# Mutation of a Lysine Residue in a Homeodomain Generates Dominant Negative Thyroid Transcription Factor 1†

Li Yang,‡ Dong Yan,‖ Molly Bruggeman,‡ Hong Du,§ and Cong Yan*,‡,‖

*Divisions of Pulmonary Biology and of Human Genetics and The Graduate Program for Molecular and Developmental Biology, Children's Hospital Medical Center and Department of Pediatrics, The University of Cincinnati College of Medicine, Cincinnati, Ohio 45229-3039*

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ABSTRACT: Thyroid transcription factor 1 (TTF-1) is a 42 kDa homeodomain (HD) containing the tissue-specific transcription factor of Nkx2 family members (also termed TEBP and Nkx2.1). TTF-1 is an essential transcription factor required for lung development and lung-specific gene expression. Transgenic mice carrying TTF-1 DNA-binding site mutations completely abolish expression of the human surfactant protein B (hSP-B) 1.5 kb *lacZ* reporter gene in the lung in vivo. Acetylation of transcription factors by nuclear receptor coactivators is an important mechanism for gene regulation. TTF-1 is acetylated by nuclear receptor coactivators including the activator of the thyroid and retinoic acid receptor, CREB-binding protein, and steroid receptor coactivator 1 (SRC-1) in cell transfection and immunoprecipitation studies. A glutathionine transferase pull-down assay shows TTF-1 direct interaction with the SRC-1 histone acetyltransferase domain. Site-specific mutagenesis identifies that the lysine residue at position 182 in the TTF-1 HD is acetylated in respiratory epithelial cells. Mutation at this acetylation site shows a dominant negative effect on SP-B gene transcription. The study supports a concept that acetylation is an important mechanism for TTF-1 activity.

Thyroid transcription factor 1 (TTF-1)[1] is a 42 kDa homeodomain containing tissue-specific transcription factor of Nkx2 family members (also termed TEBP and Nkx2.1). TTF-1 is expressed in the lung, the thyroid, and part of the forebrain (*1*−*5*). In the lung, TTF-1 is expressed in epithelial tubules during lung development and restricted to alveolar type II and conducting airway epithelial cells in the adult lungs. Gene-targeted deletion of the mouse TTF-1 gene causes severe pulmonary hypoplasia (*5*). This dramatic phenotype is partially mediated through TTF-1 regulation of lung-specific gene expression, including surfactant proteins A, B, and C (SP-A, -B, and -C) and Clara cell secretory protein (CCSP) in the respiratory epithelium (*6*−*11*). These proteins are required for maintaining surfactant homeostasis, host defenses, and the alveolar structure during respiratory cycles. For example, SP-B deficiency results in newborn death after the birth due to respiratory failure (*12*, *13*).

SP-B is a 79 amino acid amphipathic peptide, produced by the proteolytic cleavage of SP-B proprotein (proSP-B) in Clara cells and alveolar type II epithelial cells. SP-B facilitates the stability and rapid spreading of surfactant phospholipids on the alveolar surface during the respiratory cycle to prevent the lung from collapse (*14*). SP-B is essential for postnatal respiratory adaptation in newborns (*12*, *13*). SP-B gene expression is developmentally controlled and highly tissue and cell type specific. The temporal/spatial expression of the SP-B gene is primarily regulated at the transcriptional level, therefore providing an ideal system to identify transcription factors that are important for lung development and homeostasis. In the transgenic mice study, a 1.5 kb 5′-flanking regulatory region is sufficient to direct hSP-B gene tissue and cell-type-specific expression in lung development and in the adult lung (*15*). Two clustered TTF-1 DNA-binding sites have been identified within this sequence of the human SP-B gene, Figure 1. The first one is located in the −153 to −71 bp promoter region (*6*). The second one is located in the −500 to −331 bp enhancer region (*7*). These two regions are highly conserved in both human and mouse species (*16*). Deletion and mutation of TTF-1 DNA-binding sites in these regions dramatically reduce the human SP-B (hSP-B) promoter activity in vitro (*6*, *7*). The in vivo functional relevance of this observation has not been completely established yet. It is important to note that the expression patterns of TTF-1 and SP-B are overlapped in the respiratory epithelium of the developing and adult lungs (*4*, *15*).

TTF-1 contains several functional domains, including an N-terminal transactivation domain, a DNA-binding transactivation domain, and a C-terminal transactivation domain (*17*, *18*). Through biochemical analysis, several important tran-

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* To whom correspondence should be addressed. Phone: (513) 636-2996. Fax: (513) 636-7868. E-mail: Cong.Yan@cchmc.org.

‡ Division of Pulmonary Biology.

‖ The Graduate Program for Molecular and Developmental Biology.

§ Division of Human Genetics.

[1] Abbreviations: ACTR, activator of the thyroid and retinoic acid receptor; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; GST, gluthathionine transferase; HAT, histone acetyltransferase; HD, homeodomain; IP, immunoprecipitation; SP-B, surfactant protein B; SRC-1, steroid receptor coactivator; TTF-1, thyroid transcription factor 1.

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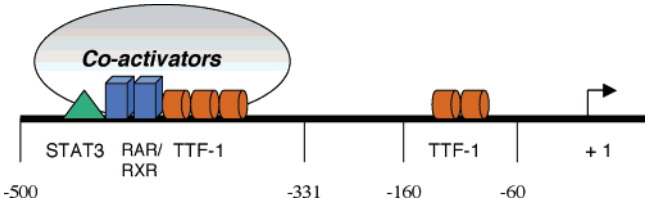


FIGURE 1: hSP-B promoter architecture.

scription factors have been identified to associate with TTF-1, including retinoic acid receptor (RAR/RXR), nuclear receptor coactivators (p160 coactivators and CBP/p300), and signal transducers and activators of transcription 3 (STAT3). They synergistically regulate hSP-B gene transcription in vitro. On the basis of biochemical analysis, it has been proposed that an enhanceosome containing TTF-1, RAR/RXR, nuclear receptor coactivators, and STAT3 is formed on the enhancer region (−500 to −331 bp's) of the hSP-B gene (*4*, *19*−*21*). These factors interact with each other in a synergistic manner. TTF-1 plays a central role in stabilization of this complex. Importantly, these protein factors are coexpressed with SP-B in the developing and adult respiratory epithelial cells by immunohistochemical staining localization (*4*, *20*).

