserved C/EBP motifs in the near-distal and proximal promoters contribute to the regulation of SP-D expression by C/EBPs. Five SP-D motifs (−432, −340, −319, −140, and −90) homologous to the C/EBP consensus sequence specifically bound to C/EBPs in gel shift assays, and four of the five sites (−432, −340, −319, and −90) efficiently competed for the binding of C/EBPα, C/EBPβ, or C/EBPδ to consensus oligomers. Cotransfection of C/EBPα, C/EBPβ, or C/EBPδ cDNA in H441 lung adenoacarcinoma cells significantly increased the luciferase activity of a wild-type SP-D promoter construct containing 698 bp of upstream sequence (SS698). Transfection of C/EBP also increased the level of endogenous SP-D mRNA in H441 cells. Transactivation of the reporter construct was abrogated by deletion of sequences upstream of −205. Independent site-directed mutagenesis of the sites at −432, −340, and −319 reduced C/EBP-mediated activation by −50%, and mutagenesis of the site at −432 in combination with either of the tandem sites at −340 and −319 blocked activation. The conserved AP-1 element at −109 was required for maximal promoter activity, but not for the transactivation of SS698 by C/EBPs. Thus, interactions among C/EBP elements in the near-distal promoter can modulate the promoter activity of SP-D.

There is increasing evidence that surfactant protein D (SP-D)‡ plays important roles in the lung’s defense against inhaled microorganisms and organic particles and in the regulation of inflammatory and immune reactions within the lung (1, 2). SP-D, like pulmonary surfactant protein A and the serum mannose-binding protein (MBL), is a member of the collagenous lectin (collectin) subfamily of mammalian C-type lectins. SP-D is secreted into the distal airways and alveoli by non-ciliated bronchiolar epithelial cells and type II pneumocytes, respectively. Although the lung appears to be a major site of SP-D expression, there is evidence that SP-D is also synthesized by epithelial cells in a variety of extrapulmonary sites, consistent with more generalized roles in innate host defense (3). Unlike other surfactant proteins, the regulation of SP-D promoter activity is dependent on the combinatorial interactions of relatively ubiquitous transcription factors, including members of the AP-1 family (4).

The expression of SP-D is increased following many forms of pulmonary injury (5). For example, the levels of SP-D mRNA and immunoreactive protein in lung lavage increase within several hours to a few days following intratracheal instillation of bacterial endotoxin in rats (6), following challenge of mice with Pseudomonas aeruginosa (7), and in rats exposed to hyperoxia (8). In addition, transgenic mice deficient in surfactant protein A or SP-D show abnormal microbial clearance or acute inflammatory responses to microbial challenge (9). Based on these and other data, it has been suggested that the lung collects contribute to a pulmonary acute-phase response, somewhat analogous to the hepatic acute-phase response (APR) to systemic injury (6). In this regard, a variety of hepatic acute-phase proteins are expressed in the lung, and at least some are elevated following lung injury (10–14). These include C-reactive protein and haptoglobin as well as the serum lipopolysaccharide-binding protein.

Molecular regulation of the systemic APR, including the response to endotoxin, is complex and involves a variety of relatively ubiquitous transcription factors. However, members of the CCAAT/enhancer-binding protein (C/EBP) family of leucine zipper transcription factors figure prominently in the regulation of many APR genes, particularly members of the so-called “Class I” group of APR proteins (15–18). Like other leucine zipper transcription factors, C/EBPs bind to DNA as homo- or heterodimers and have a diversity of effects that in part reflect tissue and developmental stage-specific expression of various C/EBPs (19). The expression and activity of different C/EBP isoforms are differentially modulated in response to inflammatory stimuli, including pro-inflammatory cytokines and glucocorticoids. In addition, the activity of these proteins can be influenced by a variety of post-transcriptional mechanisms, including “leaky translation” with the production of truncated forms and protein phosphorylation.

C/EBPα, C/EBPβ, and C/EBPδ are expressed by alveolar type II and non-ciliated bronchiolar epithelial cells, the known pulmonary sites of SP-D production (20–23). C/EBPα and C/EBPδ are particularly abundant in the lung and increase in fetal rat lung in late gestation during a time when the produc-

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‡ The abbreviations used are: SP-D, surfactant protein D; AP-1, acute-phase response; C/EBP, CCAAT/enhancer-binding protein; STAT3, signal transducer and activator of transcription-3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Rb, retinoblastoma protein; HNF-3, hepatocyte nuclear factor-3.
tion of surfactant-associated proteins, including SP-D, is increased. Mice deficient in C/EBPβ show abnormalities in alveolar development and often die secondary to the respiratory abnormalities (24). Although mice deficient in C/EBPβ and C/EBPδ also expire perinatally, they have no obvious pulmonary phenotype (15). Neonatal C/EBP-null mice show no hepatic APR and fail to induce STAT3 binding in response to systemically administered endotoxin, despite marked increases in C/EBPβ or C/EBPδ (25). However, the pulmonary APR to systemically administered endotoxin is not impaired in this model, and the levels of C/EBPβ and C/EBPδ are increased in the lung following systemic endotoxin administration (25, 26).

