Regulation of mouse SP-B gene promoter by AP-1 family members

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Regulation of mouse SP-B gene promoter by AP-1 family members. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L79–L88, 1999.—The regulatory role of activator protein-1 (AP-1) family members in mouse surfactant protein (SP) B (mSP-B) promoter function was assessed in the mouse lung epithelial cell line MLE-15. Expression of recombinant JunB and c-Jun un inhibited mSP-B promoter activity by 50–75%. Although c-Fos expression did not alter mSP-B transcription, Jun D enhanced mSP-B promoter activity and reversed inhibition of mSP-B by c-Jun or Jun B. A proximal AP-1 binding site (−18 to −10 bp) was identified by a thyroid transcription factor-1 binding site. Mutation of this proximal AP-1 site blocked both Jun B inhibition and Jun D enhancement and partially blocked c-Jun inhibition of promoter activity. Promoter deletion mutants were used to identify additional sequences mediating the inhibitory effects of c-Jun in the distal region from −397 to −253 bp. The AP-1 element in this distal site (−370 to −364 bp) is part of a composite binding site wherein AP-1, CAMP response element binding protein, thyroid transcription factor-1, and nuclear factor I interact. Point mutation of the distal AP-1 binding site partially blocked c-Jun-mediated inhibition of the SP-B promoter. Both stimulatory (Jun D) and inhibitory (c-Jun) effects of AP-1 family members on mSP-B promoter activity are mediated by distinct cis-acting elements in the mSP-B 5′-flanking region.

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SURFACTANT PROTEIN (SP) B is a 79-amino acid amphipathic polypeptide associated with surfactant phospholipids in the alveolus (for a review, see Ref. 41). SP-B enhances the rate of spreading and stability of phospholipids crucial to reducing alveolar surface tension. Both human and mouse SP-B (mSP-B) mRNAs are expressed in nonciliated bronchiolar and alveolar type II epithelial cells (9, 27). SP-B is critical for postnatal pulmonary adaptation. Transgenic mice homozygous for a null mutation in the SP-B gene failed to expand their lungs at birth (8). Likewise, human infants with mutations in the SP-B gene succumb to respiratory failure in the neonatal period (23). Lung compliance was decreased in heterozygous SP-B gene-targeted mice (7) associated with a 50% reduction in SP-B mRNA and protein, suggesting that reduction in SP-B alters pulmonary function. SP-B concentrations are reduced in various clinical conditions including respiratory distress syndrome in infants and acute respiratory distress syndrome in adults (12, 30). The temporal, spatial, and humoral regulation of SP-B synthesis are controlled at both transcriptional and posttranscriptional levels (41).

The expression of activator protein-1 (AP-1) family members is activated by phorbol esters (19). The potential role of AP-1 in the regulation of the SP-B gene is supported by the finding that phorbol ester inhibited SP-B transcription (31). SP-B mRNA and SP-B protein synthesis were inhibited by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) and by tumor necrosis factor-α in H441-4 cells and mouse lung (29, 31).

A variety of other growth factors, hormones, mitogens, and cytokines are also known to induce expression or activate members of the AP-1 family. In the lung, c-Jun mRNA is expressed at higher basal levels than in other tissues (33, 34). Agents that produce reactive oxygen radicals such as H2O2 or asbestos are strong inducers of c-Jun in lung cells (15, 39). Oxygen radicals cause injury to the lung epithelium (38) and increase SP-B expression in bronchiolar epithelial cells but decrease SP-B expression in alveolar epithelium (43).

A proximal AP-1 binding sequence has been identified in the human SP-B promoter (4, 22, 28). AP-1 binding sites have been found in the promoters of other lung-specific genes including Clara cell secretory protein (35) and SP-D (32). The present study identified two distinct regions of the mSP-B promoter bearing AP-1 binding sites that bind and function through different AP-1 family members.

MATERIALS AND METHODS

Plasmid construction and mutagenesis. The mSP-B promoter constructs were cloned in the pBLCAT6 reporter vector. This promoter template, p−1797/+42/CAT, and the primer bearing the appropriate mutation were used for site-directed mutagenesis as described by Kunkel (18). The sequence of the proximal AP-1 site was 5′-GAG CCC ATG ACT CAA GTA GGG TAC-3′ (−25 to −10 bp), and the proximal mutant TG-AP-1 primer sequence was 5′-GAG CCC Agt ACT CAA GTA GGG TAC-3′ (lowercase letters indicate bases that introduced mutation). The AP-1 mutant in the distal promoter region (p−653/−42/CAT) was generated by PCR site-directed mutagenesis as previously described (5), with the p−653/+42/CAT mSP-B promoter construct as a template. Primers bearing 5′ Hind III or 3′ Sal I sites were made to the −653 and +42 regions of the promoter, respectively. The wild-type (WT) sequence of the distal AP-1 binding site was 5′-CTT ACC CTG CGT CAA GAG CCA GGA-3′, and the sequence of the primer containing a mutation in that site was made to the −653 and +42 regions of the promoter, respectively. The wild-type (WT) sequence of the distal AP-1 binding site was 5′-CTT ACC CTG CGT CAA GAG CCA GGA-3′. 

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was 5′–CTT ACC Caa CGT CAA GAG CCA GGA–3′. All mutants were verified by sequencing.

A series of mSP-B promoter constructs containing 5′ deletions (p−1173, p−753, p−653, p−543, p−415, p−397, p−353, and p−297 SP-B(CAT)) were generated with the WT p−1797/+42/CAT plasmid template and PCR linker primers to the corresponding regions of the promoter bearing 5′-Hind III or 3′-Sal I restriction sites. PCR products were gel purified and cloned in the pBLCAT6 reporter vector. Correct mutants were verified by sequencing.

Cell culture, transfections, and reporter gene assay. The mouse dorsal cell line MLE-15 was derived from lung tumors produced in transgenic mice expressing SV40 large T antigen under the control of an SP-C lung-specific promoter (42). These cells were propagated in HITES (hydrocortisone, insulin, transferrin, estradiol, and sodium selenite) medium containing 4% fetal bovine serum (FBS; Sigma) as previously described (42). Transient transfections were done with the chloramphenicol acetyltransferase (CAT) reporter constructs containing 4% fetal bovine serum (FBS; Sigma) as previously described (3) in either 100-mm plates in duplicate or six-well Falcon plates in triplicate samples. The SP-B promoter/luciferase reporter (3,000 rpm for 5 min. The cell pellets were washed in PBS and scraped off on ice, washed in ice-cold PBS, and pelleted at 14,000 rpm for 10 min. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce). Nuclei were isolated by the high-salt procedure of Schreiber et al. (36), with modifications. Briefly, confluent monolayers of MLE-15 cells were scraped off on ice, washed in ice-cold PBS, and pelleted at 3,000 rpm for 5 min. The cell pellets were washed in PBS and lysed in two volumes of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 0.2% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) by gentle vortexing. Nuclei were isolated by centrifugation at 3,000 rpm for 5 min. Nuclear pellets were then resuspended, and the protein was extracted in one volume of high-salt buffer B (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 25% glycerol, 1 mM DTT, and 0.5 mM PMSF). Nuclear proteins were then recovered by centrifugation at 14,000 rpm for 10 min. Protein concentration was determined by the bicinchoninic acid method (Sigma) with BSA as a standard. Protein concentration in the nuclear extracts was typically 5–10 µg/µl.