Transcriptional activation is a dynamic and complex process. Nuclear receptor coactivators possess intrinsic histone acetyltransferase (HAT) activity. Upon recruiting by nuclear receptors (e.g., RAR) to the promoters of target genes, nuclear receptor coactivators acetylate histones to increase the accessibility of transcription factors to the promoter regions. In addition, nuclear receptor coactivators are able to acetlyate non-histone proteins. Examples are p53, E2F1, EKLF, TFIIE$\beta$, TCF, GATA-1, HMGI (Y), and components of the general transcription machinery such as TFIIE (*22*−*25*). The consequence of acetylation depends on where within the protein acetylation takes place. Acetylation regulates transcription factor DNA binding, protein−protein interaction, and protein stability (*23*, *26*). Since TTF-1 is in close association with nuclear receptor coactivators to regulate hSP-B gene expression, it is possible that TTF-1 is acetylated and interacts with nuclear receptor coactivators. It is not clear how acetylation affects TTF-1 transcriptional activity in regulating hSP-B gene expression. This could be an important mechanism to control temporal/spatial expression of tissue-specific gene expression (e.g., the SP-B gene) in the lung. Here, we report that TTF-1 DNA binding is essential for hSP-B gene activation in respiratory epithelial cells in vivo. TTF-1 is acetylated by nuclear receptor coactivators in respiratory cells. Site-specific mutagenesis identifies a lysine residue in HD that is essential for TTF-1 transactivation activity. Mutation at this acetylation site shows a dominant negative effect on SP-B gene transcription.

## MATERIALS AND METHODS

*Animal Care*. All scientific protocols involving the use of animals in this study have been approved by the Cincinnati Children's Hospital Institution Animal Care and Usage Committee and follow guidelines established by the Panel on Euthanasia of the AVMA. Protocols involving the use of recombinant DNA or biohazardous materials have been reviewed by the Cincinnati Children's Hospital Biosafety Committee and follow guidelines established by the NIH. Animals were housed under IACUC-approved conditions in a secured animal facility at Cincinnati Children's Hospital Research Foundation. Animals were regularly screened for common respiratory pathogens and murine viral hepatitis. Experiments involving animal sacrifice utilize $CO_2$ narcosis to minimize animal discomfort.

*Plasmid Construction*. The hSP-B 500 luciferase reporter construct was made previously (*7*). For the TTF-1 lysine mutation study, various lysine residues in the TTF-1 HD were converted to alanine, respectively, using the double PCR mutagenesis strategy as previously described (*27*). Briefly, various lysine mutations were introduced into the Flag-tagged TTF-1 HD and subcloned into the *Xba*I and *Hin*dIII sites in the PCR3.0 mammalian expression vector for the TTF-1 HD lysine acetylation study. For the transfection study, various lysine mutations were introduced into Flag-tagged full-length TTF-1 and subcloned into the *Xba*I and *Hin*dIII sites of the PCR3.0 mammalian expression vector.

*Generation of Transgenic Mice and Assay of $\beta$-Gal Activity*. The hSP-B 1.5 kb *lacZ* gene mouse line was made previously (*15*). To make the hSP-B −160 to −60 bp deletion *lacZ* gene transgenic mouse line, removal of the −160 to −60 bp region was made in the hSP-B 1.5 kb *lacZ* gene construct using the double PCR strategy and subcloned into the pSV-$\beta$-gal vector at the *Eco*RI/*Hin*dIII sites as previously described (*15*). To make the hSP-B enhancer TTF-1 DNA-binding site mutation *lacZ* gene transgenic mouse line, the same Ba point mutation (CAGGGggatCCCTGGGTTAAGAGCCAGGCA) that has been tested in vitro (*7*) was introduced into the hSP-B 1.5 kb *lacZ* gene construct using the double PCR strategy and subcloned into the pSV-$\beta$-gal vector at the *Eco*RI/*Hin*dIII sites. The expression cassette containing the deletion or point mutation of the hSP-B 1.5 kb *lacZ* gene and the simian virus 40 small T antigen poly(A) signals was dissected out, purified, and microinjected into the mouse eggs by the Transgenic Core Facility of the University of Cincinnati. Founders were identified by PCR using a pair of primers spanning from the hSP-B promoter to the *lacZ* gene. The lungs were collected from nontransgenic, wild-type or mutant hSP-B 1.5 kb *lacZ* gene transgenic FVB/N adult mice for homogenization in PBS. Approximately 1 ng of protein was used for the $\beta$-gal assay as described previously (*15*).

*Histochemistry of $\beta$-Gal Staining*. Whole lungs were prefixed with a fix solution (0.4 mL of 25% glutaraldehyde, 1.0 mL of 250 mM EGTA, 5.0 mL of 1 M $MgCl_2$, and 43.5 mL of PBS) on ice for 4 h, followed by three washes (15 min each) in a wash buffer containing 1.0 mL of 1 M $MgCl_2$, 5.0 mL of 2% sodium deoxycholate, 5.0 mL of 2% NP-40, and 489 mL of 100 mM sodium phosphate buffer, pH 7.3. Subsequently, the lungs were stained with LacZ solution (48 mL of wash buffer, 2 mL of 25 mg/mL X-gal, 0.106 g of K-ferrOcyanide, and 0.042 g of K-ferrIcyanide) overnight at room temperature. The next day, the lungs were rinsed three times (10 min each) with PBS and fixed in a postfixative solution (3.2 mL of 25% glutaraldehyde, 5 mL of 16% paraformaldehyde, 4 mL of 1 M sodium cacodylate, and 27.8 mL of water) for 10 min. The lungs were dehydrated through a graded series of ethanol washes. After fixation and embedding in paraffin, lung tissue sections were

cut to 5 $\mu$m thick. The slides were baked at 60 °C for 2 h, washed in a series of xylene and ethanol to remove paraffin from the tissues, and eoson stained.