Inspection of the upstream sequence of the SP-D gene revealed five sites consistent with the consensus sequence for C/EBP binding. We have previously utilized H441 human lung adenocarcinoma cells as a model system for studying SP-D promoter activity (27). To characterize the regulatory role(s) of the putative C/EBP elements, we examined the interactions of oligomers containing these sequences with H441 nuclear proteins from cells cotransfected with cDNAs encoding the three major C/EBP isoforms. We also compared the activity of wild-type constructs and constructs containing mutated consensus sequences in transient transfection assays using luciferase reporter constructs and examined potential functional interactions with the conserved AP-1 element in the proximal promoter.

**EXPERIMENTAL PROCEDURES**

*Genomic Clones and Reporter Constructs—* An 7-kb EcoRI fragment of the previously described human genomic clone (H5), designated H567, containing human SP-D 5′-sequence was isolated and subcloned into pGEM-3Z (Promega) as previously described (4, 27). All experiments used restriction fragments containing 5′-regulatory sequences, each terminated at a SacI site within the untranslated first exon and were numbered from the transcription start site (27). Most studies employed a SpeI/SacI fragment (SS698) of the human SP-D gene containing 698 bp upstream of the transcription start site (see Fig. 1). For some experiments, we also used a SacI/XhoI fragment containing 205 bp upstream of the start site (SS205) and a longer HindIII/SacI fragment containing 131 bp of upstream sequence (HS747A). The restriction fragments were subcloned into a luciferase reporter plasmid (pGL3-Basic, Promega) between the KpnI and SacI sites.

*Cells—* NCI H441 human lung adenocarcinoma cells were propagated outside the multiple cloning site by digestion with SacI and reverse-directed oligomers were synthesized, each containing a mutated consensus sequence. Plasmid SS698 (SS698) was linearized outside the multiple cloning site by digestion with SacI and used as template for thermal cycling reactions. Approximately 200 ng of template DNA, 200 ng of forward or reverse mutagenesis oligomer, and 200 ng of an oligomer directed to the appropriate SP6 or T7 RNA polymerase site in pGEM were combined with 200 μM dNTPs (Roche Molecular Biochemicals) and 1 unit of T7 polymerase (Fisher) in buffer supplied with the enzyme. Twenty to twenty-five cycles were performed, each consisting of 1 min at 95 °C (denaturing), 1 min at 45 °C (annealing), and 2 min at 70 °C (extension). Resultant DNA fragments were gel-purified using the QIAQuick DNA extraction kit (Qiagen). The 5′- and 3′-fragments of the mutated promoter DNA were joined together by extension thermal cycling using an overlapping internal oligomer sequence and oligomers to the flanking SP6 and T7 sites for amplification. The mutated fragments were subcloned into a luciferase reporter plasmid as described for the wild-type fragments. The orientation and sequence were verified by restriction mapping and DNA sequencing.

*Nuclear Extracts and Electrophoretic Mobility Shift Assays—* Nuclear extracts were prepared from cultured cell lines using a rapid mini-extraction technique (29) as previously described (4). The protein content was analyzed by dye binding assay, and the extracts were frozen in liquid nitrogen and stored at −70 °C. Electrophoretic mobility shift (gel retardation) and supershift assays were performed by a modification of a method employed by Bingle and co-workers (30) as previously described (4). Antibodies to C/EBPα (14AA), C/EBPβ (C-19), and C/EBPδ (M-17) were from Santa Cruz Biotechnology. Specificity of each antibody was confirmed in supershift assays using nuclear extracts from cells transfected with C/EBPα, C/EBPβ, or C/EBPδ cDNA as described below.

*Transient Transfection—* For experiments characterizing the promoter activity of mutant constructs, H441 target cells (5 × 10⁵) were transfected to 35-mm plates in RPMI 1640 medium (In VitroChem) supplemented with 10% fetal calf serum (In VitroChem), allowed to attach overnight, and washed twice with RPMI 1640 medium devoid of phenol red (4). The cells were transfected with up to 1.5 μg of the luciferase reporter construct using Lipofectin (In VitroChem) and incubated for 5 h at 37 °C in the absence of serum. The medium was replaced with fresh growth medium, and the cells were incubated overnight. Cells were harvested at 48 h, with one media change at 24 h.