EMSA. The gel shift probes were generated by annealing synthetic oligonucleotides (typically 24 mers) at a concentration of 10 µM in buffer C (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 50 mM NaCl). Double-stranded products were gel purified with 4% Biogel and Mermaid kit (BIO 101). The concentration of annealed oligonucleotides was then adjusted to 2 pmol/µl, and 1 µl was end labeled with γ-[32P]ATP and T4 polynucleotide kinase. The sequences of competitors used in the EMSAs were AP-1 collagenase (1, 21), 5′-CGG TTG ATG AGT CAG CCG GAA-3′; AP-1 osteocalcin (37), 5′-TCG ACA CCC GGT GAG TCA CCT AGA-3′; thyroid transcription factor-1 (TTF-1) thyroglobulin (6), 5′-CAC TGC CCA GTC AAC TGT TCT TGA-3′; and cAMP response element binding protein (CREB; Promega), 5′-AGA GAT TGCC CTG AC GAG AGC TAG-3′. The probes were purified with NICK G-50 columns (Pharmacia Biotech, Uppsala, Sweden) and diluted to 20,000 dpm/µl. Nuclear extracts (5 µg) were preincubated in the presence and absence of unlabeled competitors (100-fold excess or as indicated) in binding buffer [12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 12% glycerol, 4 mM DTT, 40 ng/µl of poly(dI-dC), and 0.2 mM PMSF] for 15 min at room temperature. Four microliters of probes were added, and the mixture was incubated for an additional 20 min. The reactions with recombinant proteins were performed in the absence of nuclear extract. Bacterially expressed TTF-1 homeodomain was a kind gift from Dr. R. DiLauro (Stazione Zoologica Anton Dohrn, Villa Comunale, Naples, Italy). Recombinant CREB DNA binding domain was purchased from Santa Cruz Biotechnology. All antibodies to the AP-1 family members (c-Jun, Jun B, and c-Fos) were purchased from Santa Cruz Biotechnology and typically added before the probe and incubated overnight at 4°C. Protein-bound and free probes were resolved with 5% nondenaturing gel electrophoresis. The gels were dried and exposed on X-ray film (Kodak) for 5 h at room temperature.

RESULTS

Proximal region of the mSP-B promoter contains overlapping AP-1 and TTF-1 binding sites. Conserved consensus binding sites for the AP-1 family of transcription factors are located in close proximity to the respective TATA boxes in both the mSP-B and human SP-B promoters. Previous in vitro DNase I footprinting analyses with H441 lung adenocarcinoma and HeLa cell nuclear extracts identified a protected region (+15 to +33) over a potential AP-1 binding site in the human SP-B promoter (4). Close inspection of the murine promoter sequence revealed the presence of potential overlapping binding sites for AP-1 (TGACTCA) and TTF-1 (CAAG) located at −18 to −10 bp from the transcription start site (Fig. 1A). Incubation of an oligonucleotide probe corresponding to this region with MLE-15 nuclear extracts revealed two DNA-protein complexes of similar mobility (Fig. 1B, lane 1). As shown in Fig. 1B, formation of the slower mobility complex was inhibited by 100-fold molar excess of a cold oligonucleotide competitor bearing the AP-1 (lane 3) consensus sequence from the collagenase gene promoter (1, 21). Formation of the faster mobility complex was inhibited by 100-fold molar excess of a oligonucleotide competitor containing the TTF-1 binding site (Fig. 1B, lane 4) from the thyroglobulin gene promoter. This element (−18 to −10 bp) also bound recombinant TTF-1 homeodomain protein. Therefore, both AP-1 and TTF-1 factors bind in close proximity to each other at the proximal (−18 to −10 bp) element in the murine SP-B promoter.
Fig. 1. Identification of a proximal overlapping activator protein-1 (AP-1)/thyroid transcription factor-1 (TTF-1) binding site. A: schematic representation of proximal promoter AP-1/TTF-1 overlapping binding sites (–18 to –10 bp) and their close apposition to promoter TATA box (binding sites underlined) and start of murine surfactant protein (SP) B (mSP-B) gene transcription and translation. B: electrophoretic mobility shift assays (EMSAs) were performed with wild-type (WT) probe (–25 to –2 nucleotides; lanes 1–5) and with probe containing mutations in AP-1 binding sequence (TG-AP-1; lanes 6–10). Binding to probe was competed with 100-fold excess of cold competitors: self (S), AP-1 consensus site oligonucleotide from collagenase promoter (A), and TTF-1 binding site from thyroglobulin gene promoter (T). Purified TTF-1 homeodomain (HD) was added in place of nuclear extract. Mutation in AP-1 binding site did not interfere with TTF-1 binding (lanes 6–10). C: EMSA supershift analysis with WT proximal oligonucleotide probe (–25 to –2 nucleotides). Unlabeled self-competitor was added in a 100-fold molar excess over probe. Antisera (antibodies (AB)) to indicated AP-1 family members produced supershifted complexes with WT proximal probe (arrows, Jun B and Jun D). Antisera to DNA binding domain of c-jun and c-Fos weakly reduced formation of protein-DNA complex in MLE-15 nuclear extracts. All experiments were repeated 3 times.
To further characterize the binding of AP-1 and TTF-1 to this region, we generated oligonucleotides containing point mutations to abolish the binding of the AP-1 complex. As shown in Fig. 1B, formation of the slower-mobility AP-1 complex was abolished when the oligonucleotide TG-AP-1 bearing a point mutation at the AP-1 site (TGACTCA → gACTCA) was used as a probe for EMSA analysis (lanes 6–10), leaving the faster-mobility TTF-1 complex intact. Weak competition was observed when an AP-1 cold competitor from the collagenase promoter was used (Fig. 1B, lane 8). This is probably due to a cryptic TTF-1 site in the AP-1 competitor (CTTG). Antibodies to the AP-1 family members did not supershift the TG-AP-1 probe, confirming that the point mutation abolished the ability to interact with the AP-1 site (data not shown). The TTF-1 cold competitor partially inhibited the complex formed with the TG-AP-1 probe and MLE-15 cell nuclear proteins (Fig. 1B, lane 9). The TG-AP-1 mutant retained the ability to bind the recombinant TTF-1 homeodomain (Fig. 1B, lane 10). Similarly, binding of TTF-1 was blocked by a mutation in the TTF-1 consensus site (G-TTF-1 mutant) that left the AP-1 site intact (Sever-Chroneos, Bachurski, and Whitsett, unpublished observations). These findings suggest that AP-1 and TTF-1 proteins can bind to the −18- to −10-bp site independently of one another.

Preliminary studies with S1 nuclease protection analysis showed that MLE-15 cells expressed mRNAs of AP-1 family members c-Jun, Jun B, Jun D, c-Fos, and Fos B (data not shown). Supershift assay and transient transfections with expression constructs were used to investigate the role of different AP-1 family members in the regulation of the murine SP-B gene in MLE-15 cells. Antibodies to Jun B and Jun D supershifted the WT probe containing the proximal AP-1 site sequence (Fig. 1C, lanes 4 and 5). Antibodies to the c-Jun and c-Fos DNA binding domains partially inhibited the protein–DNA complex (Fig. 1C, lanes 3 and 6, respectively), and an antibody to the NH₂ terminus of c-Jun produced a weak supershift (data not shown). Thus the complex binding to the proximal AP-1 site in MLE-15 nuclear extracts consists primarily of Jun B, Jun D, and possibly c-Jun and c-Fos in vitro.

Distinct regulation of the mSP-B promoter by different AP-1 family members. To further investigate the significance of AP-1 family members binding to the proximal AP-1 site, expression vectors for c-Jun, Jun B, Jun D, and c-Fos were cotransfected with the p1797/TG-AP-1/CAT, was cotransfected with an empty RSV vector.