*Cell Culture*. The human pulmonary adenocarcinoma cell line (H441) is originated from respiratory epithelial cells. H441 cells were cultured in RPMI supplemented with 10% fetal calf serum, glutamine, and penicillin/streptomycin. The cells were maintained at 37 °C in 5% $CO_2$/air, and passaged weekly.

*Gluthathionine Transferase (GST) Pull-Down Assay*. The GST fusion protein purification and pull-down assay followed a previously described procedure (*19*). To make GST fusion proteins, the SRC-1 HAT domain was subcloned into the pGEX4T-1 GST vector (Amersham) by PCR. The plasmids were transformed into BL21 bacterial strains for protein expression. After 3 h of induction at 37 °C by 1 mM isopropyl $\beta$-D-thiogalactoside (IPTG), the bacteria were harvested and resuspended in 1× PBS, followed by sonication and treatment with 1% Triton X-100. The proteins were purified by incubation with a 50% slurry of Glutathione Sepharose 4B beads (Amersham) for 30 min at room temperature and then eluted from the beads using glutathione (GS) elution buffer followed by dialysis. Protein expression was confirmed by Coomassie blue staining after gel electrophoresis. Protein concentrations were determined.

For the GST pull-down assay, TTF-1 full-length protein was synthesized and labeled with [$^{35}$S]methionine using Promega's in vitro transcription/translation kit. Approximately 1 $\mu$g of purified GST or GST−SRC-1 HAT was incubated with 20 $\mu$L of 50% glutathione Sepharose 4B beads at room temperature for 30 min. Approximately 25 $\mu$L of the [$^{35}$S]methionine-labeled TTF-1 full-length protein was added to the fusion protein−bead mixture and incubated at room temperature for 1.5 h. The protein−protein−bead mixtures were washed three times with 30 $\mu$L of 1× PBS. The protein−protein−bead complexes were then resuspended in 30 $\mu$L of 1× SDS sample buffer and run on 10−20% tricine polyacrylamide gels (Invitrogen-Novex). The protein gels were fixed and incubated in Amersham's Amplify reagent at room temperature. The gels were dried and exposed to X-ray films for visualization.

*TTF-1 Acetylation Assay*. The GST−TTF-1 HD fusion protein was subcloned into the pGEX4T-1 GST vector and purified as mentioned above. Nuclear protein extracts (100 $\mu$g) from H441 cells were immunoprecipitated with 2 $\mu$g of ACTR, CBP, or SRC-1 antibodies (Santa Cruz). The immunocomplexes were recovered by adding 50 $\mu$L of 50% protein A/G agarose (Santa Cruz) suspension and incubated at 4 °C for 2 h. The beads were washed sequentially for 5 min each in 1× PBS. Then acetylation reactions were performed in a 50 $\mu$L volume containing 50 mM Tris (pH 8.0), 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 $\mu$L of [$^{14}$C]acetyl-CoA (50 $\mu$Ci/mL, Amersham), 1 $\mu$g of the GST−TTF-1 HD fusion protein, and 10 $\mu$L of ACTR, CBP, and SRC-1 immunocomplexes. After incubation at 30 °C for 3 h, the reaction mixtures were analyzed by 10−20% TR gel (Invitrogen) and autoradiography.

*Anti-Acetyllysine Immunoprecipitation (IP)*. Approximately $10^6$ H441 cells were seeded in 10 cm dishes. The second day, 4.5 $\mu$g of wild-type or lysine-mutant TTF-1 HD expression constructs was transfected into the H441 cells by Fugene 6 for 2 days. The cells were lysed in a nuclei lysis buffer containing 0.5 M KCl, 1.5 mM $MgCl_2$, 20 mM Tris (pH 7.6), 0.2 mM EDTA, 25% glycerol, and 1 mM DTT. The cell lysates were immunoprecipitated with 2 $\mu$g of anti-Flag antibody at 4 °C overnight. Immunocomplexes were recovered by adding 50 $\mu$L of 50% protein A/G agarose (Santa Cruz) suspension and incubated at 4 °C for 2 h. The beads were washed sequentially for 5 min in 1× PBS. Immunoprecipitates were loaded onto 10−20% TR gel (Invitrogen) and visualized by Western blot using anti-acetyllysine antibody (Upstate Biotechnology).

*Transient Transfection*. H441 cells were seeded at a density of $2 \times 10^5$ cells per well in six-well plates. For TTF-1 mutant stimulation of hSP-B gene expression, various concentrations of TTF-1 lysine mutant expression vectors were cotransfected with hSP-B 500 luciferase reporter gene (0.25 $\mu$g) and 0.5 $\mu$g of pCMV-$\beta$-gal construct into H441 cells. For the TTF-1 and SRC-1 synergistic study, 0.5 $\mu$g of TTF-1 or 2 $\mu$g of SRC-1 or both in combination were cotransfected with the hSP-B 500 luciferase reporter construct (0.25 $\mu$g) and 0.5 $\mu$g of the pCMV-$\beta$-gal construct into H441 cells. After 72 h of incubation, the cells were lysed and the luciferase activities were determined. In each transfection study, the $\beta$-gal activities were determined for normalization of transfection efficiency. The statistical significance was determined by ANOVA.

