# JUN-CCAAT/Enhancer-Binding Protein Complexes Inhibit Surfactant-Associated Protein B Promoter Activity

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**The murine surfactant-associated protein B (*Sftpb*) gene promoter, spanning nucleotides −653 to +42, is composed of functionally distinct proximal and distal regions. Although both regions contain consensus/putative activator protein 1 (AP-1) sites, the distal, but not the proximal, region mediates the inhibition by jun proto-oncogene (JUN) of *Sftpb* promoter activity. In transient cotransfection assays, JUN inhibited the luciferase reporter activity of plasmid constructs containing *Sftpb* promoter fragments that lacked the distal putative AP-1 site, indicating that another regulatory motif mediates JUN-dependent inhibition. Electrophoretic mobility shift assays and *in silico* analyses identified a DNA target sequence (*Sftpb* nucleotides −339 to −316) and transcription factors that regulate *Sftpb* promoter activity. The identified sequence contains a CCAAT/enhancer-binding protein (C/EBP) consensus recognition element. Mutation of the site reduced *Sftpb* promoter activity and sensitivity to inhibition by JUN. Purified recombinant JUN, which did not recognize the −339 to −316 target sequence when added alone, supershifted the mobility of *in vitro* translated C/EBP-α and C/EBP-β proteins complexed with the identified cis-regulatory element. These findings support the idea that heterodimerization between JUN and C/EBP-α and/or C/EBP-β targets JUN to the *Sftpb* promoter, thereby mediating its inhibitory regulatory role.**

**Keywords:** surfactant protein B; acute lung injury; gene regulation; pulmonary surfactant metabolism dysfunction type 1; pulmonary alveolar proteinosis

The *Sftpb* gene product (SFTPB) is one of the protein components of pulmonary surfactant. The other protein components include SFTPA, SFTPC, and SFTPD. These proteins represent a small fraction (∼ 10%) of surfactant, and were initially considered contaminants (1). SFTPB and SFTPC constitute approximately 1% of the total surfactant mass (2). However, the surfactant-associated proteins, and in particular SFTPB, are essential for normal lung function and survival. Because SFTPB is essential for surfactant function, lamellar body formation, and the processing of SFTPC, SFTPB deficiency causes congential alveolar proteinosis and respiratory failure (3–6).

The expression levels of SFTPB may be reduced because of genetic disorders or during acute lung injury after exposure to toxicants. Recently, we reported that the maintenance of SFTPB expression is critical to survival during nickel-induced lung injury in mice (7). Nickel increased jun proto-oncogene (JUN) transcripts in murine lung and alveolar type II epithelial cells (MLE-15), and the induction of JUN inhibited *Sftpb* gene promoter activity. Studies aimed at understanding the molecular basis of SFTPB inhibition may thus advance our ability to develop strategies that reverse the loss of SFTPB during lung injury.

**CLINICAL RELEVANCE**

One of the key events in acute lung injury is the loss of functional surfactant. Thus, surfactant has long been investigated in the treatment of acute lung injury. However, surfactant replacement has yet to be demonstrated as an effective therapy for acute lung injury. An alternative strategy may involve the development of treatments that maintain or restore the production of endogenous surfactant. We analyzed the core surfactant-associated protein B (*Sftpb*) promoter to identify transcription factors and recognition sites that contribute to the inhibition of SFTPB.

The 5′-flanking region of the murine *Sftpb* gene contains functionally important proximal (nucleotides −132 to −1) and distal (nucleotides −382 to −283) promoter regions (8). Both promoter regions contain a number of transcription factor recognition sites, including activator protein 1 (AP-1) recognition sequences. JUN-related proteins bind to and activate AP-1 regulatory elements in the promoter and enhancer regions of several mammalian genes (9). Despite the presence of a consensus AP-1 site in the *Sftpb* proximal promoter region and a putative AP-1 site in the distal promoter region, we found that the distal, but not the proximal, *Sftpb* promoter region mediated the JUN-dependent inhibition of promoter activity (7). The mechanism of the JUN-mediated inhibition of *Sftpb* transcription is unclear.

The transcription factor JUN participates in regulating a variety of biological processes, including cell proliferation, survival, apoptosis, tumorigenesis, tissue remodeling, and development (10–16). JUN controls these diverse processes through its ability to regulate the transcription and activity of numerous target genes and gene products. JUN, which was originally identified as AP-1 (17–19), belongs to a large family of proteins known as bZIP. These bZIP proteins are functionally related proteins with homologous sequences containing a basic DNA-binding domain and a leucine zipper region. JUN interacts with more than 50 related bZIP proteins and with structurally unrelated transcription factors, forming homodimeric and heterodimeric protein complexes (20, 21). The multiplicity of combinatorial JUN–protein interactions and the sequence compositions of DNA recognition sites determine target-gene specificity and regulatory selectivity in a cell type–dependent manner.

Another group within the bZIP transcription factor family is the CCAAT/enhancer-binding protein (C/EBP) subfamily. C/EBP subfamily members (α, β, and δ), in addition to C/EBP-γ and C/EBP-ζ, are expressed and play important roles in lung development, gene regulation, and acute lung injury (22–26). The

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deletion of C/EBP-α is perinatally lethal, in part because of lung abnormalities attributable to the hyperproliferation of alveolar type II cells (27). C/EBP-α–deficient newborn mice also exhibited increased surfactant associated protein A, B, and C mRNA, indicating a role for C/EBP-α in the control of alveolar cell proliferation/differentiation and the regulation of lung-specific target genes (28). In contrast, a deficiency of C/EBP-β and C/EBP-δ did not lead to lung abnormalities (29). However, the expression levels of C/EBP-β, C/EBP-δ, and C/EBP-regulated inflammatory mediators are increased in LPS-induced, bleomycin-induced, and oxidative stress–induced acute lung injury (26). Here, we investigated the role of C/EBP proteins in the inhibition by JUN of *Sftpb* promoter activity.

