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Lung Cell-specific Expression of the Murine Surfactant Protein A (SP-A) Gene Is Mediated by Interactions between the SP-A Promoter and Thyroid Transcription Factor-1*

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Cis-acting elements determining lung epithelial cell-selective transcription of the murine surfactant protein A (SP-A) gene were identified between nucleotide positions −255 and −57. This region of the murine SP-A gene contained nucleotide sequences consistent with thyroid transcription factor-1 (TTF-1) binding motifs. An SP-A-CAT plasmid containing the TTF-1 binding sites was transcriptionally active in mouse lung epithelial (MLE-15) cells but not in HeLa, 3T3, or H441 cells. However, transcription of the SP-A-CAT construct was activated after cotransfection of HeLa cells with a vector expressing recombinant TTF-1, pCMV-TTF-1. Recombinant TTF-1 homeodomain protein bound to four distinct binding sites located between nucleotides −231 to −168. Proteins in nuclear extracts of MLE-15 cells bound TTF-1 binding sites and were supershifted by TTF-1 antibody. Mutations of three of the TTF-1 binding sites in this region reduced expression of the SP-A-CAT construct in transfected MLE-15 cells and reduced transactivation in HeLa cells. TTF-1 interacts with complex protein/DNA binding sites located in the 5′-flanking region of the murine SP-A gene enhancing lung epithelial cell-specific expression *in vitro*.

Surfactant protein A (SP-A)[1] is an abundant, 32–36-kDa glycoprotein secreted by respiratory epithelial cells in the lung. SP-A plays an important role in the aggregation, function, and regulation of pulmonary surfactant and enhances opsonization and macrophage killing (see Refs. 1 and 2 for review).

SP-A gene expression is subject to cell-specific, developmental, and humoral influences, being expressed by subsets of cells in the human tracheal-bronchial glands and in the bronchiolar and alveolar epithelium (3, 4). SP-A mRNA increases with advancing gestation and is up-regulated by cyclic AMP, glucocorticoids, γ-interferon, and epidermal growth factor (1, 5, 6) and down-regulated by phorbol esters (7), tumor necrosis factor-α (8), and transforming growth factor-β (9). Inhibitory and stimulatory effects are mediated by changes in gene transcription and mRNA stability (10). Transcriptional activity of the murine SP-A gene was previously demonstrated in pulmonary adenocarcinoma cells *in vitro* (11). The 5′-flanking sequences of the rat SP-A gene were transcriptionally active in nuclear extracts from pulmonary tissues *in vitro*, supporting the concept that these sequences contain cell-selective cis-active elements (12). DNA-protein interactions in the 5′-region of the rabbit SP-A gene were demonstrated by DNase hypersensitivity and electrophoretic mobility shift assays (13). Two E-box-like motifs, binding unique lung nuclear proteins, were identified in the rabbit SP-A gene promoter (13). These motifs required a complex interaction with cAMP to enhance lung cell-specific expression of SP-A *in vitro* (13, 14).

Recently, Bohinski *et al.* (15) demonstrated that thyroid transcription factor-1 (TTF-1) bound to and activated DNA binding sites located in the 5′-flanking region of the human surfactant protein B gene. Cotransfection of the TTF-1 cDNA and the SP-B gene promoter was sufficient to transactivate gene expression in non-pulmonary cells. In the present study, lung cell-selective transcription of the murine SP-A gene was mediated by a complex of TTF-1 binding sites located between nucleotide positions −231 and −168 from the start of transcription.

MATERIALS AND METHODS

Plasmid Constructions and Site-directed Mutagenesis—5′-Flanking sequences of the mouse SP-A gene (base pairs −255 to +45) were isolated from pCPA-1.8 (11) using polymerase chain reactions and linker primers to create a 5′-*Hin*dIII and 3′-*Pst*I sites. The product was digested with *Hin*dIII and *Pst*I and cloned into pCPA-0 to generate pCPA-0.3. To generate the TTF-1 site mutants, the pCPA-0.3 was used as template for the polymerase chain reactions. Oligomers were made to each of the three TTF-1 binding sites, replacing each with a restriction enzyme sequence. The TTF-1 site located at position −223 to −218 was changed to a *Sal*I site, the site located at −200 to −195 was changed to a *Nco*I site, and the TTF-1 site at position −190 to −185 was changed to a *Bam*HI restriction site. These oligomers were then used in polymerase chain reactions with pCPA-0.3 as template and linker primers used to generate the wild-type sequences. The products were then digested with appropriate endonucleases and cloned into pCPA-0. These SP-A promoter-chloramphenicol acetyltransferase (CAT) fusion plasmids were designated pCPA-0.3T-1,3, pCPA-0.3T-3, and pCPA-0.3T-3,4, and their identities were confirmed by dideoxy sequencing of M13 mp19 templates. The sequence originally published for the 5′-flanking sequence was incorrect at position −4. There is no C in that position. Therefore, all sequences in the present manuscript differ by −1 from the published sequences (11).

