Cyclic AMP-responsive Expression of the Surfactant Protein-A Gene Is Mediated by Increased DNA Binding and Transcriptional Activity of Thyroid Transcription Factor-1*

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Surfactant protein (SP)-A gene transcription is stimulated by factors that increase cyclic AMP. In the present study, we observed that three thyroid transcription factor-1 (TTF-1) binding elements (TBEs) located within a 255 base pair region flanking the 5'-end of the baboon SP-A2 (bSP-A2) gene are required for maximal cyclic AMP induction of bSP-A2 promoter activity. We found that TTF-1 DNA binding activity was increased in nuclear extracts of pulmonary type II cells cultured in the presence of cyclic AMP. By contrast, the levels of immunoreactive TTF-1 protein were similar in nuclear extracts of control and cyclic AMP-treated type II cells. The incorporation of [32P]orthophosphate into immunoprecipitated TTF-1 protein also was markedly increased by cyclic AMP treatment. Moreover, exposure of nuclear extracts from cyclic AMP-treated type II cells either to potato acid phosphatase or alkaline phosphatase abolished the cyclic AMP-induced increase in TTF-1 DNA-binding activity. Interestingly, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), known to activate protein kinase C, also enhanced incorporation of [32P]orthophosphate into TTF-1 protein; however, the DNA binding activity of TTF-1 was decreased in nuclear extracts of TPA-treated type II cells. Expression vectors encoding TTF-1 and the catalytic subunit of protein kinase A (PKA-cat) were cotransfected into A549 lung adenocarcinoma cells together with an SP:A human growth hormone fusion gene (255 base pairs of 5'-flanking DNA from the baboon SP-A2 gene linked to human growth hormone, as reporter) containing TBEs, or with a reporter gene construct containing three tandem TBEs fused upstream of the bSP-A2 gene TATA box and the transcription initiation site. Coexpression of TTF-1 and PKA-cat increased fusion gene expression 3–4-fold as compared with expression of TTF-1 in the absence of PKA-cat. Moreover, the transcriptional activity of TTF-1 was suppressed by cotransfection of a dominant negative form of PKA regulatory subunit RIIα. We suggest that a PKA-induced increase of TTF-1 phosphorylation and TBE binding activity mediates cyclic AMP-induced expression of the SP-A gene in lung type II cells.

Pulmonary surfactant, a phospholipid-rich, developmentally regulated lipoprotein synthesized exclusively by type II pneumocytes, acts to reduce alveolar surface tension, thereby preventing alveolar collapse upon exhalation of air (1). Four lung-specific proteins have been found to be associated with surfactant: surfactant protein (SP)1-A, SP-B, SP-C, and SP-D. These appear to serve important roles in surface activity, surfactant phospholipid reutilization, and immune function within the alveolus (2).

Expression of the gene encoding SP-A, the major surfactant-associated protein, occurs primarily in alveolar type II cells and to a lesser extent in bronchiolar epithelial (Clara) cells (3, 4). SP-A gene expression is developmentally regulated in fetal lung in concert with surfactant phospholipid synthesis (5, 6); gene transcription is initiated only after ~70% of gestation is completed in all mammalian species thus far studied (6, 7). SP-A expression is subject to multifactorial regulation; we have observed that cyclic AMP and glucocorticoids have major regulatory effects (6). In studies using rabbit, human, and baboon fetal lung in organ culture, SP-A mRNA and protein levels were found to be augmented by cyclic AMP analogues and by agents that increase the levels of intracellular cyclic AMP (8–10). Cyclic AMP also enhances the rate of type II cell differentiation and enlargement of prealveolar ducts (9). SP-A is encoded by a single copy gene in rabbits (5), rats (11), dogs (12), and mice (13), while in baboons (14) and humans (15, 16), there are two highly similar SP-A genes (the SP-A1 and SP-A2 genes). In human fetal lung in culture, expression of the SP-A2 gene is considerably more responsive to the stimulatory effects of cyclic AMP than is SP-A1 (17).

To understand the molecular basis for type II cell-specific and cyclic AMP regulation of SP-A gene expression, we have utilized differentiated type II cells in primary culture transfected with reporter gene constructs containing 5'-flanking sequences from the rabbit and human SP-A genes. Two E-box motifs were identified within the 5'-flanking sequence of the rabbit SP-A gene that are required for basal and cyclic AMP-induced expression of SP-A promoter activity (18). These elements were found to bind the basic helix-loop-helix-zipper factors USF1 (19) and USF2. Another cis-acting element, termed CRESP-A, which displays sequence similarity to both a palin-
dromic cyclic AMP-responsive element (CRE) and a nuclear receptor half-site, also was found to be required for cyclic AMP induction of SP-A promoter activity in transfected type II cells (20–22). Characterization of CRE<sub>SP-A</sub> indicated that this sequence does not bind the transcription factor CRE-binding protein, CREB, but rather may serve as a binding site for a member of the nuclear receptor superfamily that binds DNA as a monomer (22). A GT box element, located proximal to the transcription initiation site, also was found to be essential for basal and cyclic AMP regulation of the human SP-A2 gene. This element was found to bind Sp1, as well as a 55-kDa protein distinct from Sp1 (23). These findings suggest that basal and cyclic AMP induction of SP-A promoter activity are mediated by the cooperative interaction of transcription factors bound to at least three different response elements.