*Transfection with Nuclear Factor Expression Vectors—* C/EBPα cDNA was obtained by thermal cycling gene amplification of a lung cDNA library (CLONTECH) using a pair of primers: one located at the 5′-end and the other at the 3′-end according to the known human C/EBPα (NF-IL6) sequence. The full-length DNA sequence was confirmed by automated sequencing. The rat C/EBPα cDNA was a gift from Dr. Steven McKnight (University of Texas Southwestern Medical Center, Dallas, TX), and the rat C/EBPβ cDNA was a gift from Dr. Peter Rotwein (Oregon Health Sciences University, Portland, OR). All cDNAs were subcloned into the pcDNA3 vector (Invitrogen) at the HindIII and SacI sites. This vector contains the cytomegalovirus immediate-early promoter, a polylinker, and the bovine growth hormone polyadenylation sequence. The plasmid configuration was required for maximal transactivation of the wild-type reporter construct was designed in preliminary dose-response experiments. Although activation by C/EBPα was dose-dependent up to 1.5 μg, activation by C/EBPβ decreased at concentrations above 1.5 μg, whereas activation by C/EBPδ reached a plateau at 0.25 μg. Accordingly, most experiments were performed using 1.5 μg of pcDNA3 containing the desired cDNA or an equivalent weight of the pcDNA3 vector. Protein expression was confirmed by supershift assays.

*Luciferase and Chromophenicol Acetyltransferase Assays—* Cell layers were harvested, and transient transfection assays were performed using protein-equivalent amounts of cell extract containing the luciferase reporter constructs. Luciferase activity was measured using a Turner Designs Model TD2020 luminometer. Transfection efficiency was internally controlled using the pRL-tk vector (Dual-Luciferase kit, Promega). All assays were performed on duplicate or triplicate plates. Except where indicated, at least three separate experiments were performed. In some initial experiments, chromophenicol acetyltransferase assays were performed as previously described (4).

*Thermal Cycling Assays of Endogenous SP-D mRNA—* SP-D mRNA was amplified from total RNA isolated from H441 cells. Primers for the full-length product contained untranslated sequence and a series of contiguous coding sequence: 5′-primer (+), CCT GCC ATG CTG CTC TTC CTC CTG GC; and 3′-primer (−), CCA GTT GCG GCC CCC GAC GAG GC. The amplified DNA was digested for 30 min at 50 °C and then denatured for 2 min at 94 °C, followed by 10 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 68 °C. This was followed by 40 more cycles using the same denaturation and annealing conditions, but with the addition of 5 s cycle of extension at 68 °C. The identity of the amplified fragment was initially confirmed by nested PCR using a different 5′-primer (+), AAT CCT GGA GAC AAA GGA GCA AAG GGA and a 3′-primer (−) (27). AACT CGG AAC AAA GGA GCA AAG GGA GAA. For this reaction, 1 μl of the full-length PCR
product was amplified by conventional PCR. The full-length PCR product was subcloned into the pGEM-T vector (Promega), and the DNA was sequenced from both ends using T7 and SP6 primers.

Increases in endogenous mRNA were also estimated by “comparative” PCR using a commercial protocol and reagents (Comparative PCR, Ambion Inc.). This technique is a variant of competitive PCR that competes the cDNAs derived from two preparations of RNA. Each cDNA was tagged with unique reverse transcriptase primers of different length. Known amounts of the two tagged cDNAs were mixed in various proportions and then subjected to PCR using an upstream tag-specific primer and a downstream SP-D-specific 3′-sense primer ~300 nucleotides upstream from the stop codon (TGG TTT CCT GAT CAT GAC TGA T). Short and long tag primers for the amplification of control GAPDH message were provided with the kit. Purified RNA from the control and transfected cells gave essentially identical yields of cDNA/µg of RNA as assessed by incorporation of [α-32P]dATP during the reverse transcription.

RESULTS

In most cases, members of the C/EBP family specifically interact with DNA sequences fulfilling the general consensus TT(T/G)NGNAA or TKDNGNAAK (K = G/T; D = A/G/T). Computer-assisted matrix analysis of the upstream sequence of the human SP-D gene using the TRANSFAC Database revealed five consistent with this consensus within the proximal promoter (Figs. 1A and 2A). Two motifs were found in an XbaI/SacI fragment that includes 285 bp upstream of the start site, which we have previously referred to as the “proximal” promoter (4). The first three motifs are located in the region we have designated the “near-distal” promoter.