## RESULTS

*Mutation at the TTF-1 DNA-Binding Site in the −500 to −331 bp Enhancer Region Reduces Expression of the hSP-B 1.5 kb lacZ Gene in Transgeneic Mice*. Previously, a transgenic mouse line carrying the hSP-B 1.5 kb 5′-flanking regulatory sequence and the *lacZ* gene has been established. In this mouse model, the hSP-B 1.5 kb *lacZ* gene expression recapitulates the endogenous SP-B gene expression in alveolar type II epithelial cells and Clara cells (*15*). Two clustered TTF-1 DNA-binding sites have been identified within this sequence by in vitro study (*6*, *7*). These two regions are highly conserved in both human and mouse species (*16*). In the −500 to −331 bp enhancer region, there are three clustered TTF-1 DNA-binding sites (Ba, Bb, Bc). Mutation at any one of these sites significantly reduces or abolishes hSP-B transcriptional activity in vitro (*7*). The lung contains multiple cell types. Expression of a particular gene is often determined by multiple cell−cell interactions. To prove the importance of these TTF-1 DNA-binding sites in vivo, a transgenic mouse model carrying a TTF-1 DNA-binding site point mutation at the Ba site, which is critical for TTF-1 DNA binding in vitro (*7*), was generated. Four animal founder lines were obtained. Lungs were isolated from the F1 generation of each founder line ($n = 3$) and homogenized for $\beta$-gal activity analysis. In comparison with the wild-type hSP-B 1.5 kb *lacZ* gene transgenic mice, all four founders showed marked reduction in $\beta$-gal activity, Figure 2.

*Deletion of the −160 to −60 bp Region Reduces Expression of the hSP-B 1.5 kb lacZ Gene in Transgenic Mice*. The second region containing clustered TTF-1 DNA-binding sites is located between the hSP-B −160 and −60 bp's (*6*). Mutation of these TTF-1 DNA-binding sites abolished hSP-B gene transcription in vitro. Transgenic mice carrying a deletion mutation of this region were generated. Three

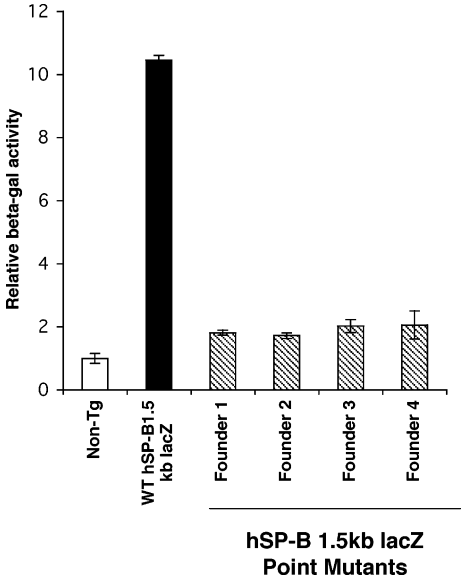


FIGURE 2: Mutation of the TTF-1 DNA-binding site in the enhancer region reduces hSP-B 1.5 kb *lacZ* reporter gene expression in transgenic mice. Lungs from nontransgenic mice (Non-Tg), wild-type (WT) hSP-B 1.5 kb *lacZ* gene transgenic mice, and point mutation mice were dissected out and homogenized. Equal amounts of proteins (1 ng) from different mouse founder lines were assayed for $\beta$-gal activity. Values are means ± SD, $n = 3$. The $\beta$-gal activities from nontransgenic mice were set as 1.

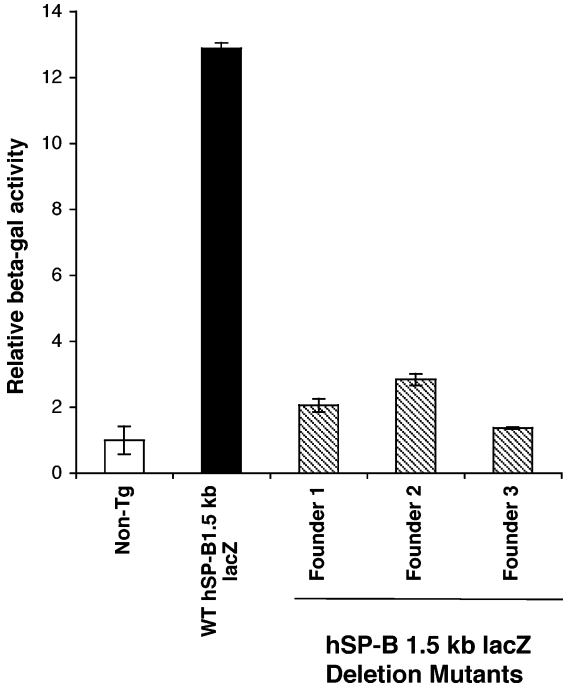


FIGURE 3: Deletion of the −160 to −60 bp promoter region reduces hSP-B 1.5 kb *lacZ* reporter gene expression in transgenic mice. Lungs from nontransgenic mice (Non-Tg), wild-type (WT) hSP-B 1.5 kb *lacZ* gene transgenic mice, and hSP-B −160 to −60 bp deletion mutation mice were dissected out and homogenized. Equal amounts of proteins (1 ng) from different mouse founder lines were assayed for $\beta$-gal activity. Values are means ± SD, $n = 3$. The $\beta$-gal activities from nontransgenic mice were set as 1.

founder lines were obtained. Lungs were isolated from the F1 generation of each founder line ($n = 3$) and homogenized for $\beta$-gal activity analysis. In comparison with the wild-type hSP-B 1.5 kb *lacZ* reporter gene transgenic mice, all founders showed marked reduction of $\beta$-gal activity, Figure 3.

*TTF-1 Point and Deletion Mutations Abolished Expression of the hSP-B 1.5 kb lacZ Gene in Alveolar Type II Epithelial Cells*. To further confirm that expression of the hSP-B 1.5 kb *lacZ* reporter gene is indeed abolished in alveolar type II epithelial cells in transgenic mice, $\beta$-gal histochemical staining was performed in the lungs of both mutant transgenic mice. In comparison with the wild-type hSP-B 1.5 kb *lacZ* gene mice, no $\beta$-gal staining was observed in the mutated transgenic mice, Figure 4, indicating that the TTF-1 DNA-binding sites in the hSP-B 5′-flanking regulatory sequence are required for hSP-B gene in vivo expression in alveolar type II epithelial cells.

