**Transcriptional Regulation of the Murine Surfactant Protein-A Gene by B-Myb**

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**Surfactant protein A (SP-A)** is selectively synthesized in subsets of cells lining the respiratory epithelium, where its expression is regulated by various transcription factors including thyroid transcription factor-1 (TTF-1). Cell-specific transcription of the mouse SP-A promoter is mediated by binding of TTF-1 at four distinct cis-acting sites located in the 5′-flanking region of the gene. Mutation of TTF-1-binding sites (TBE) 1, 3, and 4 in combination markedly decreased transcriptional activity of SP-A promoter-chloramphenicol acetyltransferase constructs containing SP-A gene sequences from -256 to +45. In contrast, the same mutations enhanced transcriptional activity in constructs containing additional 5′ SP-A sequences from -399 to +45 suggesting that cis-acting elements within the region -399 to -256 influence effects of TTF-1 on SP-A promoter activity. A consensus Myb-binding site was identified within the region, located at positions -380 to -371 in the mouse gene. Mutation of the Myb-binding site decreased activity of SP-A promoter constructs in MLE-15 cells. MLE-15 cells, a cell line expressing SP-A mRNA, also express B-Myb. B-Myb bound to the MBS in the SP-A gene as assessed by electrophoretic mobility shift assay. While co-transfection of HeLa cells with a B-Myb expression plasmid activated the transfected SP-A promoter about 3-fold, co-transfection of B-Myb with cyclin A and cdk-2, to enhance phosphorylation of B-Myb, increased transcriptional activity of SP-A constructs approximately 20-fold. Taken together, the data support activation of SP-A gene promoter activity by B-Myb which acts at a cis-acting element in the SP-A gene.

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Surfactant protein A (SP-A) is an abundant, surfactant-associated glycoprotein synthesized by tracheo-bronchial glands, nonciliated secretory epithelial cells, and Type II cells in the lung. SP-A is a member of the collectin family of polyepitides functioning as part of the innate immunity of the lung (1–3). SP-A stimulates chemotactant activity of macrophages (4), binds and enhances uptake and killing of bacteria, viruses, and fungi by macrophages and neutrophils, enhances production of free radicals (Refs. 1–3, for review, see Refs. 5 and 6), and enhances activity of the mannose receptor in macrophages (7, 8). While surfactant function and metabolism of SP-A-deficient mice are apparently normal, SP-A gene inactivated mice are more susceptible to Group B streptococcal and *Pseudomonas* lung infections than wild type mice (9, 10). The transcription of the mouse SP-A gene is regulated by complex humoral and cellular signaling mechanisms that determine the temporal and spatial expression of SP-A in the lung. Although the precise trans- and cis-active elements mediating SP-A gene expression have not been identified to date, SP-A mRNA in the lung increased with advancing gestational age and was stimulated by γ-interferon, cAMP, and epidermal growth factor in fetal lung tissues. SP-A mRNA was decreased by phorbol esters, tumor necrosis factor-α, and transforming growth factor-β (for review, see Refs. 3, 11, and 12). Glucocorticoids both stimulated and inhibited SP-A mRNA in various species and systems, the effects mediated by changes in both transcription rate and mRNA stability (13, 14). Transcription of surfactant protein genes (SP-A, -B, and -C) are dependent upon the homeodomain containing protein TTF-1, a member of the Nkx2 family of nuclear proteins (15–18). Transcription of the mouse SP-A gene is regulated by thyroid transcription factor-1 (TTF-1) which binds to four cis-active sites (TBE) (15). Activation of SP-A and SP-B gene expression by TTF-1 is further enhanced by cAMP-dependent phosphorylation (19, 20). TTF-1 functions in combination with other transcription factors including activator protein-1, nuclear factor-κ, and hepatocyte nuclear factors to regulate expression of surfactant protein genes (17, 21). In order to further study the mechanism and regulation of expression of SP-A, transfection analysis of additional 5′-flanking regions of the mouse SP-A gene was undertaken.

In the present study, a Myb-binding site (MBS) was identified at position -380 to -371 of the murine SP-A gene, mapping closely to four distinct TTF-1 binding sites at positions -159 to -120. Site specific mutation of the MBS, co-transfection analyses, and electrophoretic mobility shift assays (EMSA) demonstrated that B-Myb increased transcription of transfected murine SP-A gene constructs by binding to the MBS. Co-transfection of cyclin A and cdk-2 with B-Myb, known to mediate phosphorylation of B-Myb, resulted in markedly enhanced SP-A transcription. The data support the hypothesis that B-Myb regulates transcription of the mouse SP-A gene.

**MATERIALS AND METHODS**

**Plasmid Construction and Site-directed Mutagenesis—SP-A constructs used in this study are presented in Fig. 1. The murine SP-A gene promoter sequences −256 to +45 used to create pCPA0.3 and pCPA0.3T-1,3,4 were isolated and cloned as described previously (15). Sequences from −520 were cloned by utilizing the BamHI site present at this position to generate pCPA0.6. To create pCPA0.45 and pCPA0.45M, a 22-base pair oligonucleotide was used to generate a PCR product extending from −399 to +45. The TTF-1 site mutations were
previously described (15) (Fig. 2A). The Myb-binding site mutation was created (pCPA0.45M) by changing two nucleotides within the PCR oligonucleotide used to generate pCPA0.45 (Fig. 3). The mutants, pCPA0.6T-1,3,4, pCPA0.45T-1,3,4, and pCPA0.45MT, were generated by a modified in vitro enzyme digestion of a unique BstE1 site within pCPA0.3T-1,3,4. These mutations were expected to molecularly quantifying the cell morphology of the constructs.

Cell Culture, Transfections, and Reporter Gene Assays—MLE-15 cells were cultured in HITES media as described previously (15). MLE-15 cells express SP-A, SP-B, and SP-C mRNAs and were therefore chosen for study of SP-A gene transcription. MLE-15 and HeLa cells were transfected using calcium precipitates prepared with 7.5 pmol of test plasmid and 4 pmol of pCMVβ-gal as described previously (15). For B-Myb trans-activation experiments in HeLa cells, each 10-cm dish was treated with a precipitate prepared by using 7.5 pmol of promoter-CAT plasmid, a gift from Dr. R. Watson, Imperial College of Science, Technology and Medicine, London, United Kingdom. Precipitates from trans-activation experiments including cyclin A (pCMV/cyclin A) and cdk-2 (pCMV/cdk-2) were prepared with 2 pmol of each expression plasmid in the presence or absence of pCMV-B-myb. Cells were maintained at 37 °C for 48 h and the lysates were assayed for β-galactosidase and CAT activities as described previously (15).

Preparation of Nuclear Extracts—MLE-15 and HeLa nuclear extracts were prepared using a modified mini-extract procedure. Nuclear extraction procedures were performed in the cold with ice-cold reagents. Confluent monolayers from six, 10-cm diameter dishes were washed twice with 10 ml of ice-cold phosphate-buffered saline (pH 7.2). Harvested by scraping into 1 ml of phosphate-buffered saline, and the cells pelleted in a chilled 1.5-ml microcentrifuge tube at 3,000 rpm for 5 min. The pellet was washed once in phosphate-buffered saline and re-pelletted as described above. The cell pellet was resuspended in 1 packed cell volume of fresh (lysis) buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM MgCl2, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), and the cells were lysed during a 5-min incubation with occasional vortexing. A nuclear pellet was obtained by microcentrifugation at 3,000 rpm for 5 min which was resuspended in 1 packed nuclear volume of fresh (extract) buffer B (20 mM Hepes, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl2, 25% (v/v) glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). Nuclear extracts used in Western blot analysis were incubated as above in the presence of 5 mM sodium fluoride and 0.4 mM Na3VO4 to inhibit phosphatases. Nuclei were extracted during a 10-min incubation with occasional gentle vortexing. Extracted nuclei were pelleted in a microcentrifuge at 14,000 rpm for 10 min. The supernatant recovered was saved as the extracted nuclear protein. These nuclear extracts were quick frozen and stored at −80 °C.

Synthetic Oligonucleotides—Single-stranded oligonucleotides were annealed at 10 °C in 100 μl annealing buffer M (10 mM Tris, pH 7.5, 10 mM KCl, 0.1 mM EDTA) in a 95 °C heat block and then slowly cooled to room temperature. The A500 was determined and dilutions of this mixture were made in TE (10 mM Tris, pH 8.0, 1 mM EDTA). These double-stranded oligomers were either used directly as cold competitors in an electrophoretic mobility shift assay (EMSA) or gel purified for labeling. For use as a probe in the EMSA, 20 μl of the annealed oligomer was gel purified using a 4% Bio-Cell and a MERmaid kit as specified by the manufacturer (BIO 101, Inc.). The A500 was determined and 1.5 pmol of annealed and gel-purified oligonucleotide was end-labeled using [γ-32P]ATP and T4 polynucleotide kinase. End-labeled probe was purified from unincorporated nucleotide using a Amersham Pharmacia Biotech Nick Column and recovered in 400 μl of TE.