The potential importance of the four most upstream motifs is suggested by the spatial conservation of homologous sequences in the rat and mouse SP-D promoters (27, 31) and/or in the promoter sequences of bovine conglutinin and CL-43, hepatic host defense collectins believed to have evolved from a primordial SP-D gene (32–34). A complete C/EBP motif nearly identical to the human sequence at −432 is found in the mouse gene (TTGAGAAA, reverse) (Fig. 2B). The sequence for the corresponding region of the rat promoter is not available. Although the sequences at this position in the CL-43 and conglutinin promoters are quite highly conserved, key elements of the C/EBP motif are absent. Sequences in the region spanning −340 to −319 are highly conserved in the mouse and rat promoters, and the downstream sequences deviate from the C/EBP consensus sequence at only the first position (Fig. 2B) (27, 31). In addition, these tandem motifs are conserved in the conglutinin and CL-43 promoters (Fig. 2) (32, 34). The C/EBP motif at −140 in the proximal promoter (TTGTGAAA) is nearly identical to the corresponding mouse (cTcTGGAA) and CL-43 or conglutinin (TTcTGGAc) sequences, but diverges significantly in the rat. Interestingly, this motif overlaps an H-APF-1 motif (CTGGRAA) that is completely conserved in the mouse and rat genes and conserved at all but one position in the bovine lectins. By contrast, the motif at −90 is unique to human SP-D. It is not conserved in the bovine collectins or mouse SP-D and is interrupted by a CA repeat in rat SP-D. Thus, the motifs appear to be most highly conserved in the near-distal promoter.

C/EBP Isoforms Bind to the C/EBP Motifs—Given the low levels of endogenous C/EBP-binding activity in our H441 nuclear extracts (see “Experimental Procedures”), we were able to utilize H441 cells that were transfected with cDNA for C/EBPa, C/EBPβ, or C/EBPδ as a model system for characterizing the interactions of C/EBPs with the motifs. We observed specific binding of the C/EBPs to oligomers containing the motifs at all five sites in electrophoretic mobility shift assays (Fig. 3–5). In each case, binding was blocked by competition with the unlabeled oligomer, but not with a mutant oligomer (Table I). In supershift experiments, we also demonstrated binding of each of the three isoforms to each of the five C/EBP motifs.

As shown Fig. 3, C/EBPa, C/EBPβ, and C/EBPδ bound specifically to Oligo 432. Binding to labeled Oligo 432 was competed by unlabeled Oligo 432 (lanes 3, 7, and 11) or by a consensus oligomer (lanes 13), but not by Oligo 432m (lanes 8 and 12), which contains a mutated consensus sequence (Table I). The complexes were specifically supershifted by isoform-specific antibodies (lanes 4, 9, and 14), but not by control (normal) immunoglobulin (lanes 15, nl IgG).

The sites at −340 and −319 also showed specific binding to all three C/EBP isoforms. For example, C/EBPδ showed specific binding to Oligo 340 (Fig. 4A, lane 2) that was competed by the

FIG. 1. Upstream regulatory region of the SP-D gene. The diagram shows the proximal and near-distal regions of the human SP-D promoter. The positions of the five C/EBP motifs, regions encoded by the SS698 and SS205 plasmids, and the conserved AP-1 element at −109 are indicated. The three upstream sites within the near-distal region were the primary focus of this study. For some studies, a larger cDNA (HS1674) encoding 1674 bp of upstream sequence was also used.

FIG. 2. C/EBP motifs. A, the five C/EBP motifs are aligned and compared with the consensus (cons) sequence. B, the tandem C/EBP motifs at −340 and −319 and flanking sequences of the human SP-D (hSP-D) gene (~340 to ~308 relative to the transcription start site) are aligned with the corresponding regions of the bovine conglutinin and CL-43 (bCG/CL43) genes and the mouse (mSP-D) and rat (rSP-D) SP-D genes. The locations of cis-acting elements in the human gene identified in this study are indicated above the alignments. The positions of the conserved sequences within the mouse and rat SP-D promoters are shifted 5′ relative to the human SP-D and CL-43 sequences because of the presence of inserted repetitive elements between these sites and the TATA site. Alignments with the motif at −432 are also shown.
Fig. 3. C/EBPs bind to the motif at –432. H441 cells were transfected with C/EBPα, C/EBPβ, or C/EBPδ cDNA as indicated. Binding of nuclear proteins to a radiolabeled oligomer containing the SP-D C/EBP sequence at –432 (Oligo 432; Table I) was assessed in electrophoretic mobility shift assays as described under “Experimental Procedures.” Binding was competed by the unlabeled oligomer (Oligo 432; lanes 3, 7, and 11) and by the consensus oligomer (lane 13), but not by the corresponding mutant oligomer (Oligo 432m; lanes 8 and 12). The complexes were supershifted by specific antibodies to C/EBPα, C/EBPδ, and C/EBPβ (lanes 4, 9, and 14, respectively). A representative negative control using normal IgG (nl IgG) is shown in lane 15.

unlabeled oligomer (lane 3), but not by the corresponding mutant oligomer (lane 4) (Table I). The complex was specifically supershifted by antibody to C/EBPδ (lane 5). The complex was also competed by the NF-IL6 oligomer, but not by the mutated NF-IL6 sequence (Table I; data not shown). Likewise, C/EBPβ showed specific binding to Oligo 319 (Fig. 4B, lane 2) that was competed by the unlabeled oligomer (lane 3) or by the consensus oligomer (lane 5), but not by the mutant oligomer (lane 4) (Table I). In addition, the complex was specifically supershifted by antibody to C/EBPδ (lane 6), but not by control IgG (lane 7). Comparable results were obtained in transfection assays using C/EBPβ or C/EBPα cDNA (data not shown).