Cell Culture, Transfection, and Reporter Gene Assays—Cells were cultured and transfection experiments were performed essentially as previously described (15). MLE-15 cells were derived from lung tumors produced in transgenic mice expressing SV40 large T antigen (SV40 TAg) driven by the lung-specific human SP-C promoter (16). MLE-15 is a clonal cell line expressing SP-A, SP-B, and SP-C. For TTF-1 transactivation experiments with HeLa cells, 10-cm dishes were treated with precipitates prepared by using 7.5 pmol of promoter-CAT fusion plasmid, 4 pmol of pCMV-βgal, and 1 pmol of either the empty expression vector (pCMV/Rc) (Invitrogen) or an expression vector containing the

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[1] The abbreviations used are: SP-A, surfactant protein A; TTF-1, thyroid transcription factor-1; CAT, chloramphenicol acetyltransferase; HNF, hepatic nuclear factor; EMSA, electrophoretic mobility shift assay.

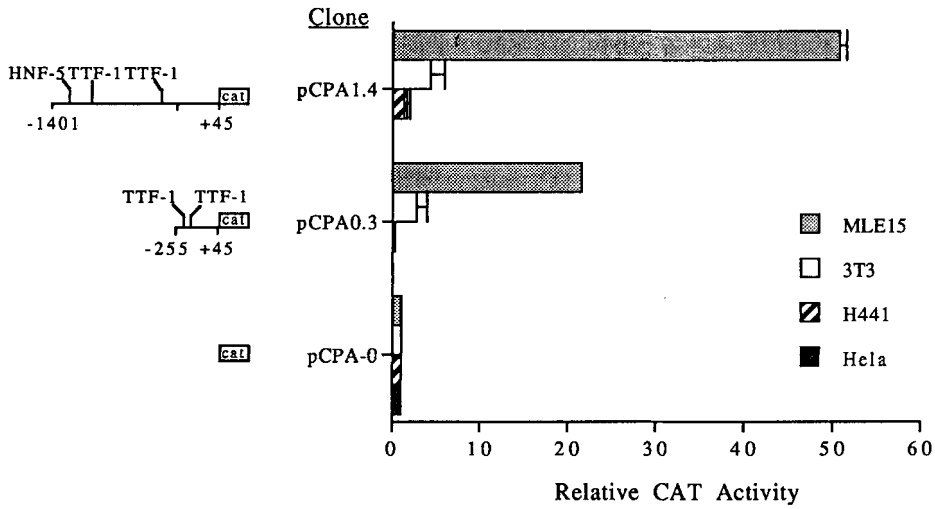

FIG. 1. **Transfection analysis of SP-A sequences.** Culture and transfection of H441, MLE-15, HeLa, and 3T3 cells were described under "Materials and Methods." To the *left*, the 5'-flanking region and portion of exon 1 of the mouse SP-A gene are depicted. Potential binding sites for TTF-1 or hepatocyte nuclear factor-5 are depicted *above* the *line*. Nucleotide positions are depicted *below* the *line*. *cat* indicates the position of the chloramphenicol acetyltransferase gene. To the *right* of each clone, CAT activity is plotted relative to the promoterless plasmid, pCPA-0. The transfection data are representative of at least five separate transfections for MLE-15 and 3T3 and two experiments for HeLa and H441. Presented data were calculated from two experiments with triplicate samples for each construct ($n = 6$). Values represent mean ± standard error. Values of pCPA 1.4 and pCPA 0.3 in HeLa or H441–4 cells were less than for pCPA-0 and therefore are not distinguished in the bar graph.

entire TTF-1 open reading frame (pCMV/TTF-1) as previously described (15). Cell lysates were assayed for β-galactosidase and CAT activities. To minimize variability, cells used for each construct were plated at the same density, transfected, and harvested at the same time.

Nuclear Extract Preparation—MLE-15 nuclear extracts were prepared by using a modified extract procedure as described by Bohinski *et al.* (15). Nuclear extraction was performed at +4 °C or on ice 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) and harvested by scraping into 1 ml of phosphate-buffered saline. Cells were pelleted in a chilled 1.5-ml microcentrifuge tube at 3000 rpm for 5 min. The pellet was washed once in phosphate-buffered saline and repelleted as described above. The cell pellet was resuspended in 1 cell volume of fresh (lysis) buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% (v/v) Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Cells were lysed in this buffer during a 5-min incubation with occasional vortexing. The nuclear pellet was obtained by centrifugation at 3000 rpm for 5 min and was resuspended in 1 volume of fresh (extract) buffer B (20 mM Hepes (pH 7.9), 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Nuclei were extracted during a 10-min incubation with occasional gentle vortexing. Extracted nuclei were pelleted by centrifugation at 14,000 rpm for 10 min. The supernatant was saved as the extracted nuclear protein. Extracts typically contained 5.0–10.0 μg of nuclear protein per μl. Nuclear extracts were quick frozen and stored at −80 °C.

Synthetic Oligonucleotides—Single-stranded oligonucleotides were synthesized on an ABI oligonucleotide synthesizer by the Oligonucleotide Synthesis Core Facility, Children's Hospital Medical Center. Single-stranded oligonucleotides were annealed at 10 μM in 100 μl annealing buffer M (10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl) in a 95 °C dry heat block and then slowly cooled to room temperature. The A_{260} 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% Biogel and a MERmaid kit as specified by the manufacturer (Bio 101, Inc.). The A_{260} was determined, and 1.5 pmol of annealed and gel-purified oligonucleotide was end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. End-labeled probe was purified from unincorporated nucleotide by using a Pharmacia nick column and recovered in 400 μl of TE.

EMSA—Nuclear extracts (5.0–10.0 μg of protein) and unlabeled oligonucleotide competitors were preincubated in 12.5 μl of buffer containing 12 mM Hepes (pH 7.9), 4 mM Tris-Cl (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 75 ng/μl poly(dI-dC) (Boehringer Mannheim), 0.2 mM phenylmethylsulfonyl fluoride for 10 min on ice. Radiolabeled oligonucleotide or DNA fragments were added to the mixture and incubated an additional 20 min on ice. For antibody supershift assays, 1 μl of TTF-1 antibody was added following addition of the nuclear extract and incubated as above. The TTF-1 antibody was previously described by Lazzaro *et al.* (17). Recombinant TTF-1 homeodomain protein (TTF-1 HD) was expressed in *Escherichia coli* and used as described by Damante and Di Lauro (18). Assays were performed with 1 μl of TTF-1 HD in place of nuclear extract. The protein·DNA complexes were resolved from free probe by nondenaturing polyacrylamide gel electrophoresis with 5% 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 electrophoresed in 0.5 × TBE buffer at constant current (30 mA) for approximately 90 min. Gels were blotted to Whatman 3MM paper, dried under vacuum, and exposed to x-ray film for 1 h at −80 °C with an intensifying screen.

RESULTS

Cell-specific Activity of SP-A Gene Constructs in Murine Lung Epithelial Cells (MLE-15 Cells)—SP-A is expressed specifically in the distal pulmonary epithelium. To determine sequences controlling SP-A gene expression, MLE-15, 3T3, H441, and HeLa cells were transfected with plasmids containing murine SP-A flanking sequences and the bacterial reporter gene, CAT (Fig. 1). MLE-15 cells are murine lung epithelial cells expressing SP-A, -B, and -C (16). Plasmids containing SP-A sequences from nucleotides −255 to +45 from the start of transcription were approximately 20-fold more active than the promoterless plasmid pCPA-0. A larger construct containing sequences from −1401 to +45 was approximately 2–3-fold more active than the −255 to +45 construct in MLE-15 cells. The SP-A-CAT constructs were no more active in 3T3, H441, or HeLa cell lines than pCPA-0.

Murine SP-A Sequences Are Transactivated by TTF-1 in HeLa Cells—The nucleotide sequences of the proximal 5'-flanking region of murine SP-A gene contained consensus motifs predicting TTF-1 binding. To determine whether these sequences were transactivated by TTF-1, deletion constructs of the 5'-flanking region of the murine SP-A gene were cotransfected into HeLa cells with pCMV-TTF-1 (Fig. 2). The SP-A-CAT construct containing −255 to +45 was approximately 15-fold more active after transfecting cells with the TTF-1 expression vector than with a promoterless plasmid, pCPA-0.

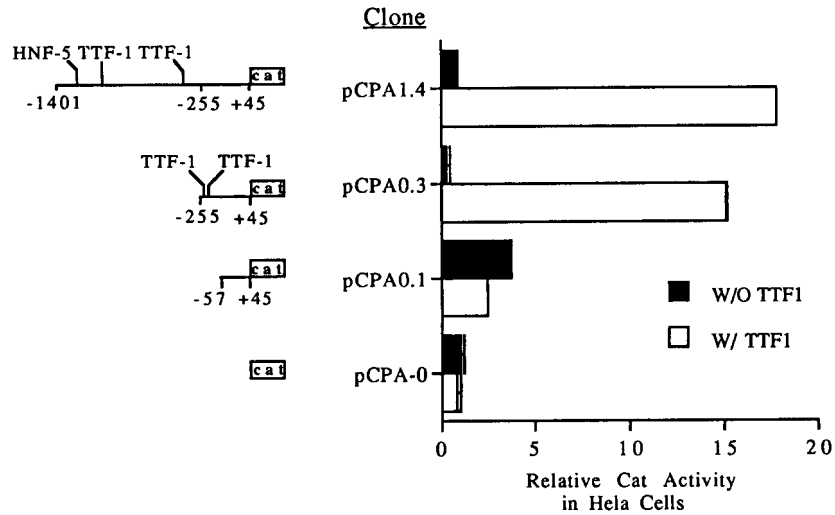

FIG. 2. **Transactivation of SP-A sequences by TTF-1 in HeLa cells.** Cell culture and transfection were described under "Materials and Methods." CAT activity is plotted relative to the activity of the promoterless plasmid. Activity was assessed with and without cotransfection with pCMV-TTF-1. CAT activity from pCPA-0.1 or pCPA-0 was not appreciably altered by cotransfection with pCMV-TTF-1. The transfection data are representative of four separate transfections. Presented data were calculated from two experiments with triplicate samples for each construct ($n = 6$). Value represents mean ± standard error. Absence of an *error bar* means that the standard error was too small to be indicated on the graph. The standard error was not greater than ±20% in those *lanes*.

Although consensus motifs for TTF-1 were present in the region from −1401 to −256, this construct was only slightly more active (20- *versus* 15-fold) than the SP-A-CAT construct containing sequences from −255 to +45. Sequences from −57 to +45 were not transactivated by TTF-1 but retained low level promoter activity in HeLa cells.

TTF-1 Binds to the SP-A Gene—Since sequences from −255 to +45 markedly activated CAT expression in transfected MLE-15 cells, we focused our studies to this region. To determine whether the TTF-1 binding motifs bound TTF-1, EMSAs were performed with recombinant TTF-1 homeodomain protein and double-stranded DNA fragments from sequences −231 to −168 as depicted in Fig. 3. The TTF-1 homeodomain had been shown to bind to TTF-1 motifs within the SP-B gene (15). TTF-1 homeodomain protein bound the SP-A DNA fragments in mobility shift assays. Four distinct TTF-1-DNA bands were identified with probe A (−231 to −168), two with probe B, and one with probes C and D (Fig. 4). The heterogeneity of complex formation with this region of the SP-A gene supported the concept that probes A and B contained multiple TTF-1 binding sites.

MLE-15 Cells Contain TTF-1 Nuclear Proteins Interacting with SP-A Sequences—To determine if MLE-15 extracts contained TTF-1 protein that bound to SP-A gene sequences, EMSAs were performed with MLE-15 extracts and a polyclonal antibody to TTF-1 (Fig. 5). This antibody was raised to three peptides of TTF-1 as described by Lazzaro *et al.* (17). In previous studies of Bohinski *et al.* (15), this antibody caused a supershift in EMSAs with the SP-B gene. As assessed by EMSA (Fig. 5), TTF-1 in nuclear extracts of MLE-15 cells bound to SP-A sequences. Since fragment B formed two bands with TTF-1 (Fig. 4), probe E was used to identify a second TTF-1 binding site. Nuclear extracts from MLE-15 cells bound to the E gene fragment, consistent with the presence of a distinct TTF-1 binding site in this region. Thus, four distinct TTF-1 binding sites were identified in the SP-A gene fragment −231 to −168.