Thyroid transcription factor-1 (TTF-1, also named thyroid enhancer-binding protein or NKs 2.1), is a homeodomain transcription factor that has been found to be involved in the tissue-specific regulation of three different thyroid-specific genes, namely thyroglobulin, thyroperoxidase, and thyrotropin receptor (24–28). TTF-1 expression is restricted to the developing thyroid gland, lung epithelia, and restricted areas of the developing brain (24, 29). The importance of TTF-1 in development was underscored by the findings of targeted deletion of its gene (30). Characterization of CRESP-A indicated that this sequence mediates the induction of SP-A promoter activity in transfected type II cells. We therefore suggest that the cyclic AMP-mediated increase in TTF-1 reporter gene activity in lung type II cells serves a major role in the cyclic AMP induction of SP-A gene expression in nonpulmonary cells.

It remains to be determined how the actions of TTF-1 to influence morphogenesis of thyroid, pituitary, and lung during early embryogenesis are regulated differently from those actions to enhance expression of specific genes at later stages of fetal development and postnatally. It was our objective, in the present study, to define the role of TTF-1 in the cyclic AMP induction of SP-A gene transcription. In type II cell transfection studies, we observed that TTF-1 binding elements (TBEs) in the 5′-flanking sequence of the baboon SP-A2 (bSP-A2) gene are crucial for cyclic AMP induction of bSP-A2 promoter activity. We also found that cyclic AMP treatment of cultured type II cells promotes increased TTF-1 phosphorylation and an associated increase in TBE binding activity. Furthermore, co-transfection of cyclic AMP-dependent protein kinase (PKA) with TTF-1 in a lung adenocarcinoma cell line caused an increase in the capacity of TTF-1 to transactivate the SP-A2 promoter. We therefore suggest that the cyclic AMP-mediated increase in TTF-1 DNA binding and transcriptional activity serves a major role in the cyclic AMP induction of SP-A gene expression in lung type II cells.

MATERIALS AND METHODS

Plasmids and Antiserum—A full-length baboon TTF-1 cDNA (~1.2 kilobase pairs) isolated from a 92-day gestational age fetal baboon lung cDNA library was ligated to EcoRI/SallI-digested pCMV5 vector to produce the TTF-1 expression vector, pCMV5/TTF-1. PKA expression vectors, RSV/VPKα-cat-α, RSV/VPKα-cat-β, and the mutated RSV/VPKα-cat-β mutants (35), were kindly provided by Dr. Richard A. Maurer (Oregon Health Sciences University). An expression vector containing the mutant form of PKA regulatory subunit RIIs under the control of the mouse metallothionein-1 promoter (MT-B-II-mat) (36) was kindly provided by Dr. Stanley McKnight (University of Washington, Seattle). bSP<sub>A2</sub> 255:1250 hGH was constructed by polymerase chain reaction amplification of a DNA fragment containing 255 bp of the 5′-flanking DNA and 40 bp of the first exon of the bSP-A2 gene, followed by ligation to the first exon of the hGH structural gene in pACsk<sub>6</sub>GH, which contains the 17% human adenovirus 5 genome and the promotorless hGH structural gene. To generate fusion genes containing TBE mutations, oligonucleotides were made containing mutations in each of the three TBEs of the bSP-A2 gene (TBE1, TBE2, and TBE3); in each case, the TBE was replaced with a restriction enzyme site sequence. The binding sequence in TBE1 was changed to a EcoRI site, that in TBE2 was changed to a KpnI site, and that in TBE3 was changed to an XhoI site. These oligonucleotides were then used in polymerase chain reaction with oligonucleotides corresponding to the 5′- or 3′-ends of the bSP-A2 genomic region from −255 to +40; bSP-A2 genomic clone was used as template. Polymerase chain reaction fragments were digested with appropriate endonucleases and cloned into pACsk<sub>6</sub>GH. To construct TBE1-III-digested pCMV5 vector to promote transactivation of heterologous reporter genes containing TBE1, TBE2, and TBE3, the mutant TBEs were inserted into the site of the 5′-flanking DNA containing TBE1 (−5′-GTGCTCCCCCTCAAGGGTGCTCA-3′) or the GT box from the human SP-A2 gene (underlined) (−3′-GACGGGTTGACCTAGCCCTT-3′), or the GT box from the human SP-A2 gene (underlined) (−5′-CTTCAGGGTTGGGAGAAA-3′) to generate a recombinant GST-TTF-1, peptide in Escherichia coli. Bacterially expressed GST-TTF-1-50 peptide was then used to immunize a rabbit to generate TTF-1 antiserum. The antiserum was absorbed with GST peptide to remove GST-specific antibodies.

Lung Type II Cells and Cell Lines—Lung tissues from mid-gestation human abortuses were maintained in organ culture in serum-free Waymouth’s MB752/1 medium (Life Technologies, Inc.) containing B<sub>e</sub>-CAMP for 3 days to promote type II cell differentiation. Phenotypically differentiated type II cells are not detected in mid-gestation human fetal lung. We previously observed that type II cells spontaneously differentiate when the human fetal lung is placed in organ culture in serum-free medium and that the rate of type II cell differentiation is enhanced by treatment with cyclic AMP analogues (9). After culture, the tissues were digested with collagenase for preparation of lung type II cells as described (20, 37). The human lung adenocarcinoma cell line A549 (ATCC CCL 185) was maintained in Waymouth’s MB752/1 medium containing fetal bovine serum (10%, v/v). Generation of Recombinant Adenoviruses—To generate recombinant adenoviruses, 293 cells, a permissive human embryonic kidney cell line, were cotransfected with recombinant pACsk<sub>6</sub>GH containing bSP-A2: hGH fusion genes and with pJM17; the latter contains the entire viral genomes of B<sub>e</sub>-CAMP for 3 days to promote type II cell differentiation. Phenotypically differentiated type II cells are not detected in mid-gestation human fetal lung. We previously observed that type II cells spontaneously differentiate when the human fetal lung is placed in organ culture in serum-free medium and that the rate of type II cell differentiation is enhanced by treatment with cyclic AMP analogues (9). After culture, the tissues were digested with collagenase for preparation of lung type II cells as described (20, 37). The human lung adenocarcinoma cell line A549 (ATCC CCL 185) was maintained in Waymouth’s MB752/1 medium containing fetal bovine serum (10%, v/v).

Expression of SP-A Fusion Genes in Transfected Type II Cells—Type II cells at a density of 5 × 10<sup>6</sup> cells/60-mm dish were maintained overnight in Waymouth’s MB 752/1 medium containing 10% fetal bovine serum. The cells were then washed twice with medium and incubated for 1 h with 1 × 10<sup>6</sup> recombinant viral particles, resulting in a multiplicity of infection of 0.1–0.2. In this manner, the same number of cells (1 × 10<sup>6</sup>) were infected in each experiment. The medium was then aspirated and replaced with fresh medium in the absence or presence of B<sub>e</sub>-CAMP (1 mM). Media from transfected cells were collected every 24 h and assayed for hGH by radioimmunoassay (Nichols Institute, San Juan Capistrano, CA).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from lung type II cells as described previously (38). Protein concentrations were determined by a modified Bradford assay (Bio-Rad). Double-stranded oligonucleotides containing TBE1 (underlined) (−5′-GTGCTCCCCCTCAAGGGTGCTCA-3′), CRE<sub>SP-A</sub>, (underlined) (−5′-GACGGGTTGACCTAGCCCTT-3′), or the GT box from the human SP-A2 gene (underlined) (−5′-CTTCAGGGTTGGGAGAAA-3′) were end-labeled using [γ-<sup>32</sup>P]ATP (ICN, Costa Mesa, CA) and used as probes. Nuclear proteins were incubated with the radiolabeled DNA probe for 30 min at room temperature in reaction buffer (20 mM Hepes, pH 7.6, 75 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.1 mM dithiothreitol (DTT), 0.1 mM dithioerythritol (DETT), 1 mM diethylpyrocarbonate (DEPC), (Pharmacia) as non-specific competitor. Protein-DNA complexes were separated on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. To determine whether TTF-1 DNA binding is phosphorylation-dependent, nuclear extracts (10 µg) were incubated either with 0.5 units of potato acid phosphatase (type III, Sigma) or 1 unit of alkaline phosphatase (Boehringer Mannheim) for 5
min at room temperature as described (39). A control, phosphatases were boiled for 15 min before incubation with nuclear extracts. Phosphatase reactions were stopped with 1 mM sodium vanadate and NaF. The phosphatase-treated nuclear extracts were incubated with \(^{32}\)P-labeled TBE1 oligonucleotides, fractionated on a 5% nondenaturing polyacrylamide gel, and visualized by autoradiography.

**Metabolic Labeling and Immunoprecipitation—**Human fetal lung type II cells were maintained either in control medium or in medium containing Bt2cAMP (1 mM) for 5 days. Parallel dishes of cells were incubated in control medium for 4 days and in medium containing TPA (10 nM) for an additional 24 h. For \(^{32}\)P-labeling, the type II cells were washed twice with Tris-buffered saline solution and incubated in phosphatase-free Dulbecco’s modified Eagle’s medium (Life Technologies) for 1 h. \(^{32}\)P-orthophosphate (NEN Life Science Products) was then added to achieve a concentration of 0.5 mCi/mL, and cells were further incubated in appropriate medium for 2 h. After washing twice with cold Tris-buffered saline, cells were lysed in 1 mL of RIPA buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 5 mM sodium fluoride, and 5 mM sodium vanadate). Cells were disrupted completely by scraping and shearing through a 27-gauge needle. After pelleting the cellular debris by centrifugation for 10 min at 4°C, aliquots of lysates containing 1 × 10⁶ cpm were precleared with 50 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 1 h at 4°C. Precleared lysates were then incubated with 5 μl of TTF-1 antisera or the corresponding preimmune serum. The immune complexes were recovered upon incubation with 40 μl of protein A/G PLUS-agarose for 1 h at 4°C. Pellets were washed twice with RIPA, twice with high salt RIPA (RIPA containing 1 mM NaCl), and twice more with RIPA. For \(^{35}\)S)methionine labeling, type II cells which had been cultured for 5 days under conditions described above were incubated in methionine-free DMEM (Life Technologies) for 30 min; \(^{35}\)S)methionine was then added to achieve a final concentration of 0.125 mCi/mL. Cells were lysed, and TTF-1 was immunoprecipitated as described for \(^{32}\)P-labeled cells. The immunoprecipitates isolated from the \(^{32}\)P-labeled and \(^{35}\)S)methionine-labeled cells were resuspended in 2 × SDS-sample buffer, resolved on a 12.5% SDS-polyacrylamide gel, and visualized by autoradiography.

**Transient Transfection—**Transient transfections were carried out using A549 cells maintained in Waymouth’s MB752/1 medium (Life Technologies) containing 10% fetal bovine serum. The plasmids used consisted of either 4 μg of BSAP-A2-255:hGH or (TBE)₃SP-A:hGH fusion genes, together with 2 μg of either an expression vector containing the entire TTF-1 open reading frame (pCMV5/TTF-1) or the empty expression vector (pCMV5) and 2 μg of either an expression vector containing the whole coding sequence of PKA-cat (RSV/PUK-caat) or the empty expression vector (RSV), with or without 2 μg of an expression vector containing a mutant form of PKA regulatory subunit RβA (MT-RβA-mut); 1 μg of RSV/B-Gal was used as the internal control. Prior to transfection, the plasmids were combined with 30 μg of DOTAP (Boehringer Mannheim) in Hanks’ balanced salt solution (pH 7.4) and incubated at room temperature for 20 min. A549 cells grown to logarithmic phase (50–70% confluence) on 60-mm diameter dishes were washed twice with phosphate-buffered saline. Plasmid-DOTAP mixtures in 2 mL of Waymouth’s MB 752/1 medium without serum were then added to the cells. The cells were then incubated for 24 h at 37°C and washed with phosphate-buffered saline. Waymouth’s MB 752/1 medium (1 mL) was then added to each dish, and the cells were incubated at 37°C for another 24 h. Media were then collected and assayed for hGH content by radioimmunoassay (Nichols Institute). Variations in transfection efficiency were corrected by normalizing hGH to β-galactosidase activity.

**RESULTS**

**TTF-1 Binding Elements Are Essential for Cyclic AMP Induction of SP-A Promoter Activity in Type II Cells—**In our initial studies of the regulation of the bSP-A2 gene, we observed that 255 bp of bSP-A2 5'-flanking DNA was sufficient to mediate high basal and cyclic AMP induction of bSP-A2 promoter activity in transfected type II cells. By DNase I footprinting and EMSA, three TBEs were characterized within the 255-bp 5'-flanking region. Whereas, TBE1 manifested the strongest DNase I footprint, the findings of EMSA have clearly indicated that TBE2 and TBE3 also serve as specific TTF-1 binding sites.\(^3\) The positions and sequences of these TBEs are shown in Fig. 1A. To analyze the functional significance of the TBEs in mediating basal and cyclic AMP induction of bSP-A2 promoter activity in type II cells, the three TBEs were individually mutated within the context of the 255-bp bSP-A2 5'-flanking region. Fusion genes were constructed composed of 255 bp of bSP-A2 5'-flanking DNA with or without TBE mutations plus 40 bp of the first exon, linked to the human growth hormone (hGH) structural gene, as reporter. These fusion genes were then incorporated into the genome of a replication-defective human adenovirus 5 for highly efficient and reproducible transfer by infection (20, 37). Equal amounts of recombinant adenoviruses were introduced into primary cultures of rat fetal lung type II cells incubated in the absence or presence of Bt2cAMP. The concentration of hGH that accumulated in the culture medium was determined by radioimmunoassay. As shown in Fig. 1B, mutagenesis of TBE1 caused a marked re-duction of bSP-A2-255:hGH fusion gene expression in transfected type II cells. Moreover, the cyclic AMP induction of fusion gene expression also was markedly decreased. Mutagenesis of TBE2 and TBE3 caused a moderate decrease in basal and cyclic AMP-induced fusion gene expression. These findings suggest that the integrity of TTF-1 binding elements is essential for maximal basal and cyclic AMP-induced bSP-A2 promoter activity in type II cells and that TBE1 plays the most critical role in this regard.

**TTF-1 DNA Binding Activity of Type II Cell Nuclear Extracts Is Increased by Bt2cAMP Treatment—**To study the mechanism(s) whereby TTF-1 mediates cyclic AMP induction of SP-A promoter activity in type II cells, we first utilized EMSA to analyze the effects of cyclic AMP on TTF-1 DNA binding activity in nuclear extracts from cultured type II cells. An oligonucleotide containing TBE1 was radiolabeled and incubated with equivalent amounts of nuclear proteins from type II cells cultured for 5 days in the absence or presence of 1 mM Bt2cAMP. This incubation time was utilized because we previously observed that SP-A gene expression is markedly increased in type II cells incubated for 5 days in medium containing Bt2cAMP, as compared with cells maintained in control medium (37). The DNA-protein complexes were separated from free probe on a nondenaturing polyacrylamide gel (Fig. 2A). As can be seen, type II cell nuclear protein-TBE1 complex formation was markedly increased in nuclear extracts from type II cells cultured in the presence of Bt2cAMP as compared with those of type II cells cultured in control medium. The finding that formation of the protein-DNA complex was abolished by co-incubation with antisera against TTF-1 indicates that the binding complex contains TTF-1 (data not shown). To determine whether the increased TTF-1/TBE1 complex formation was due to increased TTF-1 protein expression, equivalent amounts of nuclear proteins from type II cells cultured in the absence or presence of Bt2cAMP were analyzed for TTF-1 protein content by immunoblotting (Fig. 2B). Despite the increased TTF-1/TBE1 complex formation, TTF-1 protein levels were relatively unaffected by Bt2cAMP treatment. Therefore, the increased TTF-1/TBE1 complex formation in Bt2cAMP-treated type II cells was due to increased TTF-1 DNA binding activity, not to increased levels of TTF-1 protein.