Similarly, the sites at –140 and –90 showed specific binding to C/EBPα, C/EBPβ, and C/EBPδ. Representative binding data for Oligo 140 and Oligo 90 (Table I) and C/EBPδ are shown in Fig. 5. Briefly, both oligomers specifically bound to C/EBPδ (Fig. 5, A and B, lanes 1). Complex formation was blocked by the unlabeled oligomers (lanes 2), but not by the corresponding mutant oligomers (lanes 3). The complexes were also supershifted by antibody to C/EBPδ (lanes 4), but not by control IgG (data not shown). Comparable results were obtained in transfection assays using C/EBPβ or C/EBPα cDNA (data not shown). Thus, all five motifs are able to bind to the three C/EBP isoforms.

The relative affinities of the sites for transfected C/EBP were compared in parallel gel shift competition assays. Nuclear extracts from cells transfected with C/EBPα, C/EBPβ, or C/EBPδ were individually incubated with a radiolabeled commercial C/EBP consensus oligomer (Table I) in the presence of increasing concentrations of unlabeled SP-D oligomers encoding the five binding sites. As shown in Fig. 6, oligomers containing the sites at –432, –340, –319, and –90 efficiently inhibited the binding of all three C/EBPs to the consensus oligomer. The site at –432 showed the highest apparent affinity, followed by the sites at –319, –340, and –90. By contrast, the site at –140 was quite ineffective as a competitor. Oligomers encoding the near-distal motifs were particularly effective competitors of C/EBPδ binding.

Co-transfection of C/EBPs Modulates SP-D Promoter Activity—To further examine the potential modulatory roles of specific C/EBPs, we performed cotransfection studies using luciferase reporter constructs and C/EBP expression constructs. For most of our initial experiments, we used an expression plasmid encoding a human lung C/EBPβ (NF-IL6) cDNA, which we had isolated and characterized (see “Experimental Procedures”). As shown in Fig. 7, cotransfection of pGL3-SS698 with C/EBPβ cDNA gave a 4-fold stimulation of promoter activity (n = nine independent experiments). In a smaller number of experiments, cotransfection of pGL3-SS698 with plasmids encoding C/EBPα or C/EBPδ cDNA gave a maximal 5.5-fold (n = 4) and 4.7-fold (n = 3) stimulation of transactivation, respectively. Thus, there was no major difference in the level of activation when the three isomers were compared using optimal amounts of plasmid as determined in dose-response assays. Comparable transactivation was observed when a larger segment of DNA containing over 1.6 kilobase pairs of upstream sequence (HS1674) was used (Fig. 7). By contrast, cotransfection of the cDNA with the truncated construct (SS205), which lacks the three upstream motifs, showed no significant transactivation (Fig. 7). Accordingly, subsequent studies focused on the sequences in the near-distal promoter.

Additional experiments were performed to determine whether transfection of C/EBP cDNA can modulate endogenous SP-D gene expression. We have previously observed weak
signals for human SP-D mRNA by Northern blotting of H441 RNA following stimulation of the cells with glucocorticoids (27). In the present study, we employed a more sensitive thermal cycling assay to assess possible changes in SP-D message in response to increased levels of C/EBP. The full-length H441 message was amplified and cloned, with verification of its identity by DNA sequencing as described under “Experimental Procedures.” The SP-D PCR product was increased following transfection with C/EBP cDNA increased the low residual luciferase activity of the mutant reporter construct by 4-fold stimulation of SS698 (0.27 ± 0.06 (mean ± S.D.), n = four independent experiments). However, basal or C/EBP-stimulated promoter activity was decreased by 75% as compared with wild-type SS698 (0.27 ± 0.06 (mean ± S.D.), n = four independent experiments). As a result of this, the −4-fold stimulation of SS698 activity by C/EBP cDNA was largely abrogated. However, basal or unstimulated activity was decreased, and cotransfected C/EBP cDNA increased the low residual luciferase activity of the mutant reporter construct by −4-fold (4.3 ± 0.99 (mean ± S.D.), n = four independent experiments).

**Mutagenesis of the Upstream C/EBP-binding Sites Decreases C/EBP-stimulated Promoter Activity**—To study the potential functional consequences of C/EBP binding, we employed a transient transfection assay utilizing H441 cells in conjunction with wild-type SP-D and mutant SP-D luciferase reporter constructs. Substitution mutations were identical to those shown for the mutant oligomers (Table I). Representative data using a cotransfected cDNA encoding C/EBPα are shown in Fig. 9. Mutagenesis of the C/EBP elements at −340, −319, and −319 significantly decreased the level of stimulation achieved following cotransfection of C/EBPα, C/EBPβ, or C/EBP8 cDNA. Individual mutations at −340, −319, and −319 decreased activity by −50% as compared with the wild type (Fig. 7) based on at least six independent experiments.

Because mutagenesis of the individual sites gave similar but incomplete inhibition of C/EBP-stimulated promoter activity, we examined the effects of simultaneously mutating pairs of sites in the near-distal promoter (Fig. 9). Simultaneous mutation of the tandem sites at −340 and −319 did not significantly increase the extent of inhibition as compared with the single-site mutations. Activity was decreased by −50% based on four independent experiments. However, mutagenesis of the upstream site at −432 in combination with either one of the tandem sites reduced promoter activity to the level of the plasmid controls (Fig. 9).

**C/EBP Binding to the Region Spanning −340 to −319**—To further characterize the tandem binding sites in the near-distal promoter, we generated an oligomer spanning both sites (Oligo 340/319) (Table I). In this was in part motivated by the high degree of conservation of the sequence between the two binding sites (Fig. 2), which suggested that site-specific mutagenesis of the C/EBP sites might alter the binding of other transcription factors to this region. Gel shift experiments using radiolabeled wild-type Oligo 340/319 and nuclear extracts from cells transfected with C/EBPα showed a single specific band (Fig. 10, lane 10). In preliminary experiments, we observed that this complex was competed by unlabeled probe or oligomers containing the site at −340 or −319 and supershifted by antibody to C/EBPα (data not shown). In addition, no specific complexes were identified in the absence of cotransfected cDNA.

We then compared the wild-type complex with complexes formed using oligomers containing mutated sites at −340 or −319, designated Oligo 340m/319 and Oligo 340/319m, respectively (Fig. 10 and Table I). Both mutant oligomers gave a single major complex that comigrated with the complex formed on the wild-type tandem oligomer (Fig. 10, compare lanes 1 and 7 with lane 10). These complexes, which were comparable in intensity to the wild-type complex, were competed by the unlabeled oligomer (compare lane 1 with lane 2 and lane 7 with lane 8) and supershifted by antibody to C/EBPα (lanes 6 and 9). The complexes formed with the mutant oligomers were also competed by oligomers containing the individual sites or by tandem oligomers with single mutated sites. For example, the complex formed with Oligo 340m/319 (lane 1) was competed by Oligo 340, Oligo 319, and Oligo 340/319m (lanes 3–5). The findings suggest that the two sites are not simultaneously occupied within the context of the tandem oligomer and that other nuclear proteins do not interact with the intervening sequence.

**DISCUSSION**

We have previously shown that the proximal promoter of SP-D can mediate cell type-restricted, basal and glucocorticoid-stimulated promoter activities (4, 27). We further demonstrated that specific conserved sequences within this region interact with ubiquitous and lineage-dependent (but not lung-specific) transcription factors that are required for SP-D promoter activity in H441 cells. In particular, this region contains a functional AP-1 element at −109 and two interacting HNF-3-binding sites. In the present study, we identified five binding sites for three members of the C/EBP family that are expressed at varying levels by type II pneumocytes and Clara cells in normal or injured lung. C/EBP-mediated activation of AP-1-dependent promoter activity required interactions among the three C/EBP elements in the near-distal promoter.

Our current experiments demonstrate that the C/EBP motifs at −432 and the tandem motifs at −340 and −319 are functional C/EBP-binding sites capable of mediating C/EBP-dependent transactivation by C/EBPα, C/EBPβ, or C/EBP6 cDNA. Our data further suggest that the tandem sites in the human SP-D promoter, which are separated by 13 bp, constitute a compound response element that requires the integrity of both sites for function of the element. Mutagenesis of any one

| Oligomer | Site | Sequence (from 5′) | Site Orientation |
|----------|------|-------------------|-----------------|
| NF-L6    |      | GAGGCTGATTCATCCACATTCTTTTAA  |                 |
| NF-L5m   |      | GAGGCTGATTCACACTTTTGTTTAA   |                 |
| Commercial |    | TGGATCCGATCCGGATGCGA   | reverse         |
| Oligo 432 | 432 | CTCAATGCTCTCTTCTATTTTAA  | reverse         |
| Oligo 432m| 432 | CTCAATGCTCTCTTCTTTTTAA  | reverse         |
| Oligo 340 | 340 | AAGTCTTCGTTATCCACATTCTTTTAA | reverse         |
| Oligo 340m| 340 | AAGTCTTCGTTACACTTTTTTTAA  | reverse         |
| Oligo 319 | 319 | TACGATGCTCTTCTTTTTTTAA  | reverse         |
| Oligo 319m| 319 | TACGATGCTCTTCTTTTTTTAA  | reverse         |
| Oligo 140 | 140 | GTGCTGACCTTCTCTCTTTTTTTAA | reverse         |
| Oligo 140m| 140 | GTGCTGACCTTCTCTTTTTTTAA  | reverse         |
| Oligo 90  | 90  | AGTAAAGAATGTCCTCCTCTTTAAA | reverse         |
| Oligo 90m | 90  | AGTAAAGAATGTCCTCCTCTTTAAA | reverse         |
| Oligo 340/319 | 340/319 | AAGTCTTCGTTATCCACATTCTTTTAA | reverse         |
| Oligo 340m/319 | 340m/319 | AAGTCTTCGTTACACTTTTTTTAA  | reverse         |
C/EBPα binds to the motifs at −140 and −90. H441 cells were cotransfected with C/EBPα cDNA, and binding of nuclear proteins was assessed using gel retardation assays. Comparable results were obtained when H441 cells were cotransfected with C/EBPα or C/EBPβ cDNA. A, binding of C/EBPα to a radiolabeled oligomer containing the SP-D C/EBP sequence at −140 (Oligo 140; Table I). Binding was competed by the unlabeled oligomer (Oligo 140; lane 2), but not by the corresponding mutant oligomer (Oligo 140m; lane 3). The supershifted complex generated with antibody to C/EBPα is shown in lane 4. B, binding of C/EBPβ to a radiolabeled oligomer containing the SP-D C/EBP sequence at −90 (Oligo 90; Table I). Binding was competed by the unlabeled oligomer (Oligo 90; lane 2), but not by the corresponding mutant oligomer (Oligo 90m; lane 3). The supershifted complex generated with antibody to C/EBPβ is shown in lane 4.

The five sites show different affinities for C/EBPs. Parallel cultures of H441 cells were cotransfected with equivalent amounts C/EBPα, C/EBPβ, or C/EBPδ expression plasmid. Identical amounts of nuclear protein were incubated with fixed amounts of a radiolabeled commercial C/EBP consensus oligomer (Table I) either in the absence of competitor (0) or in the presence of a 4-, 20-, or 200-fold excess of unlabeled SP-D competing oligomer (−432, −340, −319, −140, or −90). The resulting complexes were resolved in gel shift assays as described under “Experimental Procedures.” The data for each C/EBP isoform were derived from two gels: one with the near-distal sites at −432, −340, and −319 and the other with the two proximal sites at −140 and −90. All the motifs, except the site at −140, were effective competitors of C/EBP binding.

The truncated construct (SS205) showed no transactivation by cotransfected C/EBPα cDNA, consistent with our ability to block C/EBP-dependent activation following mutagenesis of the site at −432 in combination with the site at −340 or −319. Thus, the binding sites at −140 and −90 are not sufficient to mediate significant C/EBP-dependent transactivation in H441 cells. In this regard, the site at −140 only very weakly competed the commercial consensus oligomer despite specific binding in gel shift assays. This appears to be consistent with the observed divergence of the motif from the consensus sequence (TTTGGAA versus TKDNGNAA, where D = A/G/T). Although oligomers containing the site at −90 showed high affinity binding, this site is not conserved in the rat or mouse.

Maximal stimulation of promoter activity by C/EBPs was dependent on the presence of a functional AP-1 element in the proximal promoter. Mutation of the conserved AP-1 element at −109 markedly decreased the stimulatory effect of C/EBPs on SS698 promoter activity. However, unstimulated promoter activity was decreased, and the low level of basal activity observed for the mutant AP-1 construct still showed stimulation by C/EBPs. Although some modulation of AP-1 activity by C/EBPs cannot yet be excluded, transactivation by C/EBPs does not require the AP-1 element.