EMSA—Nuclear extracts (5.0–10.0 μg of protein) and unlabeled oligonucleotide competitor DNA were preincubated in 12.5 μl of EMSA buffer C (12 mM Hepes, pH 7.9, 4 mM Tris-Cl, pH 7.9, 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 75 ng/ml poly(dI-dC) (Roche Molecular Biochemicals), 0.2 mM fresh phenylmethylsulfonyl fluoride for 10 min on ice. Oligonucleotide probe (100,000 cpm) was added to the mixture and incubated an additional 20 min on ice. To detect supershift of protein-DNA complex, B-Myb polyclonal antibody was added and incubated for an additional 15 min on ice. The protein-DNA complexes were resolved from free probe by nondenaturing polyacrylamide gel electrophoresis. Five percent gels (29:1, acrylamide/bisacrylamide; 0.5 × TBE (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA, pH 8.3); 2.5% (v/v) glycerol; 1.5 mm thick) were run in 0.5 × TBE running buffer at constant current (30 mA) for approximately 90 min. Gels were blotted to Whatman 3MM paper, dried, and exposed to X-ray film.

**RT-PCR**—RT-PCR reactions were performed according to the Perkin-Elmer XL PCR Kit according to the manufacturer's recommendations. Total MLE-15 cell RNA at 0.5 μg/reaction was reverse transcribed at 65 °C for 1 h. Annealing temperature for all oligonucleotides used in the PCR reactions was 65 °C. Oligonucleotides were 5′-biotinylated for use in murine B-myb. Random hexamer primer 1382–1403: AACCGAACAAGAAGCGGTG, backward primer 2606–2583: ACAGTGTAACCACGAGGACGAG. Murine C-myb, forward primer 799–821: ATCTCCCAGCACTCTCATCCT, backward primer 2020–2000: CACGTCAGGACCCATGTGTC.

**Western Blot Analysis**—Protein samples were subjected to Tris glycine acrylamide gel electrophoresis using 10–20% gradient gels and transblotted to polyvinylidine difluoride membranes (Bio-Rad), then blocked with 10 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.1% Tween 20 (TBE-T) containing 5% bovine serum albumin for 10 min at RT. B-Myb antibody (a gift from Dr. R. Lewis, University of Nebraska Medical Center, Omaha, NE) was diluted 1:5000 in TBE-T and incubated with blots at 4 °C overnight with constant agitation. Blots were washed three times for 10 min in TBE-T, then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Calbiochem) at a dilution of 1:10,000 in TBE-T for 3 h at room temperature. After washing three times with TBE-T for 10 min, bound B-Myb antibody was detected by ECL chemiluminescence reagents (Amersham).

**Analysis of Measurements**—Means, medians, and standard derivation were calculated from three or four independent experiments.
B-Myb Regulation of SP-A

Conservation of Myb binding sites in the rat and mouse SP-A genes. The region −403 to −371 of the mouse gene was 90% identical with the region −578 to −546 of the rat gene. A consensus MBS is depicted in bold print. Nucleotide differences are marked by underlining. Nucleotides CC were substituted for GG to mutate the MBS. The consensus myb-binding site is depicted on the bottom line.

FIG. 1. Schematic representation of SP-A promoter constructs. The 5′ position of each SP-A promoter construct is depicted on the left. The 3′ terminus of all constructs was at map position +45. Closed oval indicates TBE. The hatched rectangle indicates the MBS. The asterisk (*) or pound (#) indicates site-specific mutations introduced in each element.

FIG. 2. Transcriptional activity of SP-A sequences with TTF-1 mutations. Panel A, rat and mouse SP-A gene sequences from position −159 of the rat gene and −164 of the mouse gene (15, 24, 26) are compared. Consensus TBE are numbered 1–4 and printed in bold. Nucleotide differences between mouse and rat genes are underlined. The lower line indicates the mutations introduced in the TBE of the mouse SP-A gene. Panel B, SP-A gene promoter activity was assessed in MLE-15 cells. Activity of each plasmid without TTF-1 mutations is set as one; −3,4 indicates the presence of TTF-1-binding site mutations. pCPA0.6 contains sequences from −520 to +45; pCPA0.45 contains sequences from −399 to +45; pCPA0.3 contains sequences from −256 to +45. Data were derived from six separate experiments, with triplicate plates being assessed for each construct. Activity of each construct was significantly different from the respective wild type construct (p < .001).