To determine whether the increase in DNA binding activity in type II cells cultured in the presence of Bt2cAMP was specific for TTF-1, type II cell nuclear protein binding activity for TBE1 was compared with binding activity for two other elements also found to be critical for cyclic AMP induction of SP-A promoter activity, CRESP-A and GT box. Equal amounts of nuclear pro-

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\(^3\) J. Li, E. Gao, and C. R. Mendelson, unpublished observations.
teins from type II cells cultured in the absence or presence of Bt2cAMP were incubated with radiolabeled TBE1, CRESP-A, or GT box oligonucleotides and analyzed by EMSA (Fig. 3). While TTF-1/TBE1 complex formation was increased in nuclear extracts from cyclic AMP-treated type II cells as compared with extracts from type II cells cultured in control medium, the protein-DNA complexes formed with radiolabeled CRESP-A and GT box were relatively unaffected by Bt2cAMP treatment, indicating that treatment of type II cells with Bt2cAMP specifically increases TTF-1 DNA binding activity.

**TTF-1 Phosphorylation Level Is Increased in Type II Cells Cultured in the Presence of Bt2cAMP**—To determine whether phosphorylation of TTF-1 was associated with the increased TTF-1 DNA binding activity in Bt2cAMP-treated type II cells, we analyzed whether cyclic AMP altered the rate of TTF-1 phosphorylation. Type II cells were maintained in the absence or presence of Bt2cAMP for 5 days and then cultured for 2 h either with [32P]orthophosphate or [35S]methionine. The radiolabeled cell lysates were immunoprecipitated with anti-TTF-1 or preimmune serum. As can be seen in Fig. 4, incorporation of [32P]orthophosphate into immunoprecipitated TTF-1 was markedly increased in type II cells cultured in the presence of Bt2cAMP (lane 2) as compared with type II cells cultured in control medium (lane 1). By contrast, incorporation of [35S]methionine into immunoprecipitated TTF-1 was relatively unaffected by cyclic AMP treatment (lanes 3 and 4), indicating that the rate of TTF-1 synthesis was similar in Bt2cAMP-treated type II cells as compared with that of type II cells cultured in control medium. Therefore, the increased TTF-1 DNA binding activity in Bt2cAMP-treated type II cells is associated with an increase in TTF-1 phosphorylation. The broad band in the immunoprecipitation of 35S-labeled type II cell lysates may in part represent differentially phosphorylated forms of TTF-1. The faster migrating component of the band also may comprise some nonspecific interactions with the antiserum, since a portion of this was observed in immunoprecipitations using preimmune serum (data not shown).

**TTF-1 Binding Activity Is Abolished by Phosphatase Treatment**—To determine whether increased TTF-1 phosphorylation is responsible for the increase in TTF-1 DNA binding activity
Bt$_2$cAMP-treated type II cells, we analyzed the effect of phosphatases on the TTF1-TBE1 complex formation. Nuclear extracts from type II cells cultured in the absence or presence of Bt$_2$cAMP were exposed either to potato acid phosphatase or alkaline phosphatase for 5 min as described (39) prior to EMSA. In parallel, nuclear extracts were treated with heat-inactivated phosphatases as controls. As can be seen in Fig. 5, treatment either with potato acid phosphatase (lanes 4 and 5) or alkaline phosphatase (lanes 8 and 9) markedly reduced TTF1-TBE1 complex formation in nuclear extracts from control and cyclic AMP-treated type II cells to levels less than those of nuclear extracts from control cells in the absence of phosphatase treatment. By contrast, heat-inactivated phosphatases had no effect on TTF1 binding activity (lanes 6, 7, 10, and 11). These findings indicate that TTF1 binding activity is phosphorylation-dependent and that increased TTF1 phosphorylation in Bt$_2$cAMP-treated type II cells is responsible for the increased TTF1 DNA binding activity.

**Phorbol Ester Promotes TTF1 Phosphorylation but Inhibits the TTF1-DNA Binding Activity**—It was reported recently that TTF1 was effectively phosphorylated by protein kinase C (PKC) in vitro (40). On the other hand, it also was reported that TPA, a phorbol ester known to activate PKC, reduces the levels of SP-A protein and mRNA expression in H441 lung adenocarcinoma cells (41, 42). To determine whether TTF1 is phosphorylated by PKC in vivo, type II cells maintained in control medium for 4 days were treated with 10 nM TPA for 24 h and then labeled with $[^{32}P]$orthophosphate and immunoprecipitated with anti-TTF1. To compare the effects of TPA to those of Bt$_2$cAMP on TTF1 phosphorylation, type II cells were incubated for 5 days in medium containing 1 mM Bt$_2$CAMP. The TTF1 DNA binding activity of Bt$_2$CAMP-treated type II cells was increased to a level comparable with that of Bt$_2$CAMP-treated cells, suggesting that TTF1 was effectively phosphorylated by PKC in vivo. To determine the effects of TPA-induced phosphorylation on TTF1 binding activity, EMSA was used to analyze TTF1 DNA binding activity in nuclear extracts from TPA-treated type II cells as compared with nuclear ex-
FIG. 6. Effects of TPA and Bt2cAMP on TTF-1 phosphorylation
and DNA binding activity in type II cells. Type II cells were
cultured either in control or Bt2cAMP (1 mM)-containing medium for 5
days. Parallel dishes of cells were cultured in control medium for 4 days
and with TPA (10 nM) for an additional 24 h. A, immunoprecipitation of
32P-labeled type II lysates with anti-TTF-1 antiserum. The rate of
incorporation of 32P-orthophosphate into immunoprecipitated TTF-1
was analyzed as described in Fig. 4 B. TTF-1 binding activity in nuclear
extracts of type II cells incubated in the absence or presence of TPA.
Nuclear extracts from these type II cells were incubated with 32P-
labeled TBE1 oligonucleotide and analyzed by EMSA.