![Graph A](image1.png)

**Fig. 2. Role of distinct AP-1 family members in regulation of mSP-B.** A: expression of c-Jun and Jun B inhibited, whereas Jun D enhanced, mSP-B promoter (p–1797/+42/CAT) activity. Transient transfections were done in MLE-15 cells with 12 µg of [promoter/chloramphenicol acetyltransferase (CAT) construct]/100-mm plates, 25 µg of cotransfected (co-transf) expression plasmid, and 7 µg of pCMV-β-gal as an internal control. Relative CAT activity is expressed as a percentage of WT mSP-B promoter activity cotransfected with an empty RSV vector, normalized to cotransfected β-galactosidase (β-gal), and set arbitrarily to 100%. Values are means ± SE from a representative experiment. *P ≤ 0.002 relative to WT mSP-B promoter cotransfected with empty RSV vector. B: mutation in proximal AP-1 site blocked inhibition of activity by Jun B and enhancement by Jun D but did not block inhibition by c-Jun. mSP-B promoter with a mutation in proximal AP-1 site (p–1797/TG-AP-1/CAT; 12 µg) was cotransfected with 2.5 µg of c-Jun, Jun B, Jun D, or c-Fos expression plasmids, respectively. Transfections were done in triplicate and normalized to activity of cotransfected β-gal. Values are means ± SE from a representative experiment; n = 3 replicates/assay. *P ≤ 0.004 relative to activity from mutant mSP-B promoter cotransfected with empty RSV vector.
proximal AP-1 binding site. Activation of mSP-B promoter activity by Jun D was also blocked by mutation of the proximal AP-1 site. This indicates that Jun B and Jun D AP-1 family members interact with the proximal AP-1 site in MLE-15 cells. Mutation of the proximal AP-1 site partially blocked c-Jun-mediated inhibition of mSP-B promoter activity (Fig. 2B), indicating that c-Jun may act through additional AP-1 sites in the mSP-B promoter.

The AP-1 family of transcription factors binds to DNA sites as homodimers or heterodimers (2). Therefore, distinct combinations of AP-1 family members were coexpressed with mSP-B promoter p−1797/+42/CAT to determine whether their coexpression alters mSP-B gene transcription. Coexpression of both c-Jun and Jun B constructs inhibited mSP-B promoter activity by 75%. Coexpression of c-Fos did not alter the inhibitory effect of c-Jun or Jun B. However, coexpression of Jun D partially blocked the inhibitory effects of both c-Jun and Jun B (Fig. 3). Therefore, AP-1 family members have distinct effects on the activity of the mSP-B promoter.

Specificity and localization of the mSP-B promoter region mediating inhibitory effects of c-Jun. Transient transfection of the pRSV/c-Jun expression vector in MLE-15 cells inhibited SP-B promoter function in vitro. Increasing concentrations of the pRSV/c-Jun expression construct were cotransfected with the mSP-B WT

Fig. 3. Jun D reversed inhibitory effects of c-Jun and Jun B on SP-B promoter. MLE-15 cells were transiently transfected with WT mSP-B promoter p−1797/+42/CAT and an empty RSV plasmid or corresponding AP-1 expression vector. +, With; −, without. Transfection experiments were normalized to β-gal activity and WT promoter/CAT activity was arbitrarily set to 100%. Activity of mSP-B promoter cotransfected with indicated combinations of AP-1 family member expression vectors is presented relative to promoter cotransfected with empty RSV vector. Values are means ± SE; n = 3 replicates/assay. *P < 0.002 relative to mSP-B promoter replicates cotransfected with empty vector.

Fig. 4. c-Jun inhibits mSP-B promoter activity through distal 2397- to 2353-bp region. A: dose-response relationship of c-Jun inhibition of mSP-B promoter activity was assessed in MLE-15 cells that were transiently transfected with full-length mSP-B p−1797/+42/CAT construct and increasing concentrations of pRSV/c-Jun (0.2, 0.25, 0.3, 0.4, 0.5, 1.0, 2.5, and 5.0 µg/100-mm plate) expression plasmid. Transfections were done in duplicate, normalized to β-gal activity, repeated 2–4 times, and plotted as means ± SE. WT promoter activity was arbitrarily set to 100%. CAT activity without c-Jun: *P, 0.0002; **P, 0.03.

Relative CAT activity in MLE-15 cells

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Values are means ± SE. P values were calculated relative to control CAT activity without c-Jun: *P < 0.0002; **P < 0.03.
(p−1797/+42/CAT) promoter. SP-B promoter activity was inhibited in a dose-dependent manner with cotransfection of 0.2–0.5 µg of c-jun expression vector (Fig. 4A). The inhibition of promoter activity by c-jun reached a threshold (~75%) with 1.0 µg of cotransfected c-jun. Cotransfection of 2.5 and 5 µg of pRSV/c-jun did not further inhibit mSP-B promoter activity. This indicates that c-jun is a specific inhibitor of basal mSP-B promoter activity in MLE-15 cells.

Transient cotransfection of p−1797/TG-AP-1/CAT and pRSV/c-jun in MLE-15 cells suggested the presence of a distinct promoter site mediating inhibition of SP-B activity by c-jun (Fig. 2B). To identify the site of c-jun inhibitory effects, a series of five deletions of the mSP-B promoter were tested in transient transfection assays in MLE-15 cells in the presence and absence of pRSV/c-jun. As shown in Fig. 4B, most of the inhibitory effects of c-jun were lost after deletion of a region located between −397 and −353 bp from the start of transcription, indicating a potential interaction of c-jun with this region. Interestingly, basal promoter activity was lost with the deletion of promoter sequences from −415 to −353 bp. This region was also protected by nuclear proteins from MLE-15 cells in vitro DNase I footprinting assays (Sever-Chroneos, Bachurski, and Whitsett, unpublished observations).

Analysis of distal −370 to −364 bp AP-1 binding site. The mSP-B promoter in the region from −397 to −353 bp contains potential binding sites for a number of transcription factors that influence SP-B promoter activity. A potential AP-1 binding site overlaps with CREB, and both are closely apposed to a TTF-1 binding consensus (−365 to −362 bp) and a half binding site (−360 to −357 bp) for the nuclear factor I (NF-I) family members (Fig. 5A). Disruption of TTF-1 and NF-I binding to these sites abolished basal mSP-B promoter activity (unpublished observations). The interaction of TTF-1 and NF-I transcription factors with the promoter region from −397 to −353 bp is likely to be responsible for the loss of basal promoter activity with the deletion of promoter sequences from −397 to −353 bp (Fig. 4B).

To investigate binding of AP-1 factors to this region, we used a WT oligonucleotide probe to the −377 to −353-bp region in the EMSA experiment. A single complex was detected with this probe in the presence of MLE-15 nuclear proteins (Fig. 5B, lanes 1 and 2). In addition, an oligonucleotide bearing a point mutation (CTGCGTCA → CaaCGTCA) in the distal AP-1 site (−370 to −364 bp) also formed a single complex with MLE-15 nuclear proteins (Fig. 5B, lanes 3 and 4). The formation of this complex was not inhibited by the presence of the AP-1 consensus from an osteocalcin promoter, but competition with a CREB consensus oligonucleotide partially inhibited complex formation and recombinant CREB formed a complex with an AP-1 mutant probe (Fig. 5B, lanes 5–7), suggesting that CREB interacts with the −377 to −353 bp binding site independently from AP-1. Moreover, the complex formed by the AP-1 mutant probe was inhibited by both TTF-1 and NF-I unlabeled competitors (Sever-Chroneos, Bachurski, and Whitsett, unpublished observations). A point mutation in the putative TTF-1 and NF-I binding sites (TTF-1/NF-I mut) formed a complex that interacted with antibodies to both Jen D and c-jun (Fig. 5B, lanes 8–11).