Mutation of TTF-1 Consensus Motifs Decreases Activity in MLE-15 Cells—Interpretation of DNA footprint analysis of −231 to −168 was complicated by the multiple protein-DNA interactions in the region that obscured precise identification of footprint sites (data not shown). Therefore, the function of some of the TTF-1 binding sites in the SP-A gene was determined in SP-A-CAT constructs, in which multiple base changes were introduced into the likely TTF-1 sites. Mutations in each of three TTF-1 binding sites reduced expression of the SP-A-CAT constructs in transfected MLE-15 cells about 10-fold and reduced transactivation in HeLa cells (Fig. 6). TTF-1 site 3 appeared to have the highest affinity for TTF-1 in EMSA (note Fig. 5), so it was tested separately. Mutation of sites 1 or 4 in combination with site 3 did not markedly reduce the effect of the site 3 mutation. Site 2 had the least affinity for TTF-1 and was therefore not tested by mutational analysis. The combination of EMSA and mutational analysis supports the model that each of the sites indicated in Figs. 3 and 6 is required for full transcriptional activity of SP-A sequences in MLE-15 cells.

DISCUSSION

Transcriptional activity of flanking sequences of the murine SP-A gene was activated by recombinant TTF-1 through interactions with complex TTF-1 binding sites located within nucleotide positions −231 to −168. Mutations of TTF-1 motifs reduced cell-specific expression in transfected MLE-15 cells, an SV40 large TAg immortalized pulmonary adenocarcinoma cell that expresses murine SP-A, and reduced transactivation of SP-A sequences by TTF-1 in HeLa cells. TTF-1 enhances lung cell-specific expression of the SP-A gene by interactions with distinct TTF-1 binding sites.

TTF-1, a 38-kDa nuclear transcription protein, was initially identified as an important regulator of thyroid-specific gene expression and contains a highly conserved homeobox domain capable of binding to regulatory regions of target genes (19). TTF-1 activated thyroglobulin and thyroperoxidase gene transcription in thyroid adenocarcinoma cells (20, 21) and was detected in the developing and mature thyroid epithelium (17), consistent with its role in thyroid epithelial cell gene expression. TTF-1 was also detected in the embryonic forebrain and in the respiratory epithelium, where it appears in the embryonic lung buds early in gestation. A potential role of TTF-1 in lung epithelial cell gene expression was demonstrated by the finding that TTF-1 activated transcription of the human surfactant protein B gene (15). TTF-1 bound to two closely apposed TTF-1 sites positioned within nucleotides −80 to −100 of the SP-B gene in close proximity to a hepatic nuclear factor (HNF) binding site. SP-A and SP-B are coexpressed in type II epithelia cells in the alveoli and in overlapping subsets of respiratory epithelial cells in the conducting airways (4, 22). TTF-1 was detected in the same subsets of respiratory epithelial cells, consistent with the potential role of TTF-1 in the regulation of surfactant protein A expression. TTF-1 was detected in the progenitor cells of the developing bronchial tubules early in rat lung development. At the time of birth, TTF-1 is expressed in both type II cells and in subsets of respiratory nonciliated bronchiolar epithelial cells in a pattern similar to that of SP-A

FIG. 3. **Sequences of oligomer probes.** DNA probes from the SP-A 5′-flanking region were synthesized as described under "Materials and Methods." Corresponding nucleotide positions of the SP-A 5′-flanking region are listed with the top sequence (*probe A*). Position of the TTF-1 binding motifs are *underlined* and *numbered 1, 2, 3,* or *4*.

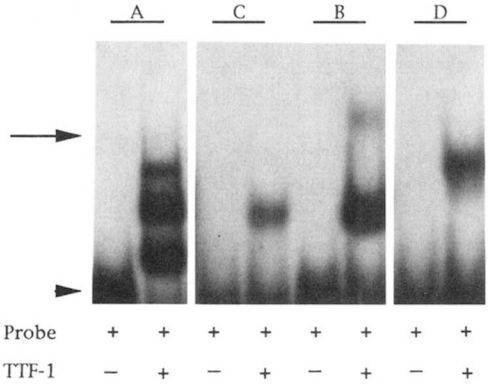

FIG. 4. **EMSA of SP-A gene probes with TTF-1 homeodomain.** Sequence of DNA probes are listed in Fig. 3. *Letters A–D* at the *top* of the figure indicate the probe used in each *lane*. Probe means the presence (+) of the labeled oligomer in each *lane*. TTF-1 is the presence (+) or absence (−) of TTF-1 homeodomain. With *probe A*, four bands were detected; two were detected with *probe B*, and one each was detected with *probes C* and *D*. The slowest migrating band for *probe A* is faint in this exposure, so its position is marked with an *arrow*. *Free probe* is marked with an *arrowhead*.

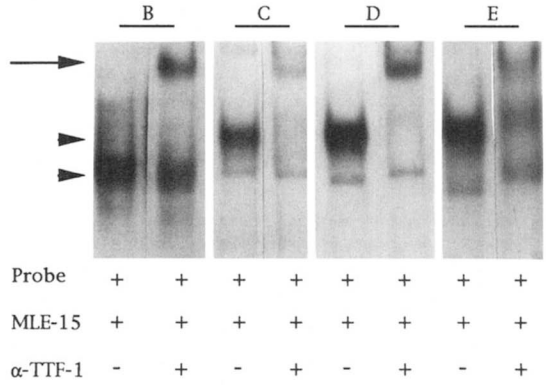

FIG. 5. **EMSA of SP-A gene probes with MLE-15 nuclear extract proteins.** Oligomer synthesis, preparation of MLE-15 nuclear extracts, and EMSA are described under "Materials and Methods." Sequences of DNA probes are listed in Fig. 3. *Letters B–E* at the *top* of the figure indicate the probe used in each *lane*. Probe means the presence (+) of labeled oligomer in each *lane*. MLE-15 means the presence (+) of nuclear extracts; α-TTF-1 means the presence (+) or absence (−) of TTF-1 antibody. Position of major bands are marked with *arrowheads*, and the supershifted band is marked with an *arrow*. Exposures are 1 h at −80 °C for *B*, 18 h at room temperature for *C*, 30 min at −80 °C for *D*, and 24 h at room temperature for *E*.

and SP-B.[2] Similarity of distribution of SP-A, SP-B, and TTF-1 in the respiratory epithelial cells provides further support for the role of TTF-1 in transcriptional control of surfactant protein synthesis.

The proximal TTF-1 binding sites in the SP-A gene are similar to but distinct from those in the human SP-B gene, the former consisting of a complex site with at least four distinct closely associated TTF-1 binding sites. These sites bound the TTF-1 protein found in nuclear extracts as well as the recombinant TTF-1 homeodomain protein as assessed by the EMSA analyses. The consensus binding sites for TTF-1 in the SP-A gene include motifs of CTCAAG, CTGAAG, TAAG, and GTTAAG. The human and murine SP-B genes contain a TTF-1 binding site CCACTCTAAGT that was critical to lung cell expression of SP-B-CAT gene constructs (15). The surfactant protein ATTF-1 sites only partially match the consensus TTF-1 binding site compiled for the thyroid-specific gene CCACTCAAGT (23). A critical TTF-1 site in the SP-B gene contained two distinct TTF-1 binding sites, each of which contribute to activation of the SP-B-CAT constructs. HNF-3α and HFH-8 bound to the proximal promoter region of SP-B in close proximity to these TTF-1 binding sites and enhanced SP-B promoter function (15, 24, 25). However, the HNF site was not critical to the lung epithelial cell gene transcription, which was entirely dependent upon the TTF-1 binding sites located −118 to −64 in the human SP-B gene. While there were no discernible HNF binding sites within the SP-A gene region from −255 to +45, a role of HNF in the regulation of SP-A remains to be more fully explored.

The TTF-1 binding sites in the SP-A 5′-flanking region were sufficient to transactivate transcription of murine SP-A in transfected HeLa cells *in vitro*. Mutation of site 3 and sites 1 and 3 or 1 and 4 in combination reduced transcriptional activity in transfected MLE-15 cells about 4–6-fold. There was also a partial reduction in transactivation of mutated TTF-1 sequences in HeLa cells. These findings support the concept that the function of the TTF-1 complex binding region was dependent upon interactions between the three TTF-1 sites. While TTF-1 is thought to bind primarily as a monomer, TTF-1 can form protein oligomers that may be important to the function of binding sites in both thyroid- and lung-specific genes. Complex interactions among these closely approximated DNA binding sites and TTF-1 may be required for lung-specific gene transcription. The heterogeneity of the TTF-1 binding sites may also confer differences in TTF-1 binding affinity and further modulate the response of the promoter to varying concentrations of TTF-1.

Although recombinant TTF-1 transactivates the expression of SP-A, SP-B, SP-C, and CC10 gene promoters in HeLa cells (15), expression of TTF-1 alone does not suffice to determine the observed heterogeneity of the expression of these four genes in the developing mammalian lung. In human lung, SP-C is expressed only in alveolar type II cells and excluded from the conducting airway (22). In contrast, CC10 is expressed in the bronchiolar but not alveolar epithelium (26). Thus, while TTF-1 appears to play an important role in lung cell-specific gene expression, other factors, such as combinatorial interactions with HNFs, may further modify gene expression, contributing to the distinct temporal and spatial pattern of gene expression.