TTF-1 Transcriptional Activity Is Increased by PKA—To
determine whether the cyclic AMP-mediated increase in TTF-1
phosphorylation and DNA binding activity in Bt2cAMP-treated
Type II cells is associated with increased TTF-1 transcriptional
activity, A549 cells were cotransfected with bSP-A2–255:hGH,
containing three TTF-1 binding elements, with a TTF-1 expres-
sion vector and expression vectors containing either the α-
or β-isofrom of the PKA catalytic subunit; the respective empty
vectors were cotransfected as controls where appropriate. A549
cells, a human lung adenocarcinoma cell line of presumed type
II cell origin (43) were chosen, since we have found that they do
not express detectable levels of endogenous TTF-1 protein
(data not shown). In previous studies, we observed that expres-
sion of rabbit and human SP-A:hGH fusion gene constructs are
not cyclic AMP-inducible in these cells. Transcription of the
reporter gene was evaluated by measuring the hGH concentra-
tion in the culture medium. As shown in Fig. 7 A, cotransfection
with TTF-1 alone increased bSP-A2–255:hGH fusion gene
expression approximately 3-fold as compared with transfection
with bSP-A2–255:hGH and control vector. Cotransfection with
PKA-cat-α (the α-isofrom of the PKA catalytic subunit) and
TTF-1 increased the activity of bSP-A2–255:hGH by 8-fold as
compared with cells transfected only with the reporter gene
construct alone; this was 2.7-fold greater than with TTF-1 in
the absence of PKA-cat. Cotransfection with PKA-cat-β (the
β-isofrom of the PKA catalytic subunit) and TTF-1 increased
the expression of bSP-A2–255:hGH by 11-fold as compared with
the reporter gene construct alone; this was 3.7-fold greater
than with TTF-1 in the absence of PKA-cat. Neither PKA-cat-α
nor PKA-cat-β had the effect of altering bSP-A2–255:hGH
expression in the absence of cotransfected TTF-1 (Fig. 7 A), fur-
ther suggesting that the effect of PKA is mediated by TTF-1. By
contrast, a mutated form of PKA-cat-β, PKA-cat-βm, had no
effect on fusion gene expression in cells cotransfected with
TTF-1. The effect of PKA-cat also was lost when the major
TTF-1 binding site TBE1 was mutated in bSP-A2–255:hGH
(Fig. 7 B). To ensure that the effects of PKA-cat observed were
due to increased TTF-1 activity, rather than to increased TTF-1
expression via PKA induction of CMV promoter activity, the
levels of TTF-1 protein were analyzed in the transfected cells
by immunoblotting. Immunoreactive TTF-1 was undetectable
in A549 cells transfected with “empty” expression vector but
readily detected in cells transfected with pCMV5/TTF-1. Co-
transfection with RSV/PKA-α, RSV/PKA-β, or RSV/PKA-βm
expression vectors had essentially no effect on the levels of
immunoreactive TTF-1 in the A549 cells (data not shown).

To determine whether the effect of PKA-cat to increase
bSP-A2 promoter activity in the presence of TTF-1 is mediated
specifically by the TBEs and occurs in the absence of other
regulatory elements, co-transfection studies in A549 cells also
were performed using the reporter construct (TBE)3bSP-A2–
hGH, a fusion gene comprised of three TTF-1 binding elements
and a basal bSP-A2 promoter linked to hGH, as reporter. As
shown in Fig. 8, (TBE)3bSP-A2–hGH expression was increased
2.8-fold upon cotransfection of TTF-1 and increased by 6.6- and
8.6-fold in cells cotransfected with TTF-1 and PKA-cat-α and
PKA-cat-β, respectively. It should be noted that the levels of
expression of the (TBE)3bSP-A2–hGH fusion gene were consid-
erable lower than those of bSP-A2–255:hGH in the A549
cells; however, the inductive effects of cotransfected TTF-1 and PKA-
cat were similar. This finding supports our previous observa-
tions regarding the importance of other regulatory elements (i.e. CRE-
SP-A, GT box) for basal and cyclic AMP induction of
SP-A promoter activity in transfected lung type II cells (20–23).

To determine whether the activation of bSP-A2–255:hGH
fusion gene expression by TTF-1 in the absence of PKA cata-
lytic subunit in A549 cells is dependent upon endogenous PKA
activity, an expression vector containing a dominant-negative
form of PKA regulatory subunit RIα (RIα-mut) was cotrans-
fected into A549 cells in the absence or presence of TTF-1.
The properties of RIα-mut, in which both cyclic AMP binding sites
are mutated, has previously been described (36). As shown in
Fig. 9, RIα-mut had no effect on basal levels of bSP-A2–255:
hGH fusion gene expression, suggesting that basal activity of
the bSP-A2 promoter in A549 cells is not dependent upon
endogenous PKA activity. Interestingly, cotransfection of RIα-
mut was sufficient to suppress the transactivation potential
of TTF-1, suggesting that phosphorylation of TTF-1 by appar-
ently low levels of endogenous PKA activity is required for
TTF-1 activation of bSP-A2–255:hGH fusion gene expression.
The increase of TTF-1 transcriptional activity by PKA-cat-β
also was inhibited by cotransfection of RIα-mut (Fig. 9).