## MATERIALS AND METHODS

### Promoter Reporter Plasmid Constructs and Transient Transfection Assays

To analyze *Sftpb* promoter regulation, the region spanning nucleotides −653 to +42 (numbering according to Bruno and colleagues (8)) and its mutant variants were inserted into the luciferase reporter pGL4–10 (catalogue number E6651; Promega, Madison, WI). The plasmids pCMV6-XL4 and pCMV-Jun (pJun; Origene Technologies, Inc., Rockville, MD) and the luciferase reporters were transfected into MLE-15 cells (a gift of Dr. Jeffrey Whitsett) (30). The efficiency of transfection was normalized using pCMV-β–Gal. To determine promoter luciferase reporter activity, cells were lysed using Glolysis buffer, assayed in 96-well plates using the Bright-Glo luciferase or Beta-Glo systems (catalogue numbers E2610 and E4720, respectively; Promega), and luminescence was measured (Fusion α; Packard Bioscience/Perkin Elmer Life and Analytical Science, Waltham, MA) (*see* the online supplement for additional details).

### Electrophoretic Mobility Shift Assays

To investigate protein–promoter binding, biotinylated oligonucleotides were used for electrophoretic mobility shift assays (EMSAs). A probe containing a binding site for AP-1 JUN homodimer and JUN/FOS heterodimeric complexes (5′-CGCTTGATGACTCAGCCGGAA-3′ annealed with 3′-GCGAACTACTGAGTCGGCCTT-5′) served as a positive control for purified recombinant JUN binding. Nuclear protein extracts (2.5 μg) were incubated with biotinylated probes and competitors, and were analyzed (see the online supplement for additional details).

### Chromatin Immunoprecipitation Assay

To examine whether the bZIP proteins D site albumin promoter binding protein (DBP), C/EBP-α, and C/EBP-β bind to the endogenous *Sftpb* promoter, chromatin immunoprecipitation (ChIP) was performed with MLE-15 chromatin (ChIP-IT Express, catalogue number 53008; Active Motif, Inc., Carlsbad, CA) (see the online supplement for additional details) and control rabbit IgG (sc-2027), anti–C/EBP-α (sc-61), anti–C/EBP-β (sc-150), or anti-DBP (sc-98411) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunoprecipitated chromatin, after repeated washings, was eluted, reverse cross-linked, and treated with proteinase K. The supernatant and eluate fractions were analyzed by PCR amplification for the *Sftpb* promoter region −159 to −541, using the primer pair forward 5′-CCACAGGGGACACAGAAATC-3′ and reverse 5′-CGATGTCGGTTCCTAGTCCT-3′ (31).

### *In vitro* Transcription/Translation

C/EBP-α and C/EBP-β cDNA expression constructs (plasmids 12550 and 12557, respectively; Addgene, Cambridge, MA) and negative control pSP72 (catalogue number P2191; Promega) were linearized to produce *in vitro* translated proteins (catalogue number L4610; Promega). In addition, full-length Dbp (catalogue number 4195116), NKX2–1 (catalogue number 3941576), POU2F1 (catalogue number 2966289), and Jund (catalogue number 4456297) cDNAs (Open Biosystems/Thermo Scientific, Huntsville, AL) were subcloned into pSP72 for coupled *in vitro* transcription/translation. The reactions were incubated for 90 minutes at 30°C. For EMSA analysis, 1 μl of the transcription/translation product that had been diluted 4-fold or 8-fold was used.

### Identification of Transcription Factor Binding Sites

The Transcription Element Search System (32) and JASPAR (33) were used to identify putative transcription factor binding sites in the *Sftpb* promoter region −339 to −316.

### Statistical Analysis

Groups were compared by one-way ANOVA with the Holm-Sidak all pairwise multiple comparison procedure (SigmaStat Program; SPSS, Inc., Chicago, IL). Relative luciferase activity units of each *Sftpb* promoter reporter construct are expressed as folds of the vector pGL4–10-Δ*Bgl* II/*Hind* III or percentages of control values. Transient reporter assays were performed at least three times each, in triplicate or more. Results are expressed as means ± SE, and $P < 0.05$ was considered significantly different from the appropriate control.

## RESULTS

### The *Sftpb* Distal Promoter Region Functions as an Independently Active Promoter

Previous studies reported that the distal *Sftpb* promoter region mediated the inhibition by JUN of *Sftpb* promoter activity (34). We further demonstrated that JUN inhibited the distal, but not the proximal, promoter region (7). Because the *Sftpb* distal promoter region contains a putative AP-1 site, whereas the proximal region contains a consensus recognition site (nucleotide sequences −370 to −364 and −16 to −10, respectively), the mechanism underlying JUN's regulatory selectivity for the distal region was not known. To investigate the mechanism of JUN's inhibition of *Sftpb* promoter activity, luciferase reporter constructs under the control of the *Sftpb* promoter region, encompassing nucleotides −653 to +42 and mutant variants (Figure 1A), were analyzed. The enzyme reporter activity of the distal promoter region −653 to −194 was greater than the activity of the proximal promoter region −198 to +42 (4.6-fold versus 1.4-fold, respectively) compared with the control vector (Figure 1B). Upon subsequent deletion of the −653 to −194 promoter fragment, the reporter activity of the −400 to −275 was enhanced (4.6-fold versus 7.2-fold, respectively), whereas reporter activity of the promoter fragments −653 to −401 and −274 to −194 was reduced compared with the −653 to −194 fragment. These results indicate the presence of inhibitory cis-acting regulatory sequences in the −653 to −401 or −274 to −194 promoter regions. In contrast to the −653 to −194 fragment, deletion of the −198 to +42 promoter region reduced reporter activity. These results demonstrate that both the proximal and distal *Sftpb* promoter regions are functionally active and distinguishable, enabling further investigation of the molecular basis of the inhibition by JUN of *Sftpb* promoter activity.

### Inhibition by JUN of *Sftpb* Promoter Reporter Activity Occurs Independent of the Distal Putative AP-1 Site

A previous study suggested that the distal promoter region contained an AP-1 element that is part of a composite binding site wherein AP-1, the cyclic AMP response element binding protein (CREB), thyroid transcription factor–1 (also known as NKX2–1), and nuclear factor I (NF1) (Figure 1C) interact (34). The nucleotide sequence of the *Sftpb* distal putative AP-1 site (TGCGTCA) differs from that of the proximal AP-1 site (TGACTCA) in the third and fourth nucleotides, raising the possibility that this property may underlie the selective JUN inhibition on the distal compared with the proximal promoter region. To investigate the contribution of the distal putative

