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Ramgopol K. Margana
University of Texas Health Science Center at Tyler

Vijayakumar Boggaram
University of Texas Health Science Center at Tyler, vboggaram@uttyler.edu

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Functional Analysis of Surfactant Protein B (SP-B) Promoter

Sp1, Sp3, TTF-1, and HNF-3α TRANSCRIPTION FACTORS ARE NECESSARY FOR LUNG CELL-SPECIFIC ACTIVATION OF SP-B GENE TRANSCRIPTION*

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Ramgopal K. Margana and Vijayakumar Boggaram‡

From the Department of Molecular Biology, The University of Texas Health Science Center at Tyler, Tyler, Texas 75710

Surfactant protein B (SP-B) is essential for maintenance of biophysical properties and physiological function of pulmonary surfactant. SP-B mRNA expression is restricted to alveolar type II epithelial cells and bronchiolar epithelial cells (Clara cells) of adult lung. We previously (Margana, R. K., and Boggaram, V. (1996) *Am. J. Physiol.* 270, L601–L612) found that a minimal promoter region (−236 to +39) of rabbit SP-B gene is sufficient for high level expression of chloramphenicol acetyltransferase reporter gene in NCI-H441 cells, a cell line with characteristics of Clara cells. In the present study we used mutational analysis, electrophoretic mobility shift assays, and DNase I footprinting to identify *cis*-DNA regulatory elements and *trans*-acting protein factors required for lung cell-specific expression of SP-B gene. We found that in addition to thyroid transcription factor 1 (TTF-1) and hepatocyte nuclear factor 3α (HNF-3α) binding sites, two spatially separate DNA sequences that bind Sp1 and Sp3 factors are necessary for the maintenance of SP-B promoter activity. Mutation of any one of the transcription factor binding sites caused a significant reduction in SP-B promoter activity suggesting that Sp1, Sp3, and TTF-1 and HNF-3α interact cooperatively with SP-B promoter to activate gene transcription.

Surfactant protein B (SP-B),[1] a hydrophobic protein of pulmonary surfactant, is essential for maintenance of biophysical properties and physiological function of surfactant (1). The critical role of SP-B in surfactant function is suggested by its deficiency in newborns with congenital alveolar proteinosis (2). Infants diagnosed with alveolar proteinosis die of respiratory failure despite maximal medical assistance. Targeted disruption of SP-B gene causes abnormalities of surfactant metabolism and respiratory failure in newborn mice, further supporting the important role of SP-B in lung function (3). SP-B mRNA is developmentally induced and in adult lung SP-B mRNA is expressed in a highly cell type-specific manner in alveolar type II cells and bronchiolar epithelial (Clara) cells (4, 5). SP-B mRNA is increased by glucocorticoids and agents that increase intracellular cyclic AMP (6–10).

Transcription initiation plays a key role in the control of gene expression during terminal differentiation of cell types. Activation of cell/tissue-specific gene transcription is dependent on interactions between transcription factors (activators and repressors), some of which are expressed widely and others are restricted in their distribution, and the general transcriptional machinery (11, 12). How interactions between various transcription factors result in cell/tissue-specific activation of gene transcription is not yet well understood.

We previously isolated and sequenced rabbit SP-B gene and determined that a minimal SP-B promoter region spanning −236 to +39 nucleotides is sufficient for high level expression of CAT reporter gene in a cell-specific manner in NCI-H441 cells, a human pulmonary adenocarcinoma cell line with characteristics of Clara cells (13). We also determined that the SP-B minimal promoter contained a lung cell/tissue-specific enhancer (13). SP-B promoter activity was enhanced in HeLa cells by co-expression of thyroid transcription factor 1 (TTF-1), suggesting that it contained sequence element(s) for TTF-1 binding (13). TTF-1 and hepatocyte nuclear factor 3α (HNF-3α) have been shown recently to play important roles in human SP-B promoter activity in NCI-H441 cells (14, 15). TTF-1 and HNF-3 are also expressed in tissues other than lung, and during lung development TTF-1 and HNF-3 are expressed before differentiation of alveolar type II cells and expression of SP-B mRNA (16, 17). These observations suggest that TTF-1 and HNF-3 are not sufficient for cell type-specific activation of SP-B gene transcription and that additional factors might be required for activation of SP-B gene transcription.

The objective of our investigation was to identify *cis*-acting DNA elements and interacting protein factors necessary for lung cell-specific expression of rabbit SP-B gene. We used mutational analysis, electrophoretic mobility shift assays, and DNase I footprinting to identify DNA sequence elements and interacting protein factors important for the functional activity of SP-B promoter. We found that in addition to TTF-1 and HNF-3 binding sites, two spatially separate DNA sequences that bind Sp1 and Sp3 factors play critical roles in maintaining functional activity of SP-B promoter. Mutation of any one of these sites resulted in a significant reduction in SP-B promoter activity, suggesting that combined or cooperative interactions of Sp1, Sp3, and TTF-1 and HNF-3α transcription factors with SP-B promoter is necessary for activation of gene transcription. Some of the findings reported in the present study have been presented in preliminary form (18).

MATERIALS AND METHODS

Nuclear Extract Preparation—Nuclear extracts from NCI-H441 cells and other cells were prepared according to the method described by Ausubel *et al.* (19). Typically cells from 10 confluent 75-cm^2 flasks were used for preparation of nuclear extracts. Nuclear extracts were aliquoted into chilled tubes and rapidly frozen in liquid nitrogen and stored at −80 °C. The protein concentration of nuclear extract was determined by Bio-Rad protein assay (20).

DNase I Footprinting—DNase I footprinting reactions were per-

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‡ To whom correspondence and reprint requests should be addressed: Dept. of Molecular Biology, The University of Texas Health Science Center at Tyler, P. O. Box 2003, Tyler, TX 75710. Tel.: 903-877-7780; Fax: 903-877-5731.

[1] The abbreviations used are: SP, surfactant protein; CAT, chloramphenicol acetyltransferase; TTF-1, thyroid transcription factor 1; HNF-3, hepatocyte nuclear factor-3; PCR, polymerase chain reaction.