*Interaction between TTF-1 and the SRC-1 HAT Domain*. Since TTF-1 is in close juxtaposition to RAR and nuclear receptor coactivators on the hSP-B −500 to −331 enhancer region (*4*), it is plausible that TTF-1 directly interacts with nuclear receptor coactivators. The HAT domains are good candidates for protein−protein interaction for their catalytic activity. The relative positions of the HAT domains on each nuclear receptor coactivator are illustrated in Figure 5A. To test this possibility, the SRC-1 HAT domain was chosen and subcloned into the GST expression vector. The GST−SRC-1 HAT fusion protein was expressed in bacteria and purified. When incubated with in vitro transcribed and translated TTF-1 that is radiolabeled with [$^{35}$S]methionine (Figure 5B), the GST−SRC-1 HAT domain pulled down $^{35}$S-labeled TTF-1 protein, Figure 5C. In comparison, a nonspecific weak signal was observed in the GST control. This observation strongly suggests that direct interaction between the HAT

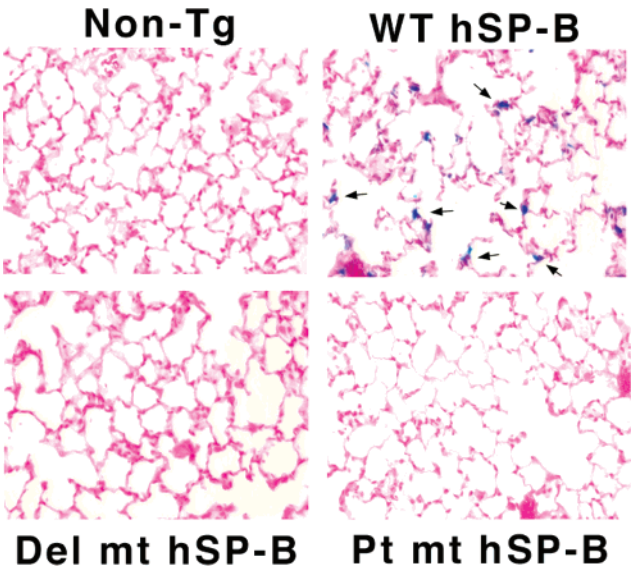


FIGURE 4: Mutation or deletion of TTF-1 DNA-binding sites in the hSP-B enhancer or promoter regions abolished hSP-B 1.5 kb *lacZ* gene expression in alveolar type II epithelial cells. Lung sections from nontransgenic (non-Tg), wild-type hSP-B 1.5 kb *lacZ* gene transgenic (WT hSP-B), hSP-B 1.5 kb *lacZ* gene promoter deletion transgenic (Del mt hSP-B), and hSP-B 1.5 kb *lacZ* gene enhancer point mutation transgenic (Pt mt hSP-B) mice were prepared and stained for $\beta$-gal activity. Original magnification 400× (reproduced at 70% of original size). Arrows point to $\beta$-gal-stained alveolar type II epithelial cells.

domains and protein substrates is required for the catalytic activity. In a transient transfection assay, SRC-1 further potentiated TTF-1 stimulation of the hSP-B 500 luciferase reporter gene in H441 cells, Figure 5D. Therefore, the

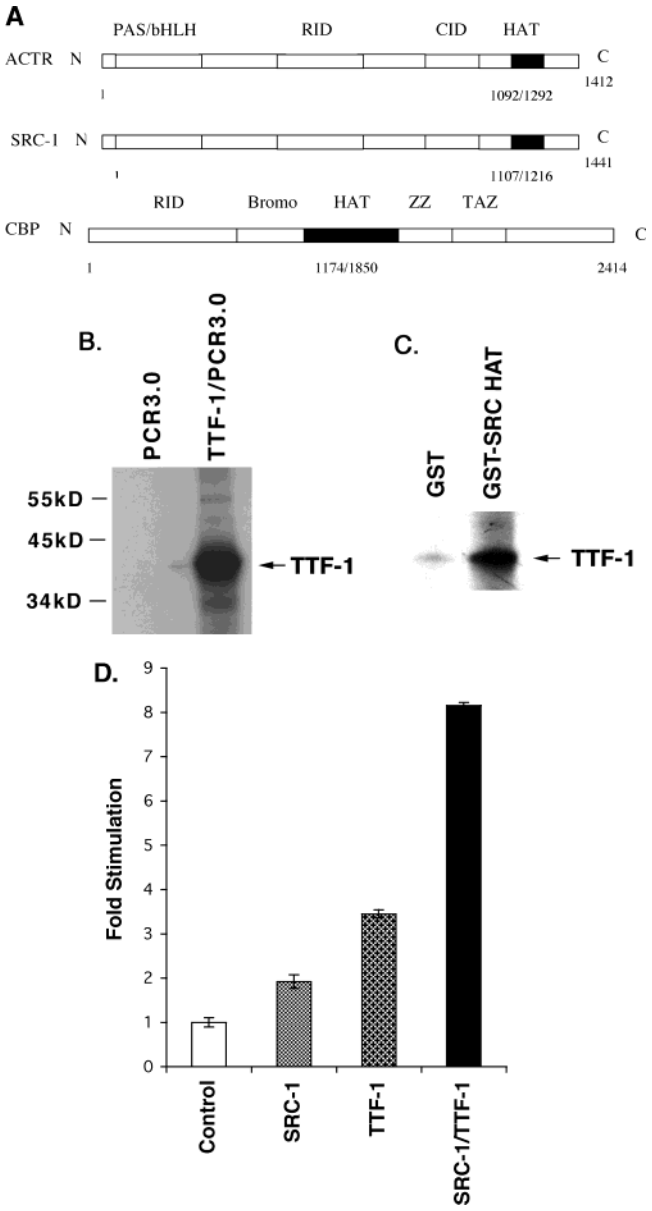


FIGURE 5: TTF-1 interaction with the SRC-1 HAT domain. (A) The schematic representation of ACTR, SRC-1, and CBP/p300 shows the position of the HAT domain. Other domains include the bromodomain (Bromo), CBP interaction domain (CID), helix−loop−helix domain (bHLH), receptor interaction domain (RID), histone acetyltransferase domain (HAT), and cysteine motifs (ZZ and TAZ) (40−43). (B) TTF-1 was radiolabeled by in vitro transcription and translation in the presence of [$^{35}$S]methionine and the vector TTF-1/PCR3.0. The empty parent vector PCR3.0 was used as a control. (C) The radiolabeled TTF-1 was subsequently incubated with GST, or GST−SRC-1 HAT fusion protein and purified by Glutathione Sepharose 4B beads. The bound GST−SRC-1 HAT complex was eluted and subjected to SDS−polyacrylamide gel electrophoresis. (D) The TTF-1 expression plasmid (0.5 $\mu$g) or the SRC-1 expression plasmid (4 $\mu$g) or both in combination were cotransfected with the hSP-B 500 luciferase reporter construct (0.25 $\mu$g) into H441 cells. Luciferase activities were measured 72 h later. The activity of hSP-B 500 without TTF-1 and SRC-1 cotransfection was defined as 1 (control). $\beta$-Gal activities were determined for normalization of transfection efficiency. Values are means ± SD ($n$ = 3).

interaction between TTF-1 and SRC-1 results in the synergistic stimulation of hSP-B transcription.

*TTF-1 Acetylation by Nuclear Receptor Coactivators in H441 Cells*. A previous study suggests that selective acetylation of lysine residues in the TTF-1 homeodomain led to conformational changes upon DNA binding (*28*). This implicates that the TTF-1 HD may be a target site for acetylation in cells. To investigate this possibility, nuclear extracts of H441 cells were immunoprecipitated with anti-ACTR, -CBP, or -SRC-1 antibodies. The immunoprecipitated coactivators were incubated with the purified GST−TTF-1 HD fusion protein (Figure 6A) in the presence of [C$^{14}$]acetyl-CoA. The protein mixtures were subjected to gel electrophoresis and visualized by autoradiography. The results showed that the TTF-1 HD indeed is acetylated by these immunocomplexes, Figure 6B. As a control, the GST−TTF-1 HD was not acetylated in the absence of the complexes.

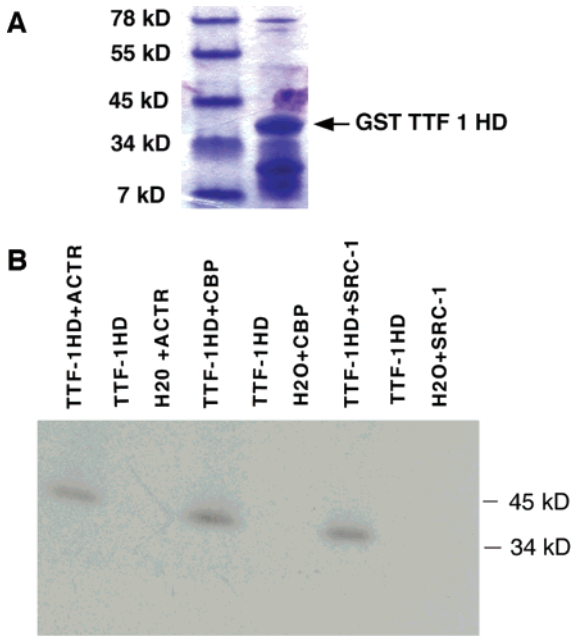


FIGURE 6: TTF-1 was acetylated by ACTR, CBP, and SRC-1 immunocomplexes. (A) Purified GST−TTF-1 fusion protein from bacteria. The gel was stained by Coomassic blue. (B) ACTR, CBP, or SRC-1 was pulled down by specific antibodies from H441 cell nuclear extracts. The immunocomplexes were incubated with the purified GST−TTF-1 HD fusion protein in the presence of [$^{14}$C]-acetyl-CoA. Controls did not include either the immunocomplexes or the purified GST−TTF-1 HD fusion protein (using $H_2O$ instead). The acetylated proteins were separated by SDS−polyacrylamide gel electrophoresis and visualized by autoradiography. ACTR, CBP, and SRC-1 represent immunocomplexes. $H_2O$ represents water.

*Identification of the Acetylated Lysine Residue in the TTF-1 HD*. There are six lysine residues in the TTF-1 HD, Figure 7A. To map the acetylating site(s), a series of Flag-tagged TTF-1 HD mutants were generated, in which the lysine residue was replaced by an alanine residue. The expression constructs of these point mutants were transfected into H441 cells for protein expression. The cell nuclear extracts from transfected cells were prepared and immunoprecipitated with the anti-Flag antibody to pull down Flag-tagged TTF-1 HD mutants and analyzed by Western blot with anti-acetylated lysine antibodies. As shown in Figure 7B, mutation at K182 abolished TTF-1 HD acetylation. Mutation at K161 also slightly reduced TTF-1 HD acetylation. Other TTF-1 HD lysine mutations remained unchanged. Therefore, K182 is the acetylation site in the TTF-1 HD. As a loading control, 10% of the cell extracts used for immunoprecipitation were monitored by Western blot using anti-

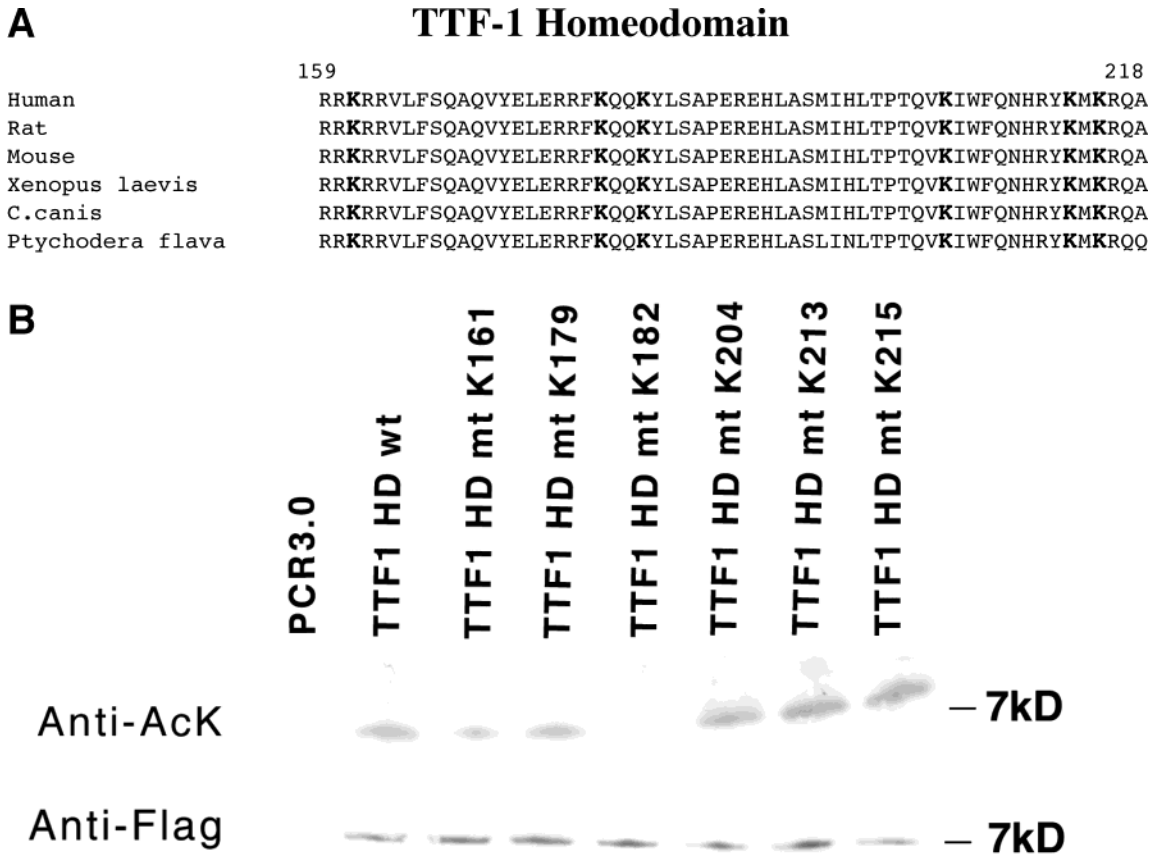


FIGURE 7: Identification of acetylated lysine residues in the TTF-1 HD by site-specific mutagenesis. (A) Homology comparison between TTF-1 homeodomains of various species. Lysine residues are highlighted by bold letters. (B) Six lysine residues in the TTF-1 HD were mutated to analine separately in the Flag-tagged TTF-1 HD mammalian expression vector. The mutated vectors were transfected to H441 cells. After 3 days of incubation, the cell nuclear extracts were immunoprecipitated with Flag antibody. Pull-down proteins were separated on polyacrylamide gel and immunoblotted with anti-acetylated lysine antibodies (upper panel). As a loading control, 10% of the extracts used for immunoprecipitation were monitored by Western blot using anti-Flag antibodies (lower panel). Abbreviations: Anti-AcK, anti-acetyllysine antibody; Anti-Flag, anti-Flag antibody; wt, wild type; mt, mutant; PCR3.0, parent empty vector with no Flag sequence.

Flag antibodies. All TTF-1 HD point mutants were expressed in H441 cells at the relatively same level, Figure 7B.

*Mutation at the K182 Position Inhibits TTF-1 Stimulation of hSP-B 500 Luciferase Reporter Gene Expression*. To evaluate the functional relevance of lysine residues in the TTF-1 HD in stimulating hSP-B gene transcription, various HD lysine mutations were introduced into the TTF-1 full-length molecule in the PCR3.0 expression vector accordingly. The expression constructs of these mutated TTF-1 molecules were cotransfected with the hSP-B 500 luciferase reporter gene construct into H441 cells. The wild-type expression construct was used as a control. After 72 h of incubation, the cells were lysed and the luciferase activities were determined. The pCMV $\beta$-gal plasmid was also included in the transfection assay for normalization of transfection efficiency. As shown in Figure 8, wild-type TTF-1 strongly stimulated transcription of the hSP-B 500 luciferase reporter gene activity in H441 cells. Interestingly, the K182 mutant down-regulated the hSP-B 500 luciferase reporter gene expression. Therefore, this mutant behaves like a dominant negative form of TTF-1. On the other hand, all other lysine mutations in the TTF-1 HD still demonstrated a stimulatory effect on the hSP-B 500 luciferase reporter gene in H441 cells, although to a much less extent. These lysine residues may be required for maintaining the proper TTF-1 HD configuration during DNA binding and transcription.

## DISCUSSION

Historically, the functional role of TTF-1 in lung biology is initiated through the study of SP-B gene regulation in respiratory epithelial cells (*6*). Subsequently, extensive biochemical and physiological characterization indicates that TTF-1 is an essential transcription factor required for lung development and respiratory-specific gene expression. Therefore, elucidation of the molecular mechanism for TTF-1 transactivation is important to understand these biological events in the lung. Given the tissue specificity and functional importance of SP-B in lung physiology, the SP-B gene is an ideal model system to study TTF-1 function in the lung. The expression patterns of TTF-1 and SP-B are overlapped in the developing and adult lung epithelium (*3*, *4*, *6*, *7*, *15*). In this study, deletion and mutation at two TTF-1 DNA-binding clustered sites (the −500 to −331 bp region and the −160 to −60 bp region) on the 5′-flanking regulatory sequence significantly reduced hSP-B gene transcription in vivo by using transgenic mouse models (Figures 2−4). This is consistent with the observations made in vitro (*6*, *7*). Therefore, TTF-1 is a key factor in controlling hSP-B gene temporal/spatial expression in the lung. By regulating distinct temporal/spatial expression of lung-specific genes, TTF-1 controls lung branching morphogenesis and organogenesis. On the other hand, the marked increase in surfactant protein synthesis occurring in late gestation is not directly related