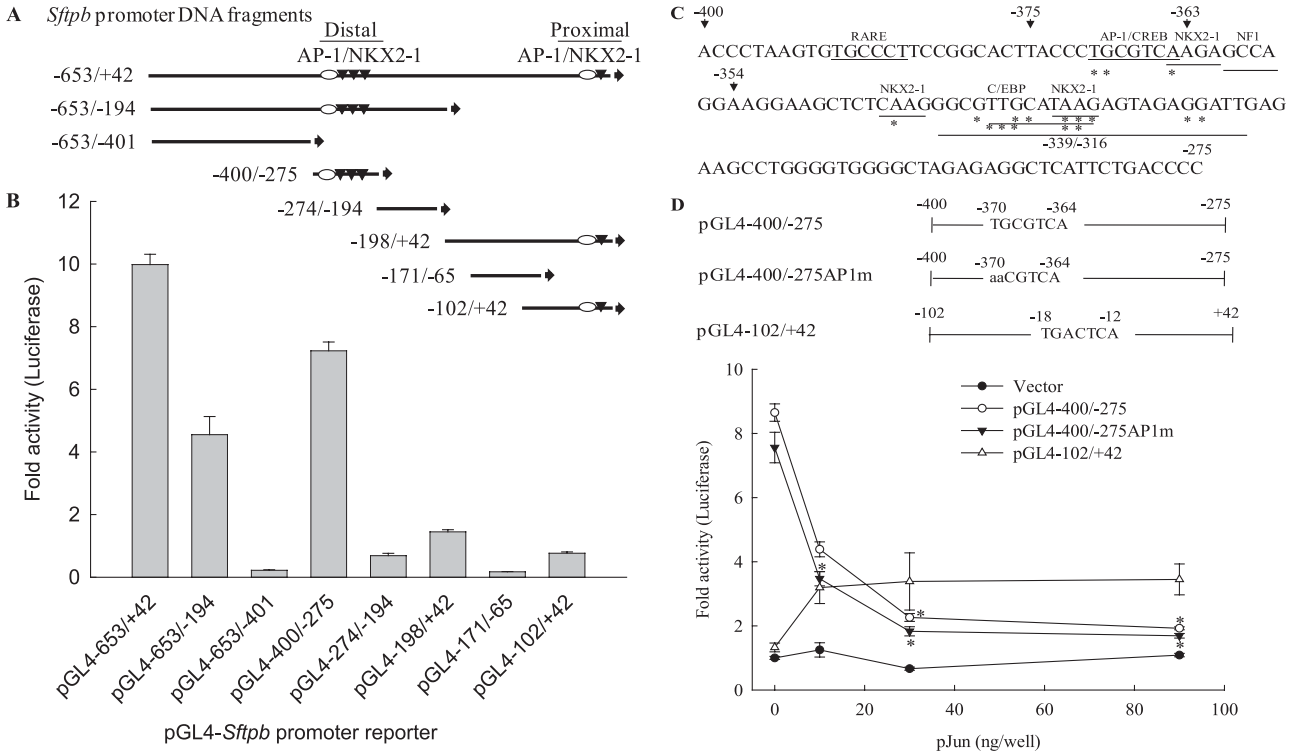


***Figure 1.*** Transient transfection analysis of surfactant-associated protein B (*Sftpb*) promoter reporter activity. (*A*) Schematic representation of the *Sftpb* promoter DNA fragments analyzed. Reporter constructs were generated by inserting promoter DNA fragments in the luciferase reporter plasmid pGL4–10. The *Sftpb* 5′ and 3′ nucleotide sequence numbers and the proximal and distal promoter regions containing the putative activator protein–1/Nk2 homeobox–1 (AP-1/NKX2.1) regions (*ovals* and *inverted triangles*, respectively) are shown. *Arrowhead* indicates the orientation of the DNA fragments ligated to the luciferase gene reporter. (*B*) The *Sftpb* distal promoter region is active, independent of the proximal promoter. *Sftpb* promoter reporter activity (fold activity versus vector control) was determined by transient transfection of MLE-15 cells ($n$ = 8–16). (*C* and *D*) JUN inhibits *Sftpb* promoter reporter activity of a construct containing site-directed mutations in the distal putative AP-1 site (nucleotides −370 to −364). (*C*) Nucleotide sequence of the mouse *Sftpb* distal promoter region −400 to −275 denotes consensus/putative recognition elements for retinoic acid receptor (RARE), AP-1/cyclic AMP-responsive element binding protein (AP-1/CREB), Nk2 homeobox 1 (NKX2–1), nuclear factor 1 (NF1), and CCAAT/enhancer-binding protein (C/EBP) (*underlined*). The 5′ ends of deletion mutants (*arrows*), the region of sequence −339 to −316, and the nucleotides altered by site-directed mutagenesis (*asterisks*) to analyze promoter activity (Figures 2–5) are also indicated. (*D*) Schematic representation of the constructs used and the mutations introduced are shown (*top*). Dose–response relationships of JUN co-expression on *Sftpb* proximal and distal promoter reporter activities were assayed by transfecting MLE-15 cells with pGL4–10Δ*Bgl* II/*Hind* III (vector), pGL4−102/+42 (proximal), pGL4−400/−275, and pGL4−400/−275AP-1m (distal putative WT and AP-1 site mutant [AP-1m], respectively) constructs. MLE-15 cells were cotransfected with reporter constructs and increasing concentrations of plasmid pCMV-Jun (pJun). The total amount of DNA was adjusted, using empty plasmid. JUN inhibited the distal putative AP-1 mutant and WT reporters ($n$ = 4). *Decreased compared with control with no pJun ($P < 0.05$), as determined by one-way ANOVA with Holm-Sidak all pairwise multiple comparisons procedure.

AP-1 site to JUN's inhibition of *Sftpb* promoter activity, a reporter construct with point mutations in the distal AP-1 site was generated. Dose-dependently, JUN inhibited both the wild-type and the AP-1 mutant −400 to −275 promoter reporters (pGL4−400/−275, pGL4−400/−275AP-1m), but not the proximal promoter reporter (pGL4−102/+42) (Figure 1D).

The protein JUN represses glucocorticoid-receptor and retinoic acid receptor gene regulatory activities by direct protein–protein interaction (35, 36). In a recent study (37), interactions of the TGF-β signaling protein SMAD3 with NKX2–1 and FOXA1 reduced binding to cognate DNA sites, leading to *Sftpb* gene repression by TGF-β. Retinoic acid and NKX2–1 recognition elements play important roles in *Sftpb* gene regulation (38–40). One of the NKX2–1 consensus core sites in the distal *Sftpb* promoter region overlaps the putative AP-1 site and retinoic acid receptor sites. To investigate the contribution of NKX2–1 recognition sites to JUN inhibition, a reporter with point mutations in the NKX2–1 sites (pGL4−400/−275 NKX2–1m; Figure 2A) was constructed. The mutant construct exhibited reduced reporter activity. The treatment of transfected MLE-15 cells with all trans-retinoic acid stimulated wild-type and mutant reporter activity, which was inhibited by JUN (Figure 2B).

To rule out the possibility that point mutations neither abolished the binding of an inhibitory AP-1 complex nor interfered with protein–protein interactions, rendering mutant reporter activity sensitive to JUN inhibition, we generated deletion mutants lacking the AP-1 and upstream NKX2–1 consensus core sites. Further deletion of the distal reporter pGL4−400/−275 reduced reporter activity. Moreover, despite the lack of a putative AP-1 site, the deletion reporters remained responsive to inhibition by JUN (Figures 3A and 3B). These results suggest that JUN exerts its inhibitory effect either through *Sftpb* promoter sites independent of the targeted putative AP-1 recognition sequence, or through protein–protein interactions.

### Recombinant JUN Enhances the Intensity of DNA–Protein Complexes Formed by Incubating the *Sftpb* −339/−316 Probe with MLE-15 Nuclear Protein Extract

After finding that the *Sftpb* distal putative AP-1 and NKX2–1 recognition sites may not be critical for JUN inhibition, we

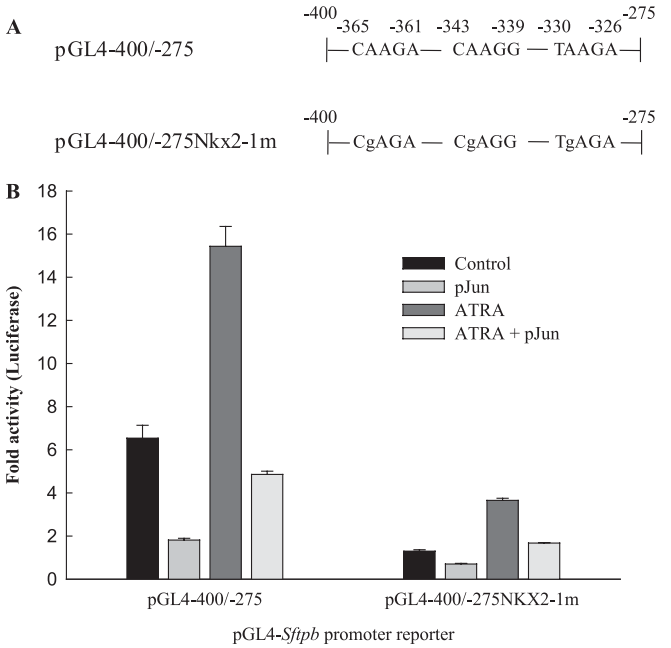


***Figure 2.*** JUN inhibits basal and all-trans-retinoic acid (ATRA)–inducible *Sftpb* promoter reporter activity. Control (pGL4−400/−275) and a mutant *Sftpb* reporter construct containing three site-directed mutations (pGL4−400/−275NKX2–1m) were cotransfected into MLE-15 cells with either empty plasmid (control) or JUN expression plasmid pJun (30 ng/well). The day after transfection, serum-containing medium was replaced with serum-free medium in the absence or presence of ATRA (1 μg/ml) for 20–24 hours. (*A*) Schematic representation of promoter fragments shows mutated nucleotides. (*B*) *Sftpb* promoter reporter activity was determined by transient transfection of MLE-15 cells ($n = 4$).

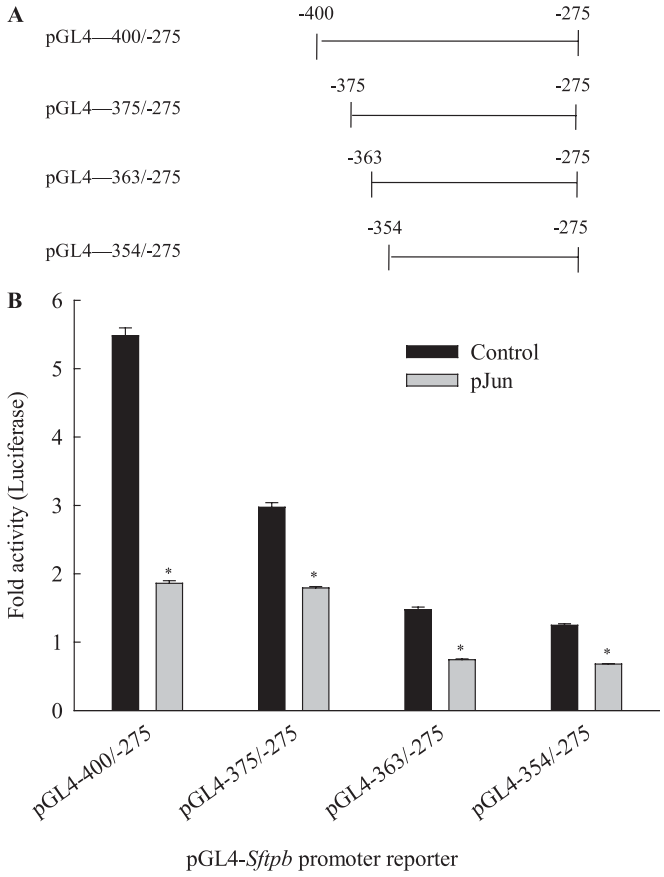


***Figure 3.*** JUN inhibits *Sftpb* promoter reporter activity of constructs lacking the distal AP-1 site. Deletion mutant reporters (pGL4−375/−275, pGL4−363/−275, and pGL4−354/−275) were constructed after exonuclease III treatment of linearized pGL4–*Sftpb*−400/−275. MLE-15 cells were cotransfected with reporter constructs plus either empty plasmid (Control) or pJun (30 ng/well). (*A*) Schematic representation of promoter DNA fragments analyzed. The *Sftpb* 5′ and 3′ nucleotide sequence numbers are indicated. (*B*) *Sftpb* promoter reporter activity was determined by transient transfection of MLE-15 cells ($n = 4$). *Decreased compared with control with no pJun ($P < 0.05$), as determined by one-way ANOVA with Holm-Sidak all pairwise multiple comparisons procedure.

sought to determine whether JUN directly or indirectly influenced the formation of DNA–protein complexes. Oligonucleotide probes (24-mer) spanning the *Sftpb* promoter region −375 to −275 were incubated in the absence or presence of recombinant JUN, with or without MLE-15 cell nuclear extracts, and were analyzed by EMSAs (Figure 4A). All probes formed DNA–protein complexes. The recombinant JUN, when used alone, interacted with an AP-1 consensus oligonucleotide probe included as a positive control (Figure 4A, *lane 16*), whereas no interaction was evident with *Sftpb* promoter–derived oligonucleotides (Figure 4A, *lanes 1, 4, 7, 10,* and *13*). However, the presence of recombinant JUN increased the intensity of the probe–protein complexes formed by incubating the *Sftpb* oligonucleotide probe −339/−316 with MLE-15 nuclear protein extract (in particular, the slow-migrating complex) (Figure 4A, *lane 9, arrow*).

To identify potential transcription factor binding sites in *Sftpb* promoter region −339 to −316, *in silico* analysis was performed using TESS (32) and JASPAR (33). Several transcription factor recognition sites were predicted (ABF1, DBP, POU1F1a, EFII, HOXA5, GAL4, DDIT3::C/EBP-α, BRCA1, NFIL3, HLF, POU5F1, C/EBP-α, FOXL1, SOX2, GATA2, HLTF, TBP, NKX2–5, FOXC1, EN1, ZNF354C, MZF1_1–4, SPIB, and ETS1). To locate regions within the 24-mer oligonucleotide that may be critical to the formation of DNA–protein complexes, mutations were introduced in the −339/−316 oligonucleotide and designated m4,7,8, m12,13, and m20,21 (Figure 4B). To test the effects of the mutations on the formation of DNA–protein complexes, a 100-fold molar excess of unlabeled wild-type and mutant oligonucleotide competitors was used. The formation of faster and slower migrating complexes was inhibited by wild-type and m20,21 oligonucleotide −339/−316 (Figure 4B, *lanes 3* and *6*). In contrast, oligonucleotides −339/−316 m4,7,8 and m12,13 inhibited the formation of complexes differentially. Whereas −339/−316 m4,7,8 inhibited the faster migrating complex, −339/−316 m12,13 inhibited the slower migrating complex (Figure 4B, *lanes 4* and *5*). These results suggested the presence of multiple protein species with partial selectivity to subregions of the −339/−316 region of the *Sftpb* promoter and the presence of exogenously added recombinant JUN modulated DNA–protein interactions.

### The Recognition Site in the *Sftpb* Distal Promoter Region −339 to −316 Plays a Key Role in the Regulation of *Sftpb* Promoter Activity

The *Sftpb* promoter region −339 to −316 contained nucleotide sequences that highly matched the consensus recognition site for the D-site albumin promoter binding protein (DBP, a member of the proline-rich and acid-rich bZIP [PAR-bZIP] protein subfamily) and C/EBP-α transcription factor (a member of the C/EBP subfamily) (Figure 5A) (41, 42). The putative DBP and C/EBP

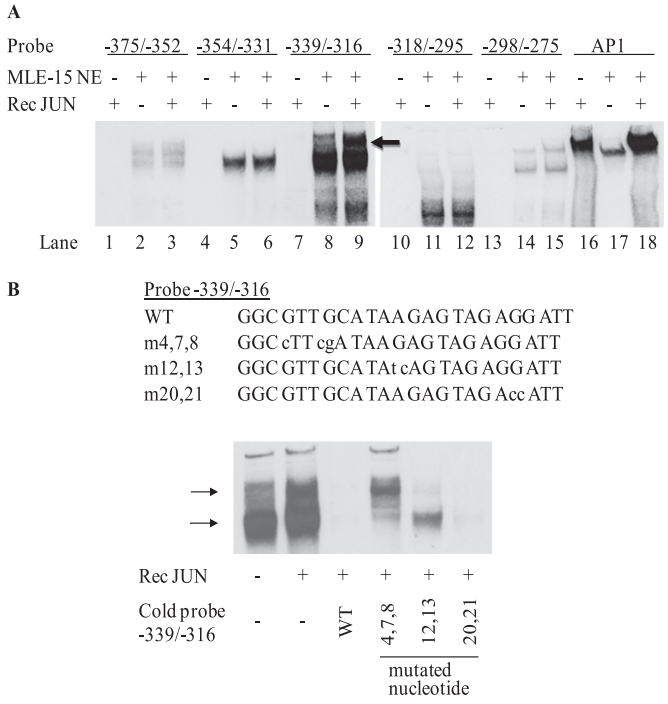


***Figure 4.*** MLE-15 cell nuclear proteins form complexes with oligonucleotides corresponding to *Sftpb* distal promoter region. (*A*) Identification of a region whose complex-forming property is altered in the presence of purified recombinant JUN. Electrophoretic mobility shift assays (EMSAs) were performed with 24-mer oligonucleotides (spanning *Sftpb* promoter nucleotide sequence −375 to −275) and an AP-1 consensus probe. The oligonucleotide probes were incubated in the presence or absence of purified recombinant JUN (Promega), with or without MLE-15 cell nuclear extract. Addition of JUN to the probe −339/−316 and MLE-15 nuclear extract mix increased the intensity of DNA–nuclear protein complexes formed, and in particular the slow-migrating complex (*arrow*). (*B*) Multiple proteins (*arrows*) bind to the *Sftpb* oligonucleotide probe −339/−316. EMSAs were performed with wild-type (WT) probe (−339/−316) without (*lane 1*) or with (*lanes 2–6*) recombinant JUN in the absence or presence of 100-fold excess unbiotinylated WT (*lane 3*) or mutant (*lanes 4–6*) oligonucleotides. The DNA–protein complexes formed in the absence and presence of JUN (*lanes 1* and *2*) were competed by excess WT and mutant (m) 20,21 oligonucleotides. Although m4,7,8 preferentially inhibited the formation of the fast-migrating complex, m12,13 inhibited the slow-migrating complex, indicating the presence of more than one protein species.

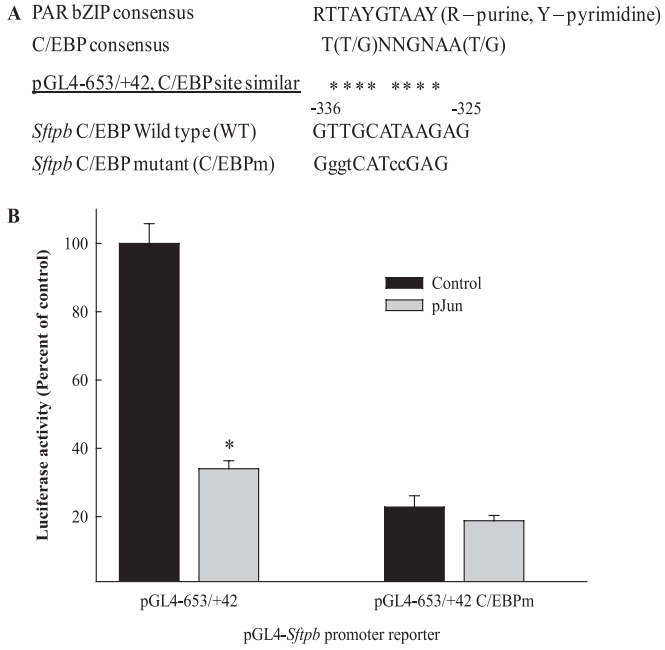


***Figure 5.*** Mutation of the C/EBP recognition site reduces *Sftpb* promoter reporter activity and sensitivity to JUN-mediated inhibition. (*A*) The *Sftpb* C/EBP recognition site and mutated nucleotides (nucleotides −336 to −325) are shown in relation to the PAR-bZIP and C/EBP consensus recognition sequences. (*B*) Wild-type (pGL4−653/+42) and C/EBP site mutant (pGL4−653/+42 C/EBPm) plasmid reporter constructs were cotransfected with either empty plasmid (Control) or the expression plasmid pJun (30 ng/well) into MLE-15 cells. The pGL4 −653/+42 C/EBPm reporter was less active (4.5-fold) and less sensitive to JUN-mediated inhibition compared with the pGL4−653/+42 reporter ($n$ = 5 and 6, respectively). *Decreased compared with control sample with no pJun ($P < 0.05$), as determined by one-way ANOVA with Holm-Sidak all pairwise multiple comparisons procedure.

recognition elements were selected for further analysis because both DBP and the C/EBP proteins belong to the bZIP protein family, of which JUN is a member. Members of different bZIP protein subfamilies homodimerize and heterodimerize, expanding the repertoire of target sites recognized by bZIP proteins.

To examine the role of the putative DBP and C/EBP site in the *Sftpb* promoter, we generated a reporter construct containing point mutations (pGL4−653/+42 C/EBPm; Figure 5A). The mutations introduced were intended to target the two halves of the recognition site, and are different from the mutations used in the competitive EMSA (Figure 4B). The wild-type and mutant reporter constructs were transfected into MLE-15 cells in the presence or absence of the JUN expression plasmid pJun (Figure 5B). Mutagenesis of the putative DBP and C/EBP site reduced *Sftpb* promoter activity by 4.5-fold, indicating that this region plays a critical role in regulating *Sftpb* promoter activity. Further, the pGL4−653/+42 C/EBPm reporter was less sensitive to JUN-mediated inhibition (Figure 5B). These results suggested that JUN forms complexes with *Sftpb* gene transactivators or repressors.

### C/EBP-α and C/EBP-β Proteins Bind to the *Sftpb* Promoter

To examine whether the bZIP proteins DBP, C/EBP-α, and C/EBP-β bind to the endogenous *Sftpb* promoter, ChIP was used. The DNA–protein complex immunoprecipitated using anti–C/EBP-α and anti–C/EBP-β antibodies was enriched compared with either control IgG or anti-DBP antibodies, as determined by PCR analysis (Figure 6). These results suggest that C/EBP-α and C/EBP-β proteins bind to the murine *Sftpb* promoter.

### C/EBP-α, C/EBP-β, and DBP Proteins Bind to the *Sftpb* Promoter Region −339 to −316

Because of the DNA fragment sizes of the chromatin sonicate used as a template for PCR analysis (100 to 1,000 bp) in the ChIP assay and the presence of multiple putative C/EBP sites in the *Sftpb* promoter, the exact location of the region mediating C/EBP binding could not be determined using ChIP. EMSA was used to demonstrate direct C/EBP protein binding to the *Sftpb* −339/−316 region in which C/EBP-α and C/EBP-β proteins were expressed, using a rabbit reticulocyte lysate system, and incubated with biotinylated probe −339/−316. The presence of C/EBP-α or C/EBP-β translation products shifted probe mobility (Figure 7A, *lanes 3* and *5*), and complex formation was competed by 100-fold excess unbiotinylated −339/−316 oligonucleotide (Figure 7A, *lanes 4* and *6*). In contrast, the nonspecific complex formation detected in the negative control sample

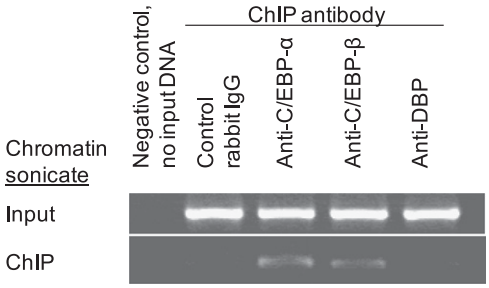


***Figure 6.*** C/EBP-α and C/EBP-β proteins bind to the endogenous *Sftpb* promoter. Cross-linked chromatin isolated from MLE-15 cells was immunoprecipitated with nonimmune rabbit IgG (*lane 2*), anti–C/EBP-α (*lane 3*), anti–C/EBP-β (*lane 4*), or anti–D-site albumin promoter binding protein (anti-DBP) (*lane 5*) antibodies. Eluates of the immunoprecipitated DNA samples were PCR-amplified with a primer pair specific to the murine *Sftpb* promoter region, spanning nucleotides −159 to −541. The supernatant fraction of the immunoprecipitated chromatin samples was used to check input DNA. A PCR reaction mix with no chromatin added served as negative control (*lane 1*). ChIP, chromatin immunoprecipitation.