DISCUSSION

We previously observed that cyclic AMP serves a major role
in the induction of type II cell differentiation (8) and SP-A gene
expression (8–10) in human, rabbit, and baboon fetal lung in
culture. In type II cell transfection studies, we found that cyclic
AMP stimulation of SP-A promoter activity is dependent upon
the cooperative interaction of transcription factors bound to at
least three types of regulatory elements. These include a CRE-
like element (20–22), which appears to bind a member of the
nuclear receptor family, an E-box (18), which binds USF1 (19)
and USF2, and a GT box, which binds Sp1 (23) and other
members of the Kruppel family. We have observed in type II cell transfection studies that mutagenesis of any one of these elements causes a marked reduction of basal and cyclic AMP stimulation of SP-A promoter activity (18, 20–23); however, the mechanisms whereby transcription factors binding to these elements mediate cyclic AMP responsiveness have not been determined.

In the present study, we observed that three TBEs within the 5'-flanking region of the bSP-A2 gene also are functionally required for cyclic AMP activation of the bSP-A2 promoter in lung type II cells. Mutation of the TBEs caused a marked reduction of basal and cyclic AMP-induced bSP-A2 fusion gene expression in transfected type II cells. These findings suggest that cyclic AMP stimulation of SP-A gene expression in lung type II cells also is dependent upon the cooperative interactions of TTF-1 with transcription factors bound these other response elements and that all of these elements are essential for cyclic AMP induction of SP-A promoter activity.

In studies to define the mechanism(s) whereby TTF-1 mediates cyclic AMP induction of SP-A gene expression in type II cells, we observed that TTF-1 DNA binding activity of type II cell nuclear extracts was increased by cyclic AMP treatment. By contrast, nuclear protein binding activities for CRESP-A and the GT box were unaffected by cyclic AMP. These findings indicate for the first time that cyclic AMP specifically increases TTF-1 binding activity in type II cells. Our finding that the

4 E. Gao, L. Wang, and C. R. Mendelson, unpublished observations.

FIG. 7. Effects of PKA on TTF-1 induction of bSP-A2-255:hGH fusion gene expression. A, A549 cells were transfected with bSP-A2-255:hGH in the absence or presence of pCMV5/TTF-1 or pCMV5 empty vector and with RSV/PKA-cat-α, RSV/PKA-cat-β, RSV/PKA-cat-β, or the corresponding empty vector plus internal control, RSV/β-Gal. Shown are the levels of secreted hGH into the medium over a 24-h period, 48 h after transfection. Data are the means ± S.E. from two independent experiments, each conducted in triplicate, normalized to β-galactosidase activity.

FIG. 8. Effects of PKA on TTF-1 induction of (TBE)3SP-A:hGH fusion gene expression. The (TBE)3SP-A:hGH construct was cotransfected into A549 cells in the absence or presence of pCMV5/TTF-1 or pCMV5 empty vector and with RSV/PKA-cat-α, RSV/PKA-cat-β, RSV/PKA-cat-β, or the corresponding empty vector plus internal control, RSV/β-Gal. Shown are the levels of secreted hGH into the medium over a 24-h period, 48 h after transfection. Data are the means ± S.E. from two independent experiments, each conducted in triplicate, normalized to β-galactosidase activity.

FIG. 9. Effect of a dominant negative mutant form of PKA regulatory subunit RIα on TTF-1 transcriptional activity. bSP-A2-255:hGH was cotransfected into A549 cells in the absence or presence of pCMV5/TTF-1 or pCMV5 empty vector, together with MT-RIα-mut or the empty vector plus internal control RSV/β-Gal. Shown are the levels of secreted hGH into the medium over a 24-h period, 48 h after transfection. Data are the means ± S.E. from two independent experiments, each conducted in triplicate, normalized to β-galactosidase activity.
levels of immunoreactive TTF-1 in nuclear extracts as well as the rate of incorporation of $[^35S]$methionine into immunisolated TTF-1 were unaffected by cyclic AMP treatment of type II cells suggests that cyclic AMP induction of TTF-1 binding activity is not mediated by changes in its nuclear localization or expression.

To begin to define the mechanisms whereby cyclic AMP increases TTF-1 binding activity, we analyzed effects on TTF-1 phosphorylation in human fetal type II cells in primary culture. We observed that, in association with its effect of stimulating TTF-1 DNA binding activity, cyclic AMP treatment markedly increased the ratio of $[^32P]$-phosphate incorporation into immunisolated TTF-1. The finding that phosphatase treatment effectively abolished the cyclic AMP induction of TTF-1 DNA binding activity indicates that cyclic AMP-induced TTF-1 phosphorylation mediates the increase in binding activity for TBEs within the bSP-A 5′-flanking sequence. While findings of previous studies (28, 39) indicated that TTF-1 DNA binding activity for the thyroglobulin and thyroperoxidase gene promoters was induced by treatment of nuclear extracts with the PKA catalytic subunit in vitro, no evidence has been presented to indicate that TTF-1-binding activity is increased by PKA-mediated phosphorylation in vitro. In fact, in studies of TTF-1 binding activity in FRTL-5 cells, it appeared that binding activity was reduced in thyrotropin-treated cells as compared with controls (28). Furthermore, the role of PKA-mediated phosphorylation in TTF-1 DNA binding activity was questioned in a later study (40) in which it was found that TTF-1 was not phosphorylated by PKA in vitro and that mutated forms of TTF-1 that could not be phosphorylated manifested normal levels of DNA binding and transcriptional activity in transfected HeLa cells. On the other hand, in a recent study, a PKA phosphorylation site near the N terminus (Thr$^9$) of TTF-1 was identified and found to be essential for PKA activation of the SP-B promoter in H441 cells, a lung adenocarcinoma cell line of Clara cell origin (44). The findings of the present study clearly indicate that cyclic AMP treatment of primary cultures of lung type II cells under conditions that increase SP-A gene transcription causes an increase in TTF-1 phosphorylation and DNA binding activity.