TABLE I
Oligonucleotides used as primers in the PCR-based mutagenesis
Putative transcription factor binding sites are underlined, and mutated nucleotides are shown in bold italics.

Primers		Sequence
Sp1 (−207)	−223	GGAGTCGG***TT***CAGGGACAAA −193
Sp1 (−130)	−138	GCTGGGAAGG***TT***CTGGTTCAAAACA −115
Sp1 (−35)	−48	CATGCTCCCCG***AA***CCCAGCTATAA −26
Myc-Max (−115)	−124	GGTTCAAAACAC***GGA***GAGGGCTCT −101
HNF-3 (−88)	(i) −96	GACAAAGGC***GTCG***ACTGAGGTCACCA −71
	(ii) −96	GACAAAGG***A***GTCG***C***CTGAGGTCACCA −71
TTF-1 (−112, −102)	−120	CAAAACAC***A***GG***T***GGGCTC***A***CCA***C***GACAAAGGC −88
ETS (−51)	−61	CAGAGCCCTG***C***TC***A***ATGCTCCCG −39
ETS (−162)	−169	CAAATCAGG***T***G***CT***G***C***GGGCAGGAA −146

formed as described by Lakin (21) with modifications. The sense and antisense strands of SP-B fragment −236 to +39 were labeled as follows: pSKCATΔS (22) containing SP-B fragment from −236 to +39 was linearized with *Bam*HI or *Hin*dIII, and the DNA was dephosphorylated. Dephosphorylated DNA was digested with *Pst*I or *Bam*HI to release the SP-B fragment, and the fragment was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. For footprinting 0.5–1 ng of labeled DNA probe (2.5–5.0 × 10^4 cpm) was incubated with H441 or HepG2 nuclear proteins or 1 footprinting unit (50 ng) of purified human recombinant Sp1 protein (Promega) for 20 min at 30 °C in 20 μl of binding buffer that contained 13 mM HEPES, pH 7.9, 13% glycerol, 80 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 67 μg of bovine serum albumin, and 0.2 μg of poly(dI-dC) as nonspecific competitor. Following incubation the reaction mixture was treated with 3 μl of DNase I (Promega) (3 μl of DNase I (1 unit/μl) was diluted to 50 μl in 5 mM $CaCl_2$ and 10 mM $MgCl_2$) for 30 s at room temperature. DNase I reaction was terminated by addition of buffer containing 8.5 mM EDTA, 8.5 μg/ml proteinase K, 85 μg/ml tRNA, and 0.07% SDS followed by incubation at 37 °C for 20 min and then at 68 °C for 2 min. The samples were phenol/$CHCl_3$-extracted, and DNA was ethanol-precipitated. DNA was dissolved in 10 μl of denaturing loading solution (90% formamide, 10 mM EDTA, pH 8.0, 0.01% bromphenol blue, and 0.01% xylene cyanol) and analyzed on a 6% denaturing polyacrylamide sequencing gel.

Plasmid Constructions and Site-directed Mutagenesis—pSKCATΔS containing SP-B promoter fragment −236 to +39 or −730 to +39 served as the template for site-directed mutagenesis by polymerase chain reaction (PCR) according to the method described by Nelson and Long (23). Briefly, the method uses four synthetic oligonucleotide primers. One oligonucleotide contained the desired mutation(s), and the other three oligonucleotides were designed to allow selective amplification of the mutated sequence by PCR. Oligonucleotides that served as primers (sense) to introduce mutations into the DNA binding sites of transcription factors are shown in Table I.

In all reactions the upstream primer (−246 sense) was 5′-CTGTTCAGAA*GGATCC*AGGAACCCAGGCCTG-3′ and the downstream hybrid primer (+29 antisense) was 5′-**ATTAACCCTCACTAAAGGGA**GCAGCCACGG*CTGCAG*GTGT. The downstream primer contained a single mutation at nucleotide +37 that generated a *Pst*I site in the amplified DNA. The sequences of *Bam*HI and *Pst*I sites in the oligonucleotide primers are shown in italics. The 5′ 20-nucleotide unique sequence of the downstream hybrid primer is shown in bold.

All reactions (100 μl) were performed in a Perkin-Elmer gene amp PCR system 2400 using 1–2 fmol of template DNA, 0.3 μM amounts of primers and AmpliTaq DNA polymerase. Unless stated otherwise the reaction conditions included denaturation at 94 °C for 5 min followed by a 30-cycle amplification that consisted of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Amplification was followed by a final extension at 72 °C for 7 min. The method consisted of four separate reaction steps. In step 1 DNA was amplified using pSKCATΔS containing SP-B promoter fragment −730 to +39 as template and mutagenic and hybrid primers. The amplified DNA was purified by agarose gel electrophoresis and used as the primer for step 2. The step 2 reaction contained approximately 0.6 pmol of the product of step 1 and pSKCATΔS containing SP-B promoter fragment −730 to +39 as template. Amplification was carried out as a single cycle that consisted of denaturation at 94 °C for 5 min, annealing at 37 °C for 2 min, and extension at 72 °C for 10 min. In step 3, the upstream sense primer and the oligonucleotide primer identical to the 5′ segment of the hybrid primer, 5′-ATTAACCCTCACTAAAGGGA-3′, were added, and 30-cycle DNA amplification was performed.

Nucleotides in the HNF-3 sequence motif were replaced by PCR mutagenesis in two steps; mutant DNA obtained using mutagenic primer (i) was used as the template with mutagenic primer (ii) to obtain SP-B promoter fragment in which all of the nucleotides in the HNF-3 binding site had been mutated. SP-B promoter fragment containing mutations in the Myc-Max binding motif at +30 was obtained by PCR amplification using pSKCATΔS containing SP-B promoter fragment −730 to +39 as the template and upstream primer (−246, sense), 5′-CTGTTCAGAA*GGATCC*AGGAACCCAGGCCTG-3′ and downstream primer (+50, antisense), 5′-CTT*GCCTGCAG*CCACGGCGG***TC-C***GTGTGACTTGGCCGT-3′. The DNA binding site is underlined, and the mutated nucleotides are shown in bold italics. *Bam*HI and *Pst*I sites are shown in italics. In all cases the final amplified DNA was phenol/$CHCl_3$ extracted and digested with *Bam*HI and *Pst*I. The digested DNA was purified by agarose gel electrophoresis and inserted upstream of CAT gene in pSKCATΔS. The sequence of insert DNA was determined to verify that it contained the desired mutations.