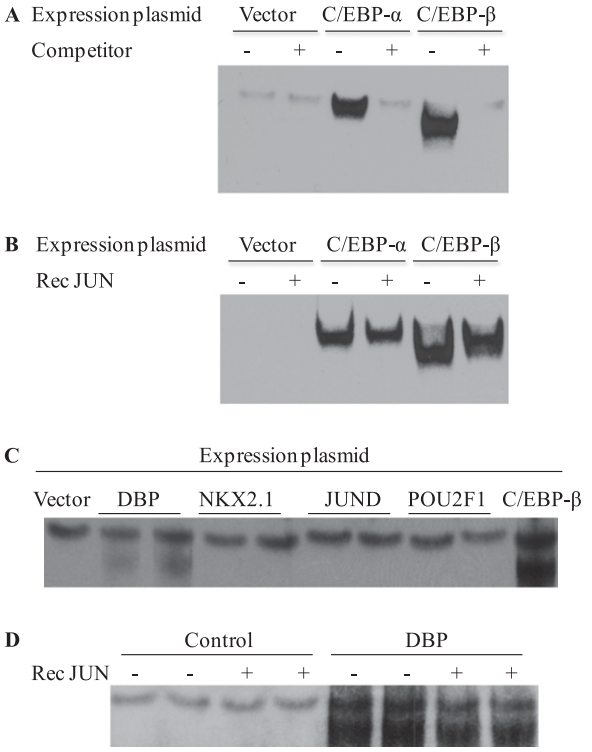


***Figure 7.*** *In vitro* translated C/EBP-α, C/EBP-β, and DBP proteins bind to the *Sftpb* promoter −339/−316 probe. (*A*) *In vitro* transcription/translation products, generated using vector (*lanes 1 and 2*), C/EBP-α (*lanes 3 and 4*), or C/EBP-β (*lanes 5* and *6*) expression plasmid DNA templates, were incubated with the biotinylated probe −339/−316. Samples were preincubated in the absence or presence of 100-fold excess unbiotinylated oligonucleotides (*lanes 2*, *4*, and *6*) to determine specificity. The nonspecific band (*lane 1*) was not competed by the addition of unbiotinylated probe (*lane 2*). (*B*) Incubation of the DNA probe −339/−316 and *in vitro* translation products in the presence of recombinant JUN supershifted the C/EBP–DNA complexes formed (*lanes 4* and *6*). (*C*) *In vitro* transcription/translation products, generated using vector (*lane 1*), DBP (*lanes 2* and *3*), NKX2–1 (*lanes 4 and 5*), jun proto-oncogene related gene d (JUND) (*lanes 6* and *7*), POU class 2 homeobox 1 (POU2F1) (*lanes 8* and *9*), or C/EBP-β (*lane 10*) expression plasmid DNA templates, were incubated with the biotinylated *Sftpb* −339/−316 probe. DBP formed a complex with the DNA probe (*lanes 2* and *3*). (*D*) The migration pattern of the DBP/DNA complex formed remained similar in the absence or presence of recombinant JUN (*lanes 5* and *6* versus *7* and *8*).

was not competed by the addition of unbiotinylated oligonucleotide (Figure 7A, *lane 2*).

To test whether C/EBP binding to the promoter region −339/−316 mediated JUN binding, the *in vitro* translation products were preincubated with purified recombinant JUN. The presence of recombinant JUN supershifted the mobility of the C/EBP-DNA complex (Figure 7B, *lanes 4* and *6*). These results, in combination with the effect of recombinant JUN on EMSAs of nuclear proteins (Figure 4), suggest that C/EBP-α and/or C/EBP-β proteins bind to the identified *Sftpb* promoter region, and JUN binding is mediated through the formation of heteromeric complexes.

In addition, DBP, NKX2–1, POU2F1, and JUND binding to the Sftpb −339/−316 probe was examined by EMSA, because one of the putative NKX2–1 sites overlaps the C/EBP site, and JUND and POU2F1 (also known as OCT1) regulate *Sftpb* and Clara cell secretory protein genes, respectively (34, 43). DBP, NKX2–1, POU2F1, and JUND translation products were incubated with the *Sftpb* −339/−316 region and analyzed by EMSA. DBP, but not NKX2.1, POU2F1, or JUND, bound the −339/−316 oligonucleotide (Figure 7C).

To examine DBP binding further, the formation of DBP–DNA complexes in the absence or presence of JUN was compared. The migration pattern of the DBP-biotinylated oligonucleotide complex remained similar in the absence or presence of JUN (Figure 7D). The ability of DBP, in addition to C/EBP-α and C/EBP-β, to interact with the *Sftpb* −339/−316 region raises the possibility that other transcription factors or bZIP family members could bind to the same site.

## DISCUSSION

SFTPB is critical in maintaining lung function, because SFTPB-deficient mice die of respiratory failure shortly after birth (3–6). One of the key events in acute lung injury is the loss of functional surfactant. Thus, surfactant has long been investigated as a treatment for acute lung injury. Surfactant replacement, however, has yet to be demonstrated as an effective therapy for acute lung injury because of immense hurdles in its administration (44). An alternative strategy may involve the development of treatments that maintain or restore endogenous surfactant production. We previously reported that in a murine model of acute lung injury, the induction of JUN was associated with a diminution of SFTPB expression (7). SFTPB expression was maintained in resistant, compared with sensitive, murine strains, and inducible SFTPB expression increased the survival of mice (7). The present work focused on analyses of the core *Sftpb* promoter, to identify transcription factor recognition sites that contribute to the JUN-mediated inhibition of SFTPB.

The murine *Sftpb* promoter, spanning nucleotides −653 to +42, contains two functionally distinguishable proximal (−132 to −1) and distal (−382 to −283) promoter regions (8). The distal promoter region can be transcriptionally active in the absence of the proximal promoter region. The distal promoter region contains a putative initiator sequence (CATTCTG) at nucleotides −286 to −280. This initiator element, which was originally identified in the TATA-less murine terminal deoxynucleotidyl transferase gene, encompasses the transcription start site and can direct basal transcription (45). As determined by primer extension (8) and expressed-sequence Tag (EST) database analyses, the SFTPB mRNA 5′ untranslated region contains ⩽14–16 nucleotides. However, basal promoter activity was lost

with the deletion of promoter sequences from −415 to −353 (34), demonstrating the critical role of the distal promoter region. Although the occurrence of an alternative SFTPB transcriptional initiation site is unknown, the functionality of the distal region as a promoter provided a useful tool for analyzing the role of its putative transcription factor recognition sites, independent of those in the proximal region.