Multiple C/EBP elements are present in many promoters, and tandem C/EBP-binding sites (referred to as contiguous, adjacent, or sequential) have been identified in the promoters of some APR proteins such as human C-reactive protein (35) and TSG-6 (tumor necrosis factor-inducible gene-6) (36), in C/EBP promoters (37), and in the promoter of the rat Clara cell-specific protein gene (38). The functional consequences of multiple C/EBP elements in such genes are quite varied, ranging from positive cooperativity to antagonism. In the case of C-reactive protein and TSG-6, it was shown that the two sequential sequences could be simultaneously occupied and that the inducibility of the intact promoter depends on a cooperative interaction between the two elements (35). This interaction was preserved when the elements were reduced from 13 to 8 base pairs. For Clara cell-specific protein, the two sites, which have 9 intervening base pairs, were required for transactivation by C/EBPα and/or C/EBPβ cDNA. Although mutagenesis of both sites blocked transactivation, mutagenesis of the high affinity distal site markedly decreased activity, whereas mu-
gels and compared with DNA standards (Promega). A representative

lanes 5 and 6). The endogenous SP-D and GAPDH mRNAs in the

H441 cells were then amplified by thermal cycling as described

under “Experimental Procedures.” Products were resolved on agarose
gels and compared with DNA standards (Promega). A representative

experiment is shown. A, RNAs from mock-transfected cells, C/EBPβ-

transfected cells, and cells treated with 50 nM dexamethasone were

amplified using full-length human SP-D primers and resolved on a 0.8% agarose gel (lanes 1, 3, and 5). The major 1.3-kb products, which

migrated at the expected positions, were reamplified using a nested human SP-D primer pair to further confirm the identity of the product.

As predicted, an ~400-bp nested fragment was generated (lanes 2, 4, and 6). Cloning and DNA sequencing definitively established the identity of the full-length PCR product as SP-D. B, as a control for the comparative PCR assays, the levels of GAPDH mRNA were assessed

using short or long primers as provided by the manufacturer. Reaction products using RNA from mock-transfected cells (lane 7) are compared with those using RNA from cells transfected with C/EBPβ cDNA (lane 8).

tageneration of the low affinity proximal site gave a smaller de-
crease. Mutagenesis of the distal site also decreased binding to the

proximal site.

In the case of SP-D, mutagenesis of either site did not de-
crease binding to the other, and mutagenesis of one or both sites comparably decreased activation. In addition, the comp-
ound element interacts with an upstream site at –432 to effect transactivation. To our knowledge, this is the first demonstra-
tion of such a tandem element interacting with other, more
distant C/EBP elements. Because only a single site was occu-

cration of the full-length PCR product as SP-D.

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mutations contributed to the positive regulatory activity of this

system.

Based on our in vitro findings, we speculate that alterations in the levels or activity of C/EBP isoforms contribute to in-
creased expression of SP-D by alveolar and bronchial epithelial cells in the setting of lung development or lung injury. As indicated in the Introduction, increases in SP-D message have been demonstrated in animal models of lung injury or infection,
considerably lower amounts of C/EBPβ were required for maximal transactivation. Thus, SP-D expression may be particularly sensitive to alterations in the levels of active C/EBPβ and/or C/EBPδ, which may be preferentially increased with injury or inflammation. In this regard, hyperoxia in rats increases the expression of SP-D by alveolar and bronchiolar epithelial cells (8) and increases pulmonary C/EBPα and/or C/EBPδ expression and binding activity (39). Likewise, intratracheal instillation of lipopolysaccharide in rats increases SP-D expression (6), and increases in both forms accompany systemic administration of lipopolysaccharide (26). Additional studies are needed to determine whether heterodimeric species, truncated forms, or post-translational modifications can differentially regulate promoter activity.

Transfection studies using cotransfected expression vectors have limitations. However, there is other evidence suggesting the physiological relevance of the current findings. In a recent study, Charles et al. (40) found that transfection of MLE-15 cells with pCMV-Rb increased the activity of the SS698 reporter construct by ~5-fold. Rb bound to the three endogenous C/EBP isoforms, with increased binding of Rb/C/EBP complexes to an oligomer containing the site at −340, which was the only site identified in our earlier studies. Deletion mutagenesis of the 9-bp C/EBP motif at −340 significantly decreased (but did not prevent) the activating effects of Rb while minimally decreasing unstimulated promoter activity. Consistent with our finding with C/EBP, a proximal promoter fragment containing the downstream sites (i.e. PS167) was insufficient to mediate activation by Rb. Thus, at least one of the near-distal sites can interact with endogenous C/EBP isoforms with activation of the promoter. Site-directed mutagenesis of the individual sites is needed to determine their specific roles in activation by Rb.

The observed 4–5-fold maximal increase in SP-D promoter activity is consistent with the increase in endogenous SP-D mRNA in response to C/EBP. It is also consistent with the magnitude of alterations in lung message and protein levels observed following lung injury in vivo. For example, lipopolysaccharide instillation causes a 50% increase in SP-D message near-distal sites can interact with endogenous C/EBP isoforms with activation of the promoter. Site-directed mutagenesis of the individual sites is needed to determine their specific roles in activation by Rb.