Electrophoretic Mobility Shift Assays—Double-stranded synthetic oligonucleotides were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Electrophoretic mobility shift assays were performed by incubating 0.5 or 1.0 ng (10^4 to 2 × 10^4 cpm) of the oligonucleotide probe (Table II) with 5 μg of nuclear protein in 20 μl of binding buffer (13 mM HEPES, pH 7.9, 13% glycerol, 80 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 67 μg of bovine serum albumin and 0.2 μg of poly(dI-dC) as nonspecific competitor) for 20 min at 30 °C. In some experiments nuclear proteins were preincubated in binding buffer prior to addition of the labeled probe. Competition experiments were performed by addition of indicated molar excess of cold wild type or mutant oligonucleotides prior to addition of the labeled probe. In case of antibody supershift assays, nuclear extracts were preincubated with preimmune IgG or polyclonal antibodies to transcription factors for 1 h or overnight at 4 °C prior to incubation with oligonucleotide probe. Polyclonal antibodies to human Sp1, Sp2, Sp3, and Sp4 were purchased from Santa Cruz Biotechnology. Polyclonal antibodies to forkhead domains of mouse HNF-3α, HNF-3β, and HNF-3γ were kindly supplied by Drs. Gunther Schutz and Wolfgang Schmid, German Cancer Research Center, Heidelberg, Germany. Polyclonal antisera to the N-terminal portion of rat T/EBP (TTF-1) was kindly supplied by Dr. Shioko Kimura, National Cancer Institute. After incubation the DNA-protein complexes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel containing 0.5 × TBE (0.045 M Tris borate and 0.001 M EDTA) using 0.5 × TBE as running buffer. Electrophoresis was performed at constant current (30–35 mA) for 1.5–2 h. Gels were vaccum-dried and exposed to autoradiographic film.

Cell Culture and Transfections—Cell lines were maintained in culture medium supplemented with 10% fetal bovine serum, penicillin (100 units), streptomycin (100 μg), and amphotericin B (0.25 μg) at 37 °C in a humidified atmosphere of 5% CO_2 and air. NCI-H441 (24), a human pulmonary adenocarcinoma cell line with characteristics of Clara cells which expresses SP-B endogenously, was maintained in RPMI 1640 medium. HeLa, a human cervical cancer cell line, was maintained in Dulbecco's modified Eagle's medium.

Plasmid DNAs were transiently transfected into cells by liposome-mediated DNA transfer using LipofectAMINE (Life Technologies, Inc.) as described previously (13). At least two independent preparations of plasmids were used for transfection. pCH110 (Pharmacia Biotech Inc.), a β-galactosidase expression vector served as an internal control for normalization of transfection efficiency.

CAT and β-Galactosidase Assays—CAT activity of cell extracts was determined by the liquid scintillation counting assay (25) using [^{14}C]chloramphenicol and *n*-butyryl coenzyme as described previously (13). β-Galactosidase activity was determined by chemiluminescent assay using Galacto-Light Plus (Tropix, Bedford, MA) substrate accord-

TABLE II

Wild type and mutated double-stranded oligonucleotides used in gel mobility shift assays

Mutated nucleotides are shown in bold italic. *wt* = wild type; *mt* = mutant; *cons* = consensus.

Primers	Sequence
Sp1 (−207) *wt*	−215 TGGGAGTCGGGGCAGGGACAAAGG−190 ACCCTCAGCCCCGTCCCTGTTTCC
Sp1 (−130) *wt*	−138 GCTGGGAAGGGGCTGGTTCAAAACA −114 CGACCCTTCCCCGACCAAGTTTTGT
Sp1 (−30) *wt*	−50 TCCATGCTCCCGCCCCCAGCTAT −28 AGGTACGAGGGCGGGGGTCGATA
Sp1 *cons*	5′- ATTCGATCGGGGCGGGGCGAGC-3′ 3′- TAAGCTAGCCCCGCCCCGCTCG-5′
Sp1 *mt*	5′- ATTCGATCGG***TT***CGGGGCGAGC-3′ 3′- TAAGCTAGCCAAGCCCCGCTCG-5′
TTF-1 *wt*	−118 TCAAAACACCTGGAGGGCTCTCCAGGACAAAG −90 AGTTTTGTGGACCTCCCGAGAGGTCCTGTTTC
TTF-1 *mt*	−118 TCAAAACACC***A***GG***T***GGGCTC***A***CCA***C***GACAAAG −90 AGTTTTGTGGTCCACCCGAGTGGTGCTGTTTC
HNF-3 *wt*	−96 GACAAAGGCAAACACTGAGGTC −75 CTGTTTCCGTTTGTGAGTCCAG
HNF-3 *mt*	−96 GACAAAGG***AGTCGC***CTGAGGTCACC −72 CTGTTTCCTCAGCGGACTCCAGTGG

ing to the recommended protocol. CAT activities were normalized for transfection efficiency based on β-galactosidase activity.

RESULTS

SP-B Proximal Promoter Region Contains Binding Sites for Multiple Transcription Factors—Proximal promoter regions of rabbit (−236 to +39) (13) and human (−218 to +41) (26) SP-B genes display high degree of sequence conservation and support high level expression of CAT reporter gene in NCI-H441 cells (13, 27). These observations suggest that SP-B proximal promoter regions contain binding sites for transcription factors necessary for expression in NCI-H441 cells. DNA sequence alignment of rabbit and human SP-B promoter regions (13) showed that rabbit SP-B promoter contains sequence motifs that are nearly identical to TTF-1 and HNF-3 motifs identified in human SP-B promoter. As in the case of human SP-B promoter (14), the TTF-1 binding site in rabbit SP-B promoter has limited identity to the consensus binding sequence for TTF-1 (GNNCACTCAAG) (28). The TTF-1 binding site consisted of two closely juxtaposed elements with the sequence CTGGAG and CTCCAG that resemble the 3′ segment of consensus TTF-1 binding site. The putative HNF-3 binding site is characterized by the sequence, 5′-TGTTTG-3′, that occurs in the regulatory elements of a wide variety of liver-specific genes (29).

We searched rabbit SP-B proximal promoter (−236 to +39) for the presence of other putative transcription factor binding sites using a transcription factor data base (MacDNASIS 3.0) and by comparison with the consensus binding sequences for vertebrate-encoded transcription factors (30). Results showed that the SP-B promoter, besides containing TTF-1 and HNF-3 sites, also contained putative binding sites for Sp1, ETS, and Myc-Max transcription factors (Fig. 1).

DNase I Footprinting Reveals Multiple Interactions between NCI-H441 Nuclear Proteins and SP-B Promoter—SP-B proximal promoter contained putative binding sites for several transcription factors. To map out regions of SP-B promoter that bind transcription factors, we analyzed the interactions of nuclear proteins present in NCI-H441 cells with SP-B promoter by DNase I footprinting assay. In DNA samples complexed with NCI-H441 nuclear proteins, several protected regions were observed (Fig. 2), suggesting that multiple proteins present in nuclear extracts of NCI-H441 cells interact with SP-B promoter. The protected regions of SP-B promoter included binding sites for Sp1, ETS, TTF-1, HNF-3, and Myc-Max transcription factors (Fig. 1).

Sp1, TTF-1, and HNF-3 Factors Are Critical for SP-B Promoter Activity—We investigated the functional importance of

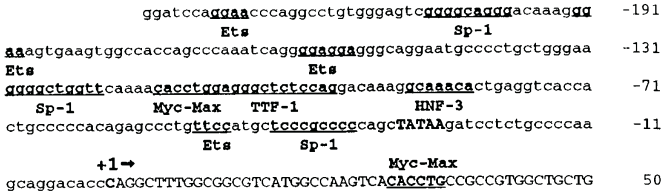

FIG. 1. **Nucleotide sequence of the rabbit SP-B promoter.** Nucleotide sequence of the promoter region (−236 to +39) that supports high level expression of CAT reporter gene in NCI-H441 cells is shown. The transcription start site is marked with an *arrow*, and the nucleotide numbering is relative to transcription start site (+1). Nucleotide sequences of putative transcription factor binding sites are shown in *bold* and are *underlined*.

putative binding sites for Sp1 (−207, −130, −35), ETS (−161, −51), TTF-1 (−112, −102), HNF-3 (−88) and Myc-Max (−115, +30) transcription factors by mutational analysis (Fig. 3).

Sp1 binding sites were mutated by GG to TT substitution in the 5′ portion of the binding site. Mutation of these nucleotides drastically reduces binding of Sp1 to its binding site (31). Results showed that mutation of Sp1 binding site at −207 did not significantly alter SP-B promoter activity, whereas mutation of binding site at −130 or −35 caused 80% or greater reduction in SP-B promoter activity. TTF-1 binding sites were mutated by substituting nucleotides in DNA elements that closely resemble the 3′ portion of consensus TTF-1 binding sequence. Mutation of TTF-1 sites at −112 and −102 caused approximately 70% reduction in SP-B promoter activity and 60% reduction in trans-activation of promoter by TTF-1 in HeLa cells. Mutation of the HNF-3 binding sequence by substituting all six nucleotides resulted in nearly 70% reduction in SP-B promoter activity.

SP-B promoter contains several purine-rich sequence elements that are similar to the sequence 5′-GGAA/T-3′ that is known to bind the Ets family of transcription factors (32). Electrophoretic mobility shift assays with SP-B promoter oligonucleotides showed that among various Ets binding sites, the ones located at −162 and −51 displayed strong binding to NCI-H441 nuclear proteins. We therefore analyzed the functional importance of Ets binding sites at −162 and −51 in SP-B promoter activity by mutational analysis. Mutation of the ETS binding site at −162 did not alter SP-B promoter activity, whereas mutation of the ETS site at −51 caused approximately 30% increase in promoter activity. Mutation of both the ETS sites caused approximately 30% increase in SP-B promoter activity. Two sequence elements that closely resemble the con-

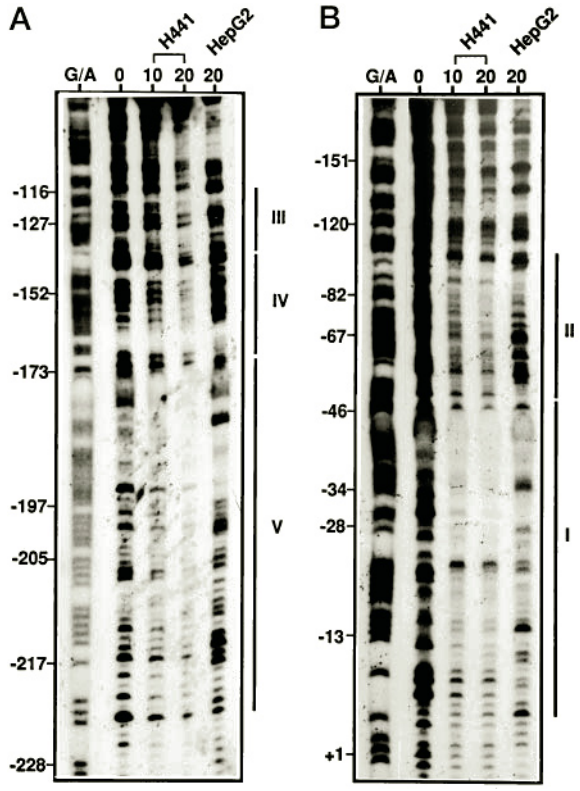

FIG. 2. **DNase I footprinting analysis of interaction of nuclear proteins from NCI-H441 cells and HepG2 cells with SP-B promoter.** The coding (*A*) and noncoding (*B*) strands of SP-B promoter region −236 to +39 were labeled and incubated with 0, 10, and 20 μg of nuclear proteins from NCI-H441 or HepG2 cells before partial digestion with DNase I. The same DNA fragments were subjected to guanosine- and adenine-specific cleavage by the Maxam and Gilbert method. The reaction products were electrophoresed on a 6% denaturing polyacrylamide gel. Protected DNA regions are indicated by *bars* and *roman numerals*. Nucleotides are numbered relative to the transcription start site (+1).