FIG. 3. Conservation of Myb binding sites in the rat and mouse SP-A genes. The region −403 to −371 of the mouse gene was 90% identical with the region −578 to −546 of the rat gene. A consensus MBS is depicted in bold print. Nucleotide differences are marked by underlining. Nucleotides CC were substituted for GG to mutate the MBS. The consensus myb-binding site is depicted on the bottom line.
FIG. 4. Mutation of the MBS inhibits activity of the SP-A promoter. Panel A, relative CAT activity was determined after transfection of SP-A-CAT constructs in MLE-15 cells. All constructs consist of the parental DNA sequence, pCPA0.45 from −399 to +45 with the following mutations: pCPA0.45M (MBS mutation); pCPA0.45T-1,3,4 (TBE-1,3,4 mutations); pCPA0.45MT (MBS and TBE-1,3,4 mutations). Data are representative of five separate experiments with triplicate plates tested for each construct. Note that mutation of both MBS and TTF-1,3,4 does not completely abolish transcriptional activity. Activity of each construct was significantly different from pCPA0-0, p < .01. Panel B, a representative CAT assay is presented. Each construct was transfected onto three separate plates of MLE-15 cells. Forty-eight hours after transfection, cells were collected and lysed and CAT activity determined as described under “Materials and Methods.” Following thin layer chromatography to separate acetylated from unacetylated \([^{14}C]\)chloramphenicol, the TLC was exposed to X-AR film for 16 h.

extracts and the MBS from the SP-A gene sequences (Fig. 7B).

B-Myb Trans-activates SP-A Gene Sequences—To determine whether B-Myb trans-activates SP-A sequences containing the MBS, the pCPA0.45 SP-A construct was co-transfected with a B-Myb expression plasmid in HeLa cells. Transcriptional activity of pCPA0.45 was relatively weak in HeLa cells, however, B-Myb increased activity approximately 3-fold (Fig. 8). B-Myb has been previously shown to be phosphorylated when co-transfected with cyclin A and cdk-2 (31–34). Co-transfection of the pCPA0.45-CAT construct with the B-Myb expression plasmid and plasmids expressing cyclin A and cdk-2 markedly enhanced activity. While co-transfection of cyclin A and cdk-2 alone slightly enhanced activity of pCPA0.45, co-transfection of B-Myb, cyclin A, and cdk-2 enhanced transcriptional activity of the SP-A construct more than 20-fold (Fig. 8) in HeLa cells. In MLE-15 cells, transcriptional enhancement with B-Myb was approximately 2-fold, most likely due to the presence of excess B-Myb saturating available cis-active sites, preventing further enhancement (data not shown).

DISCUSSION

TTF-1 binds to four distinct binding sites (TBE) located at positions −159 to −120 in the mouse SP-A gene. TBE-1,3,4 site-specific mutations in SP-A sequences from −256 to +45 markedly reduced transcriptional activity whereas the same mutations tested in a construct consisting of nucleotides −399 to +45, enhanced transcriptional activity, revealing a context specific inhibitory effect of the TTF-1-binding sites. The enhancement of transcription detected in the presence of the TBE mutations of the murine SP-A gene is dependent on protein-DNA interactions in sequences from −399 to −256, and at least a portion of the transcriptional activity related to this region is mediated by B-Myb binding to an MBS consensus element within this region. Activation of the SP-A promoter by B-Myb was markedly enhanced by co-transfection with cdk-2 and cyclin A kinase. Co-transfection of cdk-2 and cyclin A have been previously shown to phosphorylate B-Myb (31–34), suggesting that phosphorylation of B-Myb enhanced its activity on the SP-A promoter. Activity of MBS was influenced by TTF-1-binding elements located 3′ to the myb-binding site within the 5′-flanking region of the mouse SP-A gene.

The murine pulmonary adenocarcinoma cell line, MLE-15, used in this study expressed B-Myb protein and mRNA, consistent with a role for B-Myb in the regulation of SP-A gene expression. Nuclear proteins of MLE-15 cells bound to oligonucleotides containing the MBS and protein binding was competed with a known MBS from the adenosine deaminase gene. An antibody to B-Myb generated a supershifted band on EMSA with MLE-15 nuclear extracts. Mutation of the MBS decreased transcriptional activity of both the wild type (about 50%) and the SP-A constructs with TBE mutations (about 3-fold) in MLE-15 cells, demonstrating that MBS enhances activity of the SP-A promoter. Thus endogenous B-Myb binds to the SP-A MBS enhancing SP-A promoter activity in MLE-15 cells.