The *Sftpb* promoter contains AP-1, NKX2–1, trans-acting transcription factors 1 and 3 (SP1, SP3), hepatocyte nuclear factor 3 (HNF3), retinoic acid receptor, and other recognition elements (8, 34, 38, 40, 46). Although JUN can activate numerous genes by binding to AP-1 recognition elements, it can also inhibit the induction of other genes by a different mechanism. JUN inhibits the induction of the bone γ-carboxyglutamate protein (osteocalcin) gene by retinoic acid and vitamin D3 (35) and the induction of the kallikrein-related peptidase 3 (prostate-specific antigen) gene by androgen (47). Direct interactions between JUN and the receptors for vitamin D3 or androgen inhibit the induction of target genes.

The *Sftpb* proximal and distal promoter regions contain putative AP-1 sites. The proximal AP-1 site is identical to the optimal consensus AP-1 site (TGACTCA), whereas the distal AP-1 site (TGCGTCA) differs by two nucleotides. In addition, the distal CREB site differs from the consensus CREB site (TGACGTCA). However, these nucleotide sequence differences could not explain JUN's ability to inhibit the distal, but not the proximal, promoter region. The patterns of JUN inhibition of the distal AP-1 wild-type and point mutant reporters were comparable. To rule out the possibility that the introduced mutations may not have sufficiently altered the mode of protein–DNA interactions, we investigated the effects of JUN co-expression on reporter constructs that lacked the distal putative AP-1 site. Despite the absence of the putative AP-1 site, JUN inhibited reporter activity. These results suggest that the presence of the putative AP-1 site in the distal promoter region is not required for the inhibition by JUN of *Sftpb* promoter activity, raising the possibility of protein–protein interactions as a mediator of JUN's inhibitory effect.

Our conclusion about the role of the *Sftpb* AP-1 sites contrasts with those of previous studies (34). Sever-Chroneos and colleagues (34) observed that mutation in the distal AP-1 binding site increased basal promoter activity fivefold in MLE-15 cells. In addition, they concluded that the distal AP-1 element is involved in, but is not sufficient for, the inhibition by JUN of promoter activity. In our analysis, although deletion of the distal element containing the overlapping AP1/NKX2–1 recognition sites reduced promoter activity, mutation of the AP-1 site did not induce basal promoter activity or reverse JUN inhibition. It is unclear whether the use of the luciferase reporter (half-life of approximately 0.84 hours) (48) versus the CAT reporter (half-life of approximately 16 hours) (49) and other experimental variations (e.g., the use of different promoter fragments) contributed to the discrepancies observed. In support of our observations, a genome-wide analysis of the frequency and distribution of AP-1 sites indicated that the number of AP-1–regulated genes identified is far smaller than the number of genes containing potential AP-1 sites, and that not all AP-1 sites are activated in a given cell under a given condition (50, 51).

In addition, the interaction of JUN with NKX2–1 appears unlikely to inhibit *Sftpb* promoter activity. Point mutations at the NKX2–1 sites in the distal promoter reduced reporter activity, but the mutants remained sensitive to inhibition by JUN, suggesting that other recognition sites or regulatory factors mediated the JUN-mediated inhibition of *Sftpb*.

Mobility shift assays indicated that JUN may target the *Sftpb* promoter by binding to a site within the −339/−316 region. Nucleotide sequence analysis for potential transcription factor binding sites in the −339/−316 *Sftpb* promoter region predicted recognition sites for DBP and C/EBP. This was a pertinent finding because DBP and the C/EBPs, like JUN, belong to the bZIP protein family. The contribution of the identified site to the regulation of *Sftpb* promoter activity was demonstrated in MLE-15 cells transfected with pGL4−653/+42 wild-type and mutant reporters. Mutagenesis of the putative recognition site reduced *Sftpb* promoter activity and sensitivity to JUN-mediated inhibition.

Endogenous DBP or C/EBP binding to the *Sftpb* promoter was investigated by chromatin immunoprecipitation. Immunoprecipitation of MLE-15 chromatin sonicate with anti-DBP antibodies did not enrich the *Sftpb* promoter PCR amplification product, suggesting that DBP does not associate with *Sftpb* promoter *in vivo*, or that the abundance of DBP–*Sftpb* promoter complexes formed *in vivo* does not permit detection by this method. In contrast, C/EBP-α and C/EBP-β binding to the *Sftpb* promoter was evidenced by the enrichment of chromatin immunoprecipitation.

JUN modulates gene transcription by forming homodimeric or heterodimeric complexes (52, 53). Our results indicate that JUN may not inhibit *Sftpb* promoter activity by forming homodimers. The recombinant JUN protein formed a complex with the consensus AP-1 oligonucleotide, but with none of the *Sftpb*-derived oligonucleotides spanning the *Sftpb* promoter region −375 to −275. However, in the presence of MLE-15 nuclear protein extract, the addition of JUN increased the intensity of DNA–protein complexes formed with the −339/−316 oligonucleotide probe. Further analysis, using point mutant oligonucleotides, suggested that multiple transcription factors bound to the −339/−316 probe.

Previous studies demonstrated that C/EBP-β and JUN interacted through their bZIP region in the absence of DNA. Such interactions altered the specificity of DNA binding (54). In our EMSA analyses, C/EBP binding to the *Sftpb* −339/−316 region, to which JUN by itself cannot bind, was shifted in the presence of purified recombinant JUN protein. These results suggest that the *Sftpb* −339/−316 region is recognized by C/EBP-α and/or C/EBP-β dimers or C/EBP-α and/or C/EBP-β/JUN heterodimers, and that the formation of heteromeric complexes is key to *Sftpb* promoter targeting by JUN. The PAR-bZIP DBP also bound to the −339/−316 probe, raising the possibility that other transcription factors or bZIP proteins could bind to the same site.

In conclusion, we analyzed the murine *Sftpb* promoter encompassing nucleotides −653 to +42. Deletion and site-directed *Sftpb* promoter reporter mutants and EMSAs indicate that the distal *Sftpb* promoter region −339 to −316 is a critical regulatory element. Mutagenesis in this region, which contains a C/EBP recognition site, reduced *Sftpb* promoter activity and sensitivity to JUN-mediated inhibition. Endogenous C/EBP-α and C/EBP-β bind to the *Sftpb* promoter. The transcription factor JUN can partner with C/EBP-α or c/EBP-β, bind to the identified cis-acting regulatory DNA site, and inhibit *Sftpb* promoter activity. Thus, the inhibition by JUN of the *Sftpb* promoter is likely indirect and dependent on heteromeric complex formation.

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