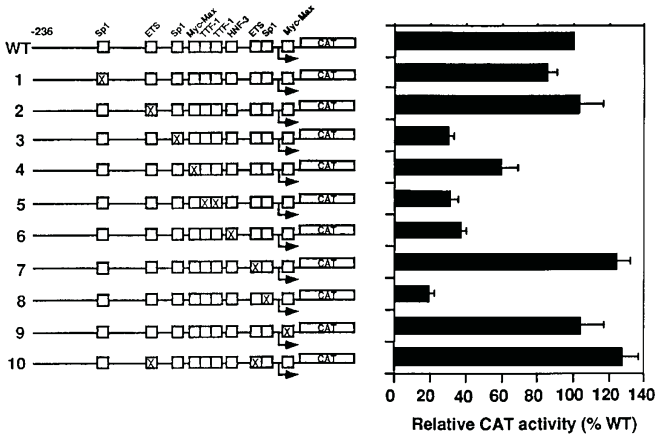

FIG. 3. **Mutational analysis of SP-B promoter region.** Putative transcription factor binding sites in SP-B promoter region −236 to +39 were mutated as described under "Materials and Methods." SP-B -CAT chimeric genes containing mutations were transiently transfected into NCI-H441 cells by liposome-mediated DNA transfer. pCH110, a β-galactosidase expression plasmid, was included to serve as an internal control. CAT activity in cell extracts was determined and normalized to β-galactosidase activity to correct for variations in transfection efficiency. Mutations in putative transcription factor binding sites in the SP-B promoter map are represented by *X*. CAT activity of promoter constructs is expressed relative to activity of the wild type SP-B-CAT construct (*WT*). The transcription start site is marked by an *arrow* in the promoter map. Data represent mean ± S.E. of four to six independent experiments. Data of the effect of mutation of Myc-Max binding site at +30 represent mean ± S.D. of two independent experiments.

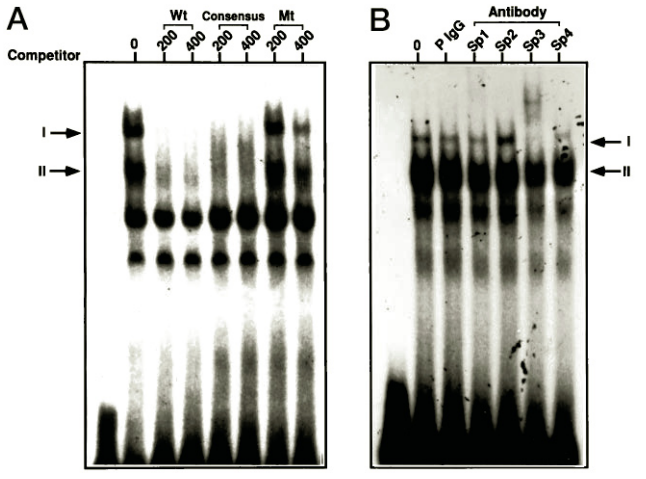

FIG. 4. **Electrophoretic mobility shift analysis of NCI-H441 nuclear proteins binding to the Sp1 site at −130 in the SP-B promoter.** The ^{32}P-labeled double-stranded SP-B promoter oligonucleotide (−138 to −114) was incubated with 5 μg of NCI-H441 nuclear proteins in the presence of unlabeled oligonucleotides as competitors or in the presence of antibodies to different members of the Sp1 family of transcription factors as described under "Materials and Methods." The sequences of oligonucleotides used are shown in Table II. *A*, effects of indicated molar excess of wild type (*Wt*) or mutant (*Mt*) or consensus Sp1 oligonucleotides on complex formation. *B*, effects of preimmune IgG (*P IgG*) or monospecific antibodies to Sp1, Sp2, Sp3, and Sp4 on the mobilities of retarded complexes. Incubation of the SP-B promoter oligonucleotide produced two complexes (*I* and *II*) with different mobilities. Sp1 and Sp3 antibodies reacted with proteins bound to the oligonucleotide to produce supershifted complexes.

sensus binding motif for Myc-Max transcription factors (33) are located at −115 and +30. Mutation of the Myc-Max binding site at −115 resulted in approximately 40% reduction in SP-B promoter activity, whereas mutation of the site at +30 had no significant effect on promoter activity.

Transcription Factors Sp1, ETS, TTF-1, and HNF-3 Bind to the SP-B Promoter—To verify if Sp1, TTF-1, HNF-3, and ETS factors are components of the binding activity in nuclear extracts, we analyzed binding of factors present in nuclear extracts by electrophoretic mobility shift assays. Results showed that SP-B promoter oligonucleotide that contained Sp1 binding site at −207 did not form any complex, indicating absence of interaction of nuclear proteins (data not shown). However, SP-B promoter oligonucleotides that included binding sites for Sp1 at −130 or −35 formed two complexes that were competed by excess amounts of unlabeled wild type oligonucleotide and by an oligonucleotide containing a consensus Sp1 binding site (Figs. 4*A* and 5*A*). However, an oligonucleotide that contained a mutant Sp1 site failed to prevent formation of complex, suggesting the identity of binding factors as Sp1 or Sp1-like proteins (Fig. 4*A*). Electrophoretic mobility shift assays in the presence of monospecific antibodies to different members of the Sp1 family of proteins revealed the identity of proteins binding to Sp1 sites as Sp1 and Sp3 (Figs. 4*B* and 5*B*). Results also showed that the lower mobility complex (complex I) was completely supershifted by Sp3 antibody, indicating that complex I results from binding of Sp3. We further confirmed binding of Sp1 to SP-B promoter by DNase I footprinting assays with purified recombinant Sp1. Results demonstrated that purified Sp1 binds to Sp1 binding sites at −130 and −35 in SP-B promoter (Fig. 6).