The sequence of the rat SP-A gene differs from the mouse in containing a brain identifier sequence from position −316 to −211 (12). This sequence likely represents an insertion occurring after the divergence of the mouse and rat lineages and suggests that conserved sequences from position −210 to +1 of the rat gene may be critical to gene expression. Within this

[2] Ikeda, K., Clark, J. C., Stahlman, M. T., Boutell, C. J., Whitsett, J. A. (1995) *J. Biol. Chem.*, in press.

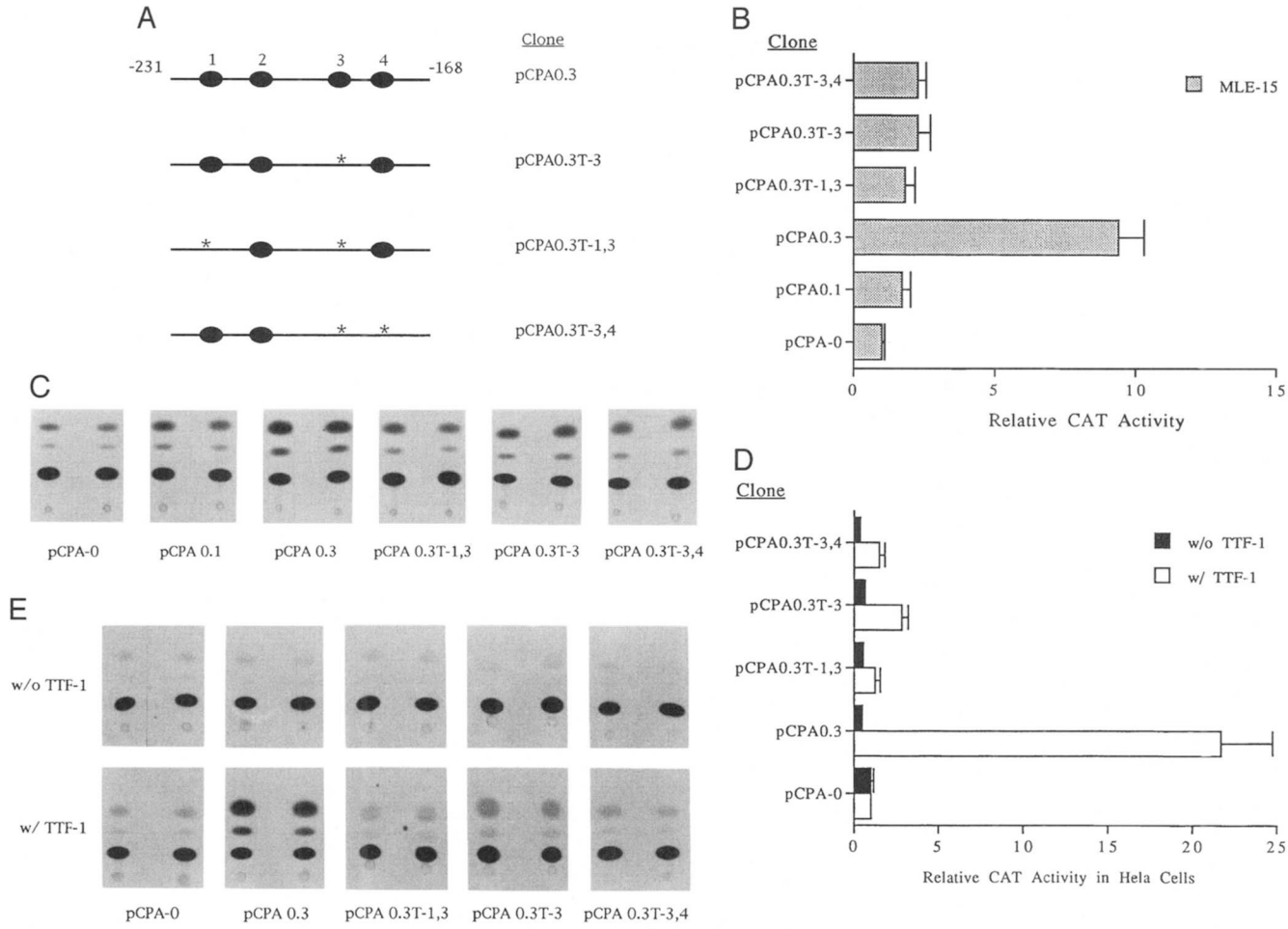

FIG. 6. **Transfection analysis of TTF-1 binding site mutations.** Cell culture, transfection, and plasmid construction were described under "Materials and Methods." *Panel A* is a schematic representation of the TTF-1 sites with mutated sequences indicated with *asterisks*. *Panel B* is transfection analysis of MLE-15 cells, and relative CAT activity is presented relative to the activity of the promoterless pCPA-0 plasmid. The transfection data are representative of four separate transfections. Presented data were calculated from two experiments with triplicate samples for each construct ($n = 6$). Value represents mean ± standard error. *Panel C* is an autoradiogram of representative CAT assays of MLE-15 cells. Each construct is presented in duplicate. *Panel D* is transactivation with TTF-1 in HeLa cells. The transfection data are representative of two separate transfections. Relative CAT activity is presented relative to the activity of the promoterless pCPA-0 plasmid. Presented data were calculated from both experiments with triplicate samples for each construct ($n = 6$). Value represents mean ± standard error. *Panel E* is an autoradiogram of representative CAT assays of HeLa cells. Each construct is presented in duplicate. Absence of *error bars* means that the standard error was too low to be represented in the graph. Standard error did not exceed ±20% in those *lanes*.

region of the rat gene, five potential TTF-1 binding sites are present. Allowing for degeneracy in TTF-1 binding motifs as identified in the present study, the following potential sites are present: CTGGAG at −194 to −189, CTCGAG at −162 to 157, TAAG at −151 to −148, GTTAAG at −137 to −132, and CTGAAG at −127 to −122. These sites are present in the regions active in cell- free transcription (12). Four of these regions were encompassed in protected regions identified by DNase I footprint analyses of the rat gene (12). Sites from −130 to −169 of the rat gene formed a single footprint on the coding strand called P5 (12). Similarly, we were unable to distinguish individual footprints (data not shown) because of the multiple DNA-protein interactions in a comparable region of the mouse SP-A gene. Footprints in this region of the rat SP-A gene were distinct in liver and lung, suggesting that these