B-Myb is expressed widely in vertebrate cells and its expression is closely linked to the cell cycle. B-Myb concentrations are increased in proliferating cells (28) and both B-Myb and C-Myb have been identified in other respiratory epithelial cells, while A-Myb has been detected in the basal layer of the olfactory epithelium (29). In the present study, B-Myb was readily detected in MLE-15 cells, a model cell line of the distal respiratory epithelium.

B-Myb enhances expression of target genes by interactions of its N-terminal domains with the MBS or other unidentified binding sites (for review, see Refs. 33, 34, and 37). Recently, cdk-2 and Acl were shown to phosphorylate B-Myb during the S-phase of proliferating cells (31–34). Phosphorylated B-Myb was a potent trans-activator of the thymidine kinase promoter containing copies of the MBS from the chicken mim-1 promoter A site. Trans-activation of the TK promoter with MBS was cell specific, detected in U-2 OS and CV-1 cells but not Saos-2 osteosarcoma, or C33A cervical carcinoma cells (34). The present study supports the concept that cdk-2/cyclin A phosphorylated B-Myb is also a potent activator of SP-A reporter constructs in HeLa cells.

The present study demonstrated that SP-A transcription
was most strongly enhanced by myb in the context of alterations in the TTF-1-binding sites, suggesting that the TTF-1 binding to TBE elements influenced the binding and activity of Myb on the MBS. The TBE mutations may alter binding at the MBS through alterations in chromatin structure. Alternatively, the TBE mutations may make accessible binding sites for other nuclear proteins that, in turn, influence Myb binding.

Mutation of the MBS and TBE-1,3,4 in combination did not completely abolish transcriptional activity of the pCPA0.45 SP-A construct suggesting that other trans-active proteins may influence SP-A gene transcription from cis-active sites within the SP-A gene sequences.

Although mitotic rates of the bronchiolar Clara cells and alveolar Type II cells are low in a healthy adult lung, epithelial cell proliferation is high during fetal and early postnatal development, and increases during recovery from injury. Oxygen injury enhances both cell proliferation and SP-A expression in the lung. For example, SP-A mRNA was enhanced 5–6-fold after exposure of adult rats to 95% oxygen (38). Chronic oxygen exposure of human fetal lung explants caused an increase in SP-A mRNA that was mediated by both transcriptional and post-transcriptional mechanisms (39). Cyclin A was increased dramatically and cdk-2 modestly in rat alveolar epithelial cells...
isolated from rat lungs exposed to hyperoxia in vivo at the same time that proliferative activity was increasing in the alveolar epithelial cells (40). Thus, SP-A, cdk-2, and cyclin A mRNAs increase during hyperoxic injury to the lung, in association with increased mitotic activity involved in lung repair. Taken together, the previous and present studies suggest that phosphorylated B-Myb may play a role in SP-A gene regulation in proliferating epithelial cells following lung injury.

In other species, in addition to the mouse, transcriptional activity of the SP-A gene has been reported to be strongly influenced by both TTF-1 and other transcription factors. TTF-1-binding sites were identified in SP-A genes from several species, although the number of TBE, precise spacing, and sequences vary (15, 20). TTF-1 and other transcription factors including upstream factor-1, SP-1, and cAMP response element-binding protein/ATF family members act combinatorially with cAMP to enhance transcription of human, baboon, or rabbit SP-A genes (20, 41–43). In contrast, SP-A genes of the rat or mouse are not activated by cAMP. TTF-1 binding and/or transcriptional activity of TTF-1 is increased by cAMP-dependent phosphorylation mediated, at least in part, by PKA-dependent phosphorylation (19, 20). In the present study, cdk-2/cyclin A phosphorylation of B-Myb markedly enhanced its activation of the mouse SP-A gene promoter constructs.

The present study demonstrates complex, combinatorial interactions of a myb-binding site with TTF-1-binding sites in the mouse SP-A gene. An MBS was identified in the SP-A gene and activity of the constructs was enhanced by B-Myb. Mutations of TTF-1-binding sites in the mouse gene either enhanced or reduced activity of the SP-A promoter depending on whether other cis-active binding sites, including the MBS, were present. Activation by B-Myb is observed in wild type and TBE mutant promoter constructs. The present study suggests a hypothetical model where phosphorylated B-Myb may bind to and regulate SP-A gene expression in proliferating respiratory epithelial cells following lung injury.

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