In electrophoretic mobility shift assays, the SP-B promoter oligonucleotide containing the TTF-1 binding site formed two major complexes (Fig. 7). Formation of these complexes was significantly reduced in the presence of excess wild type

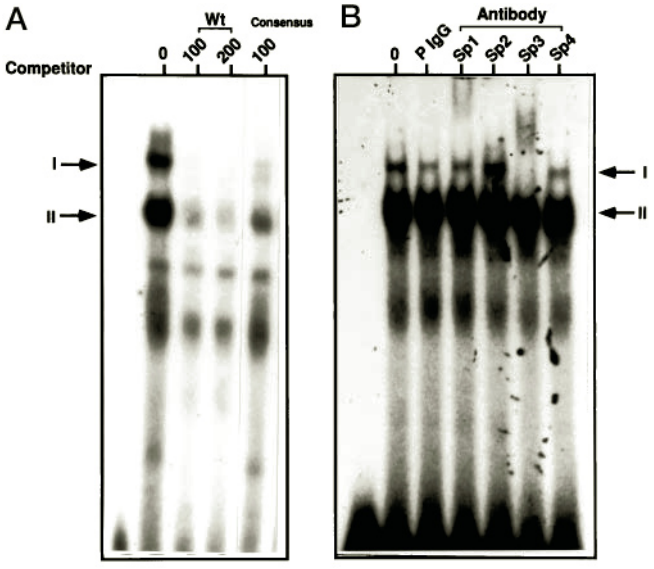

FIG. 5. **Electrophoretic mobility shift analysis of NCI-H441 nuclear proteins binding to the Sp1 site at −35 in the SP-B promoter.** The ^{32}P-labeled double-stranded SP-B promoter oligonucleotide (−50 to −28) was incubated with 5 μg of NCI-H441 nuclear proteins in the presence of unlabeled oligonucleotides as competitors or in the presence of antibodies to different members of Sp1 family of transcription factors as described under "Materials and Methods." The sequences of oligonucleotides used are shown in Table II. *A*, effects of indicated molar excess of wild type (*Wt*) or mutant (*Mt*) or consensus Sp1 oligonucleotides on complex formation. *B*, effects of preimmune IgG (*P IgG*) or monospecific antibodies to Sp1, Sp2, Sp3, and Sp4 on the mobilities of retarded complexes. Incubation of the SP-B promoter oligonucleotide produced two complexes (*I* and *II*) with different mobilities. Sp1 and Sp3 antibodies reacted with proteins bound to the oligonucleotide to produce supershifted complexes.

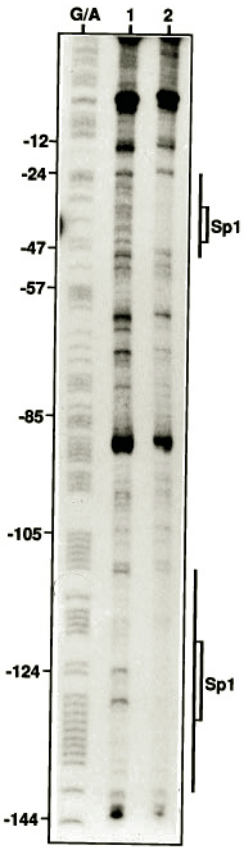

FIG. 6. **DNase I footprinting analysis of the SP-B promoter region −236 to +39 in the presence of purified Sp1 protein.** The coding strand of SP-B promoter fragment −236 + 39 was ^{32}P-labeled and incubated with no protein (*lane 1*) or with 1 footprinting unit (50 ng) of purified recombinant Sp1 (*lane 2*) and subjected to DNase I digestion. The same DNA fragment was subjected to guanosine- and adenine-specific cleavage by the Maxam-Gilbert method (*lane G/A*). Digested products were analyzed by electrophoresis on a 6% polyacrylamide sequencing gel, and an autoradiogram was obtained. Nucleotide numbering relative to transcription start site (+1) is shown on the *left side*. The protected regions of DNA are indicated by *lines*, and the location of the Sp1 core binding element is indicated by *rectangular boxes*.

oligonucleotide but not in the presence of excess mutant oligonucleotide (Fig. 7). In the presence of monospecific antibodies to TTF-1 the higher mobility complex (complex II) was specifically supershifted, indicating the identity of the protein species present in complex II as TTF-1 or TTF-1-related protein (Fig. 7). The lower mobility complex (complex I) was not supershifted in the presence of antibodies to TTF-1, indicating that complex I arises from interaction with protein(s) unrelated to TTF-1.

Electrophoretic mobility shift analysis with the SP-B promoter oligonucleotide containing the HNF-3 binding site demonstrated formation of two complexes (Fig. 8). Formation of these complexes was significantly reduced in the presence of excess wild type oligonucleotide. In the presence of excess mutant oligonucleotide formation of complex I was not reduced but formation of complex II was reduced, suggesting that complex I likely results from interaction with HNF-3 (Fig. 8). In the presence of monospecific antibodies to different members of the HNF-3 family of transcription factors, formation of a supershifted complex was observed only in the presence of antibodies to HNF-3α (Fig. 8). Furthermore in the presence of HNF-3α antibodies complex I was specifically supershifted, indicating that complex I arises from interactions with HNF-3α or a related protein. The identity of proteins present in complex II remains to be determined.

Electrophoretic mobility shift assays with SP-B promoter oligonucleotides containing binding sites for the ETS family of transcription factors showed formation of three distinct complexes that were competed by excess wild type oligonucleotide but not by a mutant oligonucleotide (data not shown). The complexes were not recognized by polyclonal ETS antibody (Santa Cruz Biotechnology) capable of cross-reacting with ETS family proteins.

DISCUSSION

SP-B mRNA is induced during fetal lung development, and in adult lung its expression is restricted to alveolar epithelial (type II) cells and bronchiolar epithelial (Clara) cells (4, 5). Molecular mechanisms that mediate cell type-specific activation of SP-B gene transcription are not well understood. Recent studies have shown that TTF-1 and HNF-3/forkhead proteins play important roles in the functional activity of human SP-B promoter (14, 15). During lung development HNF-3α and TTF-1 proteins are expressed at the onset of lung morphogenesis (16, 17) at which time expression of SP-B mRNA is not detected, and in fully developed lung the expression of HNF3α, TTF-1, and SP-B co-localize to cells of distal epithelium. These data suggest that TTF-1 and HNF-3α are not sufficient for cell-specific activation of SP-B promoter and that additional factors are required for activation of the promoter.