In the present study, we observed that the rate of TTF-1 phosphorylation also was increased when type II cells were cultured in the presence of TPA. This is consistent with previous findings that TTF-1 may serve as a substrate for PKC-induced phosphorylation in vitro (40). However, in contrast to the inductive effects of cyclic AMP, we found that TPA treatment caused a reduction of TTF-1 DNA binding activity. Interestingly, TPA has been found to decrease SP-A protein and mRNA levels in H441 cells in a time- and dose-dependent manner (41) and to decrease the rate of SP-A gene transcription (42). Based on these findings, we suggest that the inhibitory effect of TPA on SP-A gene expression is mediated, in part, by a decrease in TTF-1 DNA binding activity. It is possible that phosphorylation of specific residues by PKC interferes with the DNA binding activity of TTF-1 in TPA-treated type II cells. It should be noted that it is not known whether the increased TTF-1 phosphorylation that we observed in the TPA-treated type II cells is directly mediated by PKC or whether it is an indirect effect via activation of another protein kinase. Since the TTF-1 protein level in nuclear extracts of TPA-treated type II cells was found to be comparable with that of type II cells cultured either in control medium or in the presence of Bt$_3$AMP, phosphorylation of TTF-1 does not seem to alter the nuclear localization or steady state levels of TTF-1 protein. This is in contrast to recently published findings of Kumar et al. (45), which suggest that TPA treatment of H441 cells cause cytoplasmic trapping of TTF-1, resulting in a loss of TTF-1 from the nucleus.

A549 is a lung adenocarcinoma cell line of presumed type II cell origin (43) that lacks endogenous TTF-1. In the present study, A549 cells were transfected with a reporter gene construct (bSP-A$_2$-255:hGH) comprised of 5′-flanking sequence from the bSP-A2 gene containing three TBEs; cotransfection of a TTF-1 expression vector caused an induction of bSP-A2 promoter activity. The response to TTF-1 was increased further by cotransfection of the PKA catalytic subunit. The finding that PKA-promoter had no effect of increasing bSP-A2 promoter activity in the absence of cotransfected TTF-1 and that mutation of the major TTF-1 binding site abolished PKA induction of TTF-1 transcriptional activity suggests that the action of PKA to induce bSP-A2 gene expression is mediated, at least in part, through TTF-1. To further substantiate the role of TTF-1 in PKA induction of SP-A promoter activity, A549 cells transfected with a reporter gene containing three tandem TBEs fused upstream of the bSP-A2 gene TATA box and transcription initiation site (TBE)$_5$SP-A2:hGH were cotransfected with PKA-promoter and TTF-1 expression vectors. The finding that PKA-promoter enhanced transactivation of (TBE)$_5$SP-A:$hGH$ by cotransfected TTF-1 indicates that the action of PKA to increase SP-A promoter activity is mediated specifically by TTF-1 binding to TBEs in the absence of other response elements. These findings, together with those indicating that cyclic AMP specifically increases TTF-1 binding activity in type II cell nuclear extracts, suggest that TTF-1 is the cyclic AMP-responsive transcription factor in lung type II cells. It should be noted, however, that basal and TTF-1/PKA-stimulated levels of expression of the (TBE)$_5$SP-A2$hGH$ fusion gene were considerably reduced as compared with those of the bSP-A2-255:hGH fusion gene construct, indicating the importance of the cooperative interaction of the TBEs with other response elements in basal and cyclic AMP regulation of SP-A promoter activity.

In A549 cell transfection studies, we also observed that the TTF-1 induction of bSP-A$_2$-255:hGH fusion gene expression in the absence of cotransfected PKA catalytic subunit was prevented by cotransfection of a dominant negative form of PKA RII. This finding suggests that the inductive effect of TTF-1 on bSP-A2 promoter activity in A549 cells is dependent upon phosphorylation by endogenous PKA activity. Interestingly, Bt$_3$AMP had no effect of increasing TTF-1 transcriptional activity in A549 cells cotransfected with TTF-1 and bSP-A$_2$-255:hGH (data not shown). These findings suggest that A549 cells may be defective in some component of the cyclic AMP-mediated signaling pathway.

In conclusion, the findings presented in this study suggest that cyclic AMP-responsive expression of the SP-A gene is mediated by an increase in TTF-1 transcriptional activity, which is associated with increased TTF-1 phosphorylation and DNA binding activity. It appears that the PKA-mediated increase in TTF-1 phosphorylation and DNA binding activity may constitute the primary mechanism whereby cyclic AMP induces SP-A gene expression. We suggest that the increase in TTF-1 phosphorylation and DNA binding activity may, in turn, facilitate its interaction with transcription factors bound to other cis-acting elements found to be essential for cyclic AMP induction of SP-A promoter activity, including CRP$_{SP-A}$ (20–22), the GT box (23), and E-box sequences, which bind USF1 (18, 19) and USF2, as well as with components of the basal transcription complex.

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Cyclic AMP-responsive Expression of the Surfactant Protein-A Gene Is Mediated by Increased DNA Binding and Transcriptional Activity of Thyroid Transcription Factor-1

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