Mutation of the distal AP-1 binding site (p−653/AP-1/CAT) increased basal promoter activity fivefold in MLE-15 cells (Fig. 6). This indicates that the distal AP-1 (−370 to −364 bp) site is an inhibitory promoter element. To test whether c-jun inhibition is mediated through the distal AP-1 binding site, the WT p−653/+42/CAT and distal mutant p−653/AP-1/CAT mSP-B promoter constructs were transfected with and without the pRSV/c-jun expression vector (Fig. 6). Transient expression of c-jun in MLE-15 cells inhibited WT promoter activity by 80% (Fig. 6). Similar to the cotransfection of the proximal AP-1 site mutant promoter, CAT activity of the distal AP-1 mutant promoter was inhibited by 50% with c-jun, indicating that the distal AP-1 element (−370 to −364 bp) is involved in, but is not sufficient for, c-jun-mediated inhibition of promoter activity. Interestingly, coexpression of Jen D with c-jun overcame the c-jun un-mediated inhibition of promoter activity. Interestingly, coexpression of Jen D with c-jun overcame the c-jun un-mediated inhibition of promoter activity. In summary, AP-1 family members interact with both proximal (−18 to −10 bp) and distal (−370 to −364 bp) mSP-B promoter sites. mSP-B promoter activity is inhibited by the interaction of Jen B with the proximal AP-1 binding site and by the interaction of c-jun with both the proximal and distal AP-1 binding sites. Cotransfection with Jen D stimulates SP-B promoter function and antagonizes the inhibitory effects of c-jun and Jen B on mSP-B promoter activity.

**DISCUSSION**

Two distinct sites of AP-1 family member interaction with the mSP-B promoter were identified. A proximal AP-1 binding site that overlaps with a TTF-1 binding site is located from −18 to −10 bp from the transcription start site (Fig. 1A). Similarly, an AP-1 consensus is situated at +20 to +26 bp in the human SP-B promoter (4), demonstrating the conservation of this motif in the proximal region of human SP-B and mSP-B promoters. Inhibition of mSP-B promoter activity by Jen B was mediated by the proximal (−18 to −10 bp) AP-1 site, whereas the inhibitory effect of c-jun was mediated by both the proximal and distal regions from −397 to −253 bp in the mSP-B promoter. A cis-acting element located at −370 to −364 bp in the distal promoter region contained superimposed AP-1 binding/CREB sequences overlapping with binding sites for other transcription factors. Jen D interacted with the proximal (−18 to −10 bp) AP-1 binding site to enhance promoter activity and block the inhibitory effects of both c-jun and Jen B. These results suggest a complex
interaction of distinct AP-1 family members binding to at least two regions of the mSP-B promoter.

In this work, we show that c-Jun inhibits mSP-B promoter activity in a dose-dependent manner by interacting with both the proximal (−18 to −10 bp) and distal (−397 to −253 bp) regions of the SP-B promoter. Moreover, the proximal AP-1 binding site mediated inhibition of the mSP-B promoter by Jun B. Jun D enhanced promoter activity through the proximal AP-1 site in vitro. In another system, different AP-1 family members also have distinct effects on estrogen-dependent transcription. In that system, the expression of c-Jun, Jun B, and c-Fos inhibited estrogen-induced estrogen receptor activity, whereas Jun D did not affect promoter activity (10).

Deletion mutants of the mSP-B promoter cotransfected with c-Jun expression vector pinpointed an additional site of c-Jun inhibitory effects between −397 and −253 bp. However, with the use of MLE-15 cell extracts, the binding of endogenous c-Jun to the proximal and distal promoter elements was weak. Consistent with this observation, MLE-15 cells expressed...
lower levels of c-jun mRNA compared with Jun B and Jun D mRNAs (Sever and Whitsett, unpublished observations). In addition, the level of Jun D mRNA in the mouse lung was previously estimated to be 5–10 times higher than the level of c-jun mRNA (13). Therefore, the high levels of endogenous SP-B expression in MLE-15 cells (42) correlate with the low levels of c-jun and increased Jun D expression in these cells.

Sequence analysis of the −370- to −364-bp promoter element identified an imperfect AP-1 and a half CREB site. A previous study (44) suggested that CREB inhibits human SP-B promoter activity. Another potential CREB site is conserved at −78 bp of human SP-B (28) and at −80 bp of the murine promoter. Interestingly, recombinant CREB DNA binding domain also binds to the WT proximal AP-1 mSP-B oligonucleotide probe from −25 to −2 bp (data not shown). Similar findings were reported wherein CREB sites mediated c-jun inhibition of the placentomal hormone chorionic gonadotropin α and β gene transcription (26) and c-jun and c-Fos inhibition of Myo D transcription (25). Jun D activates the proenkephalin promoter through the CREB site, and that activation is dependent on protein kinase A activation but is blocked by cotransfected Jun B (16).