DNA footprinting analysis showed that multiple proteins present in nuclear extracts of NCI-H441 cells bind to the SP-B promoter and that protected regions contain binding sites for Sp1, ETS, Myc-Max, TTF-1, and HNF-3 transcription factors. TTF-1 and HNF-3 binding sites are nearly identical in rabbit, human, and mouse SP-B promoters and so is their placement relative to the TATAA element (13). Human SP-B promoter is transactivated by HNF-3α/HFH-8 proteins in HepG2 cells (15),

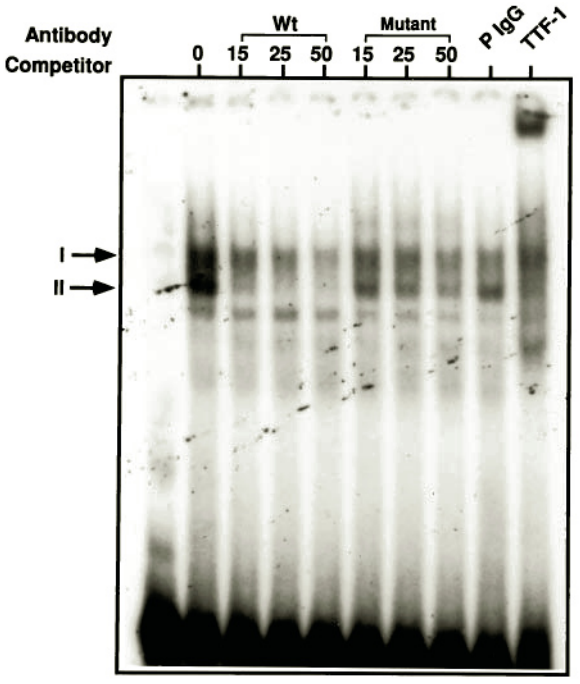

FIG. 7. **Electrophoretic mobility shift analysis of NCI-H441 nuclear proteins binding to the TTF-1 site in the SP-B promoter.** The ^{32}P-labeled double-stranded SP-B promoter oligonucleotide (−118 to −90) was incubated with 5 μg of NCI-H441 nuclear proteins in the presence of indicated molar excess of unlabeled wild type (*Wt*) or mutant (*Mt*) oligonucleotides as competitors and in the presence of preimmune IgG (*P IgG*) or monospecific antibodies to TTF-1. The sequences of oligonucleotides used are shown in Table II. Protein-DNA complexes were resolved by electrophoresis as described under "Materials and Methods." On incubation with NCI-H441 nuclear proteins two major retarded complexes were formed. Complex II was specifically recognized by TTF-1 antibodies.

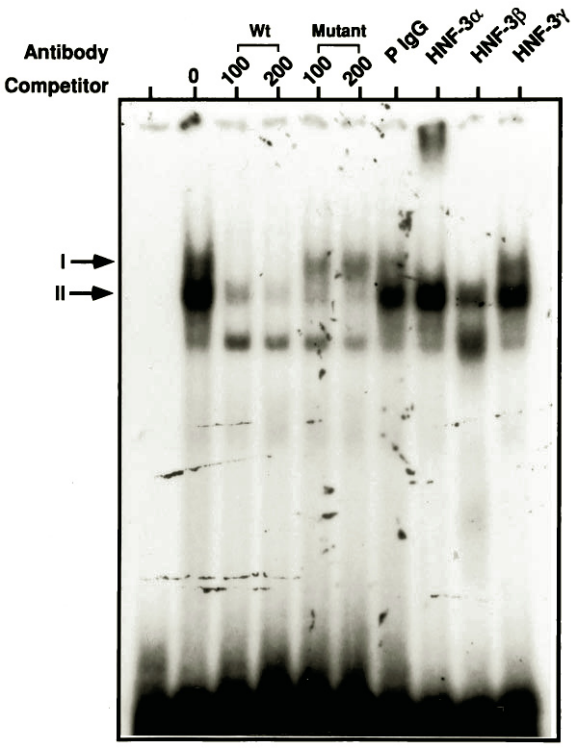

FIG. 8. **Electrophoretic mobility shift analysis of NCI-H441 nuclear proteins binding to the HNF-3 site in the SP-B promoter.** The ^{32}P-labeled double-stranded SP-B promoter oligonucleotide (−to −75) was incubated with 5 μg of NCI-H441 nuclear proteins in the presence of indicated molar excess of unlabeled wild type (*Wt*) or mutant (*Mt*) oligonucleotides as competitors and in the presence of preimmune IgG (*P IgG*) or monospecific antibodies to different members of the HNF-3 family of proteins. The sequences of oligonucleotides used are shown in Table II. Protein-DNA complexes were resolved by electrophoresis as described under "Materials and Methods." On incubation with NCI-H441 nuclear proteins, two major retarded complexes were formed. Complex I was specifically recognized by HNF-3α antibodies.

and human (14) and rabbit (13) SP-B promoters are transactivated by TTF-1 in HeLa cells, further supporting functional roles for TTF-1 and HNF-3 binding sites in rabbit SP-B promoter. We further assessed the functional roles of TTF-1 and HNF-3 binding sites in SP-B promoter activity by mutational analysis. Mutation of TTF-1 and HNF-3 binding sites significantly reduced SP-B promoter activity as did activation of mutant SP-B promoter by TTF-1, demonstrating the importance of these sites in the functional activity of SP-B promoter. Electrophoretic mobility shift analysis demonstrated that TTF-1 and HNF-3 binding sites, in addition to binding TTF-1 and HNF-3α, also bind other nuclear proteins whose identities remain to be determined.

Putative Sp1 binding sites are located at −207, −130, and −35 in SP-B proximal promoter. We determined the functional importance of these elements in SP-B promoter activity by mutational analysis. Mutation of the site at −207 had no significant effect on SP-B promoter activity, but mutations of the site at −130 or −35 caused a significant reduction in promoter activity. These data demonstrated that Sp1 elements at −130 and −35 play equally important roles in the functional activity of SP-B promoter. Gel mobility shift and DNase I footprinting experiments indicated the identities of proteins interacting with these sites as Sp1 and Sp3.

The occurrence and role of Sp1 elements in the promoter functions of human and mouse SP-B genes have not been investigated. Examination of human (26) and mouse (34) SP-B proximal promoter sequences reveals that the human and mouse SP-B promoters contain putative Sp1 binding sites at −36 and −42. The putative Sp1 binding sequences in human and mouse promoters, 5′-GCCCGCCCA-3′ and 5′-TCCAGCCCC-3′, display a high degree of similarity to Sp1 element at −35 in rabbit SP-B promoter. The high degree of conservation of sequence as well as similar placements of Sp1 binding sites in rabbit, human, and mouse SP-B promoters undescores the importance of Sp1 binding element in the functional activity of SP-B promoter. Sp1 binding site at −130 in rabbit SP-B promoter appears to be unique to rabbit SP-B gene, since a similar sequence element could not be found in human and mouse SP-B promoters.

Sp1 binds to GC boxes present in promoters of a wide variety of genes and modulates promoter activity. To date three Sp1-related proteins, Sp2, Sp3, and Sp4, have been described (35–37). Similar to Sp1, all three Sp1-related proteins are expressed widely and contain zinc finger structures and glutamine- and serine/threonine-rich amino acid stretches. The DNA binding domains of Sp1, Sp3, and Sp4 proteins are highly conserved and display similar binding affinity to GC boxes. Of the different members of Sp1 family proteins, Sp3 (37, 38) was found to act as a potent repressor of basal and Tat-mediated activation of the human immunodeficiency virus promoter. Although Sp1 is expressed ubiquitously, several lines of evidence suggest a regulatory role for Sp1. The recent identification of several Sp1-related proteins (35–37) with similar binding affinities, the differential expression of Sp1 in various cell types, as well as developmental regulation of Sp1 (39) support a regulatory role for Sp1. In the lung notable expression of Sp1 was detected in alveolar epithelial cells (39), and Sp1 mRNA levels increased during postnatal development of mice.

Our data suggest that besides TTF-1 and HNF-3α, Sp1 and

Sp3 serve as important regulators of SP-B gene expression. The critical role of Sp1 and Sp3 binding sites in the functional activity of SP-B promoter suggests that cell type-specific and developmental induction of SP-B gene expression is dependent on expression of Sp1 and Sp3 proteins/or activity. Sp1 is modified by phosphorylation, and modification by phosphorylation modulates binding of Sp1 to its target sites (40). The role of cell type-specific and developmental control of Sp1 and Sp3 expression, and the putative role of phosphorylation of Sp1 in the regulation of SP-B gene expression in fetal lung, remain to be investigated. Since Sp3 has the potential to function as a transcriptional repressor, developmental and cell type-specific regulation of SP-B gene expression might be maintained by a dynamic positive and negative regulation exerted by Sp1 and Sp3. The putative role of Sp1-related transcription factors in the control of other lung-specific genes, particularly other surfactant protein genes, is not known. The recent identification of binding sites for Sp1 and Sp3 proteins in the minimal promoter of rat Clara cell-specific protein (41) suggests that Sp1-related transcription factors might serve as important regulators of lung-specific gene expression.

Recent studies have suggested that ETS proteins can interact with other transcription factors to modulate promoter activity (32). Studies have also suggested that ETS proteins play important roles in the control of growth and differentiation (32). Specifically ETS 1 expression increases during fetal development, and high levels of expression are found in fetal lung (42). The role of ETS 1 in control of lung growth and differentiation is not known. Rabbit SP-B promoter contains a number of putative binding sites for ETS proteins. Results of mutational analysis of ETS binding sites showed that the element at −51 can function as a weak suppressor of SP-B promoter activity. Proteins that bound to ETS sites were not recognized by an antibody capable of cross-reacting with members of ETS family transcription factors, suggesting that the proteins are either unrelated to ETS proteins or that they represent new members of the ETS family of transcription factors. Further investigations are needed to define the role of the ETS binding site at −51 and of other putative ETS sites in the control of SP-B promoter activity.

SP-B promoter contained putative Myc-Max binding sites at −115 and +30. Mutation of the site at +30 did not alter SP-B promoter activity, but mutation of site at −115 reduced SP-B promoter activity by nearly 40%. The site at −115 overlaps with TTF-1 binding site; whether reduction in SP-B promoter activity as a result of mutation in the Myc-Max element is due to interference with binding of TTF-1 remains to be investigated.

TTF-1 activates surfactant protein (SP)-A (43), SP-B, SP-C, as well as Clara cell-specific protein (14) promoters, suggesting that it plays an important role in the control of lung-specific gene expression. TTF-1 has also been shown to be a key regulator of thyroid-specific gene expression (44). Our studies of the control of SP-B promoter activity has demonstrated that significant differences exist between TTF-1-regulated control of gene expression in thyroid and lung. Whereas lung-specific expression of SP-B is controlled by combined interactions of multiple transcription factors with the promoter, thyroid-specific expression of thyroglobulin and thyroid peroxidase genes is dependent on mutually exclusive interactions of TTF-1 and Pax-8 factors (45).

Functional analysis of 5′-flanking regions has shown that human (27, 46) and rabbit (13) SP-B proximal promoter regions comprising nucleotides −218 to +436 and −236 to +39 are sufficient for high level expression of the CAT reporter gene in NCI-H441 cells, but further deletion of 5′ DNA to −130 nucleotides significantly reduces CAT expression. Functionally important transcription factor binding sites thus far identified in human and rabbit SP-B promoter regions, namely TTF-1, HNF-3α, and Sp1 and Sp3 sites, are located within −130 nucleotides, suggesting that factors binding to upstream sequences are necessary for activation of the promoter.

In summary our studies have identified Sp1 and Sp3 transcription factors as important regulators of SP-B promoter activity and that combined or cooperative effects of Sp1, Sp3, and TTF-1 and HNF-3α proteins on SP-B promoter is required for activation of promoter. Further characterization of regulatory DNA elements and interacting proteins of SP-B promoter region −236 to −136 will aid in understanding mechanisms that mediate lung cell-specific activation of SP-B gene transcription.

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