regions bind a combination of specific and ubiquitous factors (12) supporting the concept that combinatorial interactions of endodermally expressed factors control SP-A gene expression. The 5′-flanking region of the human SP-A gene diverges from the mouse and rat genes. However, four sites in the human gene with the consensus motif CTNNAG are present at positions −213 to −208, −180 to −175, −160 to −155, and −102 to −97 (27), consistent with a potential role for TTF-1 in control of the human SP-A gene.

Recently, two cis-active sites involved in cAMP-induced cell-specific expression of the rabbit SP-A gene were described (13, 14). These sites correspond most closely to E-box motifs. TTF-1 is specific to endodermal derivatives of the foregut epithelium (17), whereas E-box motifs are generally involved in regulation of cell-specific expression of mesodermal derivatives. A consensus sequence for an E-box binding site (CACTTG) was identified at position −8 to −3 of the murine SP-A gene. However, sequences from −57 to +45 of the murine gene were expressed at low levels in multiple cell types (Fig. 2). Thus, this E-box motif does not appear to play a regulatory role in lung cell-specific gene transcription of the murine SP-A gene assessed by transfection of MLE cells. Murine SP-A gene expression did not require cAMP and was induced only 1.5–2.0-fold by 1 mM dibutyryl cAMP (data not shown).

We previously demonstrated non-cell-specific expression of SP-A-CAT constructs (−1401 to +455) in transient transfections of H441–4 cells (11). These studies included the sequences used in the present study, but the constructs also contained intron 1 and a portion of exon 2. Sequences from +46 to +455 of the mouse gene (11), excluding the known transcription start site and 5′-flanking sequences, were tested in tran-

sient assays of MLE-15 and 3T3 cells. Expression from this construct was not lung cell-specific (data not shown), consistent with the presence of an alternate transcription site initiating downstream from the sequences tested in the present study. Since transcription initiation sites of the SP-A gene do not map to this region (11), the function of this alternative transcription start site likely occurs only in the context of the chimeric gene constructs in transfection assays and is not active in the endogenous murine SP-A gene.

In summary, biochemical, site-specific mutagenesis, and functional assays demonstrate that 5′-flanking sequences of the murine SP-A gene from nucleotides −231 to −168 play an important role in determining its lung cell-specific gene expression. This region contains at least four distinct TTF-1 binding sites, supporting the important role of this homeodomain protein in control of SP-A gene expression in the lung.

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