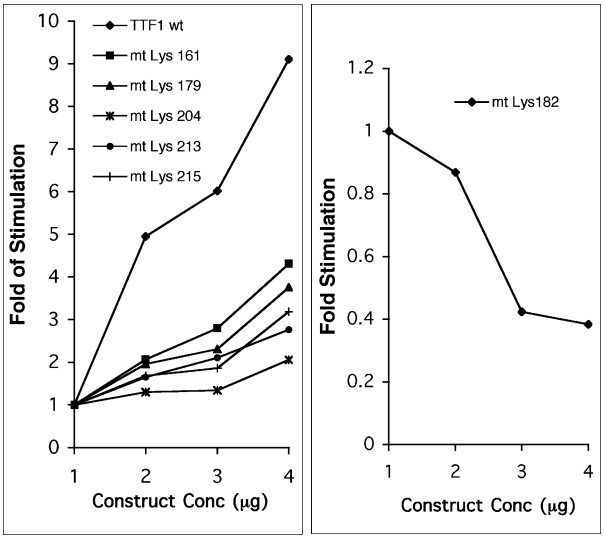


FIGURE 8: TTF-1 mutation at the K182 position inhibits expression of the hSP-B 500 luciferase reporter gene in H441 cells. Various lysine mutations in the TTF-1 HD were introduced into the full-length TTF-1 expression construct. The wild-type and mutated TTF-1 expression constructs at different concentrations (0, 1, 2, and 4 $\mu$g) were cotransfected with the hSP-B 500 luciferase reporter gene construct into H441 cells. Luciferase activities were measured 72 h later. The activity of the hSP-B 500 luciferase reporter gene without wild-type and mutant TTF-1 cotransfection was defined as 1. $\beta$-Gal activities were determined for normalization of transfection efficiency. Values are means ± SD ($n = 3$). Abbreviations: wt, wild type; mt, mutant.

to the level of the TTF-1 mRNA change (*3*). Therefore, other mechanisms must be involved in TTF-1 regulation of downstream genes.

The first mechanism involves the protein−protein interactions of TTF-1 with other transcription factors and coactivators. The interactions stabilize transcriptional complexes that synergistically stimulate activation of downstream genes. TTF-1 has been identified to interact with RAR on the −500 to −331 bp enhancer region to recruit nuclear receptor coactivators (*4*, *19*, *21*, *29*). The interaction between TTF-1 and RAR is mediated through their DNA-binding domains (*19*). RAR and nuclear receptor coactivators are coexpressed with TTF-1 in the developing and adult respiratory epithelium (*4*). In the present studies, the GST pull-down analysis demonstrates that TTF-1 directly interacts with the SRC-1 HAT domain (Figure 5C). Interestingly, this domain possesses the acetyltransferase activity. The interaction leads to SRC-1 potentiated TTF-1 stimulation of the hSP-B 500 luciferase reporter gene in the transient transfection assay in H441 cells (Figure 5D). In addition, a $Ca^{2+}$-binding calreticulin with chaperone activity binds to the TTF-1 HD to promote its folding and increases its transactivation (*30*).

The second mechanism involves TTF-1 posttranslational modification. TTF-1 is phosphorylated by multiple kinases, including PKA, (*27*, *31*), PKC (*32*), and MST2 (*33*). These kinases cause TTF-1 conformational and functional changes. Lacking TTF-1 phosphorylation has a severe physiological consequence. Mice bearing mutations at serine phosphorylation sites result in death immediately following birth (*34*). Redox regulation also affects TTF-1 DNA binding and dimerization ability (*35*). Here, we report that TTF-1 is subject to acetylation modification. Besides histone modification, nuclear receptor coactivators modify non-histone protein to influence gene expression. The balance between acetylation and deacetylation of protein factors is an important mechanism for gene regulation. Multiple nuclear receptor coactivator complexes, including ACTR, SRC-1, and CBP, are able to acetylate TTF-1 in vitro (Figure 6). This modification may explain why SRC-1 further enhances TTF-1 stimulation of hSP-B transcription in H441 cells in the transient transfection assay (Figure 5D). Furthermore, site-specific mutagenesis identifies that K182 in the TTF-1 HD is the site for acetylation (Figure 7). It has been suggested that a glycine or a serine residue immediately before the acetylated lysine is important for CBP acetylation (*36*) and that a positively charged residue (either lysine or arginine) at either the −3 or +4 position relative to the acetylated lysine is required (*37*). The sequence surrounding the K182 fits the pattern of this description. Interestingly, mutation at this site results in TTF-1 down-regulation of hSP-B promoter transcription in respiratory epithelial cells (Figure 8). To our knowledge, this is the first example that mutation at a lysine acetylation site of a transcription factor causes a dominant negative effect. Therefore, through acetylating TTF-1, nuclear receptor coactivators can influence lung-specific gene expression and lung development.

The detailed molecular mechanism for the TTF-1 dominant negative effect is not clear. Acetylation is a dynamic process and regulates many diverse functions of transcription factors. These include DNA recognition, protein−protein interaction, and protein stability (*23*, *25*, *38*, *39*). Association of nuclear receptors with ACTR is inhibited by acetylation (*24*). In addition to the requirement for stabilizing the DNA-binding activity, acetylation at TTF-1 K182 may be involved in protein−protein interactions with other associated factors to stabilize transcriptional complexes. TTF-1 without acetylation may lead to the conformational change that disrupts transcriptional complex formation to inhibit hSP-B gene transcription.

Taken together, our results show that acetylation by nuclear receptor coactivators directly regulates the TTF-1 transactivation activity. This change along with other changes (e.g., phosphorylation) is a crucial step in determining the architecture of promoter-bound transcription complexes to increase the nuclear retention, DNA binding, and transcriptional activation at the TTF-1 DNA-binding sites by facilitating formation of a stable transcription complex on the hSP-B 5′-flanking regulatory sequence. In addition, the recruitment of the HAT activity of nuclear receptor coactivator complexes modifies histone proteins to release transcriptional repression and further increase the accessibility of other transcriptional machinery to the SP-B promoter, resulting in binding of RNA polymerase II and activation of SP-B gene transcription in respiratory epithelial cells.

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