The expression of Jun D alone in MLE-15 cells enhanced SP-B promoter activity, and Jun D antagonized the c-jun- and Jun B-mediated inhibition of SP-B promoter activity. Cotransfection of Jun D with c-jun restored promoter activity to 75% of the control value. Cotransfection of Jun D with Jun B abolished the inhibitory effect of Jun B on SP-B promoter activity. This observation is likely explained if, for example, heterodimers of c-jun and Jun B with c-Fos are active inhibitors of SP-B gene transcription. On the other hand, heterodimers of Jun B with either c-jun or Jun B are likely inactive as repressors of SP-B promoter activity. Similarly, in liver cells (14), heterodimerization of Jun B with c-Fos activate, whereas Jun B and liver regenerating factor-1 dimers repress the c-Fos/c-jun-mediated activation. We speculate that expression of distinct combinations of AP-1/jun and CREB family members in the alveolar and bronchiolar epithelia may have distinct effects on mSP-B gene regulation.

In the mSP-B promoter, AP-1/CREB and TTF-1 share overlapping binding sites located from −18 to −10 and −370 to −364 bp and are likely to compete for binding to their respective sites. It is well established that repression of transcription may occur by the prevention of binding (24). The repression is indirect when a repressor binds to the activator binding site and thus prevents the activator from binding (for a review, see Ref. 20). For example, c-jun and c-Fos inhibit osteocalcin gene transcription by preventing retinoic acid receptor binding to the same sequence (37). It is conceivable that the binding of the negative regulators c-jun, Jun B, and CREB may compete for binding with TTF-1 or NF-I activators. We speculate that binding of distinct AP-1 heterodimers to the proximal and distal AP-1 sites of the mSP-B promoter may either allow or interfere with binding of other transcriptional activators such as TTF-1 to overlapping sites.

The repression of mSP-B gene transcription may thus be regulated by a variation in the concentration or activity of factors such as c-jun, Jun B, CREB, and Jun D. Because Jun D is highly expressed in the lung (13), formation of AP-1 heterodimers with Jun D may allow other transcriptional activators such as TTF-1 to bind to the overlapping site(s), enhancing SP-B gene expression. The dynamic equilibrium between the relative abundance of AP-1/CREB and TTF-1 factors therefore may provide a mechanism by which SP-B gene regulation may be modulated during cell differentiation, proliferation, or repair.

Recently, TPA has been shown to regulate human SP-B expression by cytoplasmic trapping of TTF-1 and hepatocyte nuclear factor-3 in A549 and H441 cells, respectively (17). Inhibitory effects of TPA on human SP-B transcription were localized to the proximal promoter in H441 cells (40). This is consistent with a region containing TTF-1, hepatocyte nuclear factor-3,
and AP-1 binding sites in the human SP-B promoter (4). Most commonly, AP-1 activity is associated with the activation of the protein kinase C signal transduction pathway. However, in other systems, AP-1 activity is also induced by a number of cytokines (tumor necrosis factor-α), growth factors (transforming growth factor-β and fibroblast growth factor), and bacterial products (lipopolysaccharide) (reviewed in Ref. 2), some of which are also known inhibitors of SP-B expression (29). Moreover, in mouse models of oxygen lung injury and adenoviral infection, SP-B mRNA expression is decreased (43, 45). A modest reduction in SP-B in heterozygous SP-B knockout mice alters lung function. Therefore, the elucidation of factors regulating SP-B concentrations in the lung may have important clinical implications (7). The AP-1 family members may interact with the SP-B promoter to influence SP-B expression during development or after lung injury.

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