GATA-6 Activates Transcription of Thyroid Transcription Factor-1*

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Thyroid transcription factor-1 (TTF-1) is expressed in respiratory epithelial cells, where it regulates the transcription of target genes expressed in a cell-selective manner. GATA-5 and -6, members of the zinc finger family of transcription factors, are also expressed in various cell types within in the developing lung. In the present work, GATA-6 mRNA was detected in adult mouse lung, purified mouse type II epithelial cells, and differentiated mouse pulmonary adenocarcinoma cells (MLE-15 cells), being co-expressed with TTF-1 mRNA. In order to test whether GATA factors regulated TTF-1 gene transcription, GATA-5 and -6 expression vectors were co-transfected with TTF-1 luciferase expression vectors. GATA-6, but not GATA-5, markedly activated TTF-1 gene transcription in HeLa cells. EMSA and supershift analysis with GATA-6 antiserum demonstrated that GATA-6 in MLE-15 cell nuclear extracts bound to an element located 96–101 base pairs from major start of TTF-1 gene transcription. Site directed mutagenesis of the GATA element in the TTF-1 promoter region inhibited trans-activation by GATA-6 in HeLa cells. GATA-6 is co-expressed with TTF-1 in the respiratory epithelium in vivo and respiratory epithelial cells in vitro. GATA-6 strongly enhanced activity of the human TTF-1 gene promoter in vitro. These findings support the concept that GATA-6 may play an important role in lung cell differentiation and gene expression, at least in part by altering the expression of TTF-1 and its potential targets.

Thyroid transcription factor-1 (TTF-1) is a 38-kDa member of the Nkx family of transcription factors (1). TTF-1 is expressed in developing thyroid and respiratory epithelium, as well as in subsets of cells in the embryonic diencephalon (2). In the fetal lung, TTF-1 is expressed in highly regulated pattern in subsets of respiratory epithelial cells, both in the conducting and peripheral airways and lung sacculles (3–5). The level of expression of TTF-1 mRNA is generally higher in fetal than in postnatal lung, being confined primarily to subsets of bronchio- and type II epithelial cells in the postnatal human and mouse lung. TTF-1 binds to and activates the transcription of a number of genes expressed selectively in thyroid (e.g. thyroperoxidase, thyroglobulin, and the sodium-iodide transporter) (6, 7) and in lung (surfactant proteins A, B, and C (SP-A, SP-B, and SP-C) and Clara cell secretory protein (CCSP)) (8–12). TTF-1 also plays a critical role in lung morphogenesis. Deletion of TTF-1 in transgenic mice causes severe thyroid and lung hypoplasia (13). Thus, TTF-1 regulates both lung morphogenesis and respiratory epithelial cell gene expression in the developing and postnatal lung.

TTF-1 is expressed in the progenitor cells of the lung buds early in rat, mouse, and human development. TTF-1 is encoded by a single gene in humans located on chromosome 14q13 (1). While the elements governing TTF-1 expression have not been defined with certainty, three distinct transcriptional start sites within the proximal promoter region and the expression of alternatively spliced TTF-1 cDNAs have been described in the rat and human TTF-1 genes (14–18). The 5′ regulatory regions of the human and rat TTF-1 genes have been studied in vitro (15, 19). TTF-1 transcription was enhanced by HNF-3β, which bound to two distinct sites located at bp –135 to –124 and –14 to –3 relative to the major start of transcription in the human gene, suggesting that TTF-1 regulation may be influenced by other transcription factors expressed in the foregut endoderm (19).

The GATA family of transcription factors includes a family of zinc finger domain-containing nuclear proteins that includes at least six distinct proteins that are expressed in a variety of tissues. GATA family members, particularly GATA-1, -2, and -3, were initially identified as important in the control of gene regulation during hematopoiesis (20). Gene targeting of GATA-1, -2, and -3 demonstrated their important roles in the development of the hematopoietic system, being critical for gene expression and development of erythroid cell lineages in the developing mouse embryo (21–24). More recently, several of the GATA family members were identified in nonhematopoietic sites including the heart, gastrointestinal, and genitourinary tracts. The finding that GATA-4, -5, and -6 are expressed in the foregut or cardiac regions of the developing embryo suggested their potential role in organogenesis and gene expression in the heart and lung (25–27). GATA-5 and -6 are expressed in developing mouse lung, GATA-6 being detected primarily in epithelial cell respiratory tubules of the fetal lung, in a pattern similar to that previously described for TTF-1. The finding that, in the heart, Nkx2.5 (structurally closely related to TTF-1) and GATA-4 interact in the regulation of cardiac specific gene expression, suggested that GATA family members and TTF-1 might interact in respiratory epithelial cells (28).

The present work was undertaken to determine whether GATA-5 or -6 was expressed in respiratory epithelial cells in vivo and in vitro and whether they might play a role in the regulation of TTF-1 gene expression, thereby influencing TTF-1 transcriptional targets. GATA-6, but not GATA-5, was expressed at high levels in the mouse pulmonary adenocarcinoma cells in vitro and in freshly isolated rat type II epithelial cells. GATA-6 activated a regulatory region of the mouse TTF-1 gene in HeLa cells and in human pulmonary adenocarcinoma cells (H441 cells).

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The abbreviations used are: TTF, thyroid transcription factor; nTTF, mouse TTF; EMSA, electrophoretic gel shift assay; SP-A, -B, and -C, surfactant protein A, B, and C, respectively; CCSP, Clara cell secretory protein; kb, kilobase pairs; bp, base pair(s); CMV, cytomegalovirus; HNF, hepatocyte nuclear factor.
RESULTS

GATA-6 Activates Transcription of TTF-1

Cell Transfection, Luciferase, and β-Galactosidase Assays—Cell transfection was performed by the calcium phosphate coprecipitation method except that glycerol shock was not used (31). Transfections were done in six-well plates with 0.5 pmol of reporter luciferase construct, 0.2 pmol of transactivator, and 200 μg/ml salmon sperm DNA at 42° C.

Preparation of Nuclear Extracts—Cell lines were maintained in standard tissue culture conditions prior to making the nuclear extracts. Cells were seeded to 4° C, washed in phosphate-buffered saline and scraped off of the plates. Cells were spun at 3000 rpm at 4° C for 5 min and then washed again twice with phosphate-buffered saline before lysing. Cells were then resuspended in three packed cell volumes of lysis buffer consisting of 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, and 0.2% Nonidet P-40, 100 μM dithiorethiol, and 50 μM phenylmethylsulfonyl fluoride. Cells were then vortexed at room temperature for 10 s and incubated on ice for 5 min. Nuclei were then pelleted at 3000 rpm and 4° C for 5 min. The pellet fraction contained the nuclei. One packed volume of extract buffer consisting of 20 mM HEPES, pH 7.9, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl2, 25% glycerol, 100 μM dithiorethiol, and 50 μM phenylmethylsulfonyl fluoride was added to the nuclei and vortexed at medium speed every minute for 10 min. The nuclear extracts were then centrifuged at 15,000 rpm for 5 min, aliquoted into chilled tubes, quick frozen in liquid nitrogen, and stored at –80° C. Protein concentration was determined by the method of Lowry (32).

Annealing of Synthetic Oligonucleotides—Oligonucleotides were annealed at a concentration of 250 μM in 250 μM Tris (pH 7.8) at 95° C for 5 min and then cooled slowly to room temperature overnight and stored at –20° C. The annealed oligonucleotides were diluted to 5 μM in the same buffer. Probes were labeled using [γ-32P]ATP and T4 polynucleotide kinase. End-labeled probes were purified from unincorporated [γ-32P]ATP by an Amersham Pharmacia Biotech Nick column eluted in a 400-μl volume. Unlabeled probe was placed on a Nick column at a 10-fold greater concentration and also eluted in 400 μl. Oligonucleotides used in shifts consisted of nucleotides –110 to –87 and their complement –87 to –110, and the GATA mutant consisting of the nucleotide –110 to –87 region (and complement containing the mutation TTATCT → TGAATT). Oligonucleotides were generated from the published sequence of the histidine decarboxylase promoter (33) consisting of the sequence TACGTGCTAGAGGAA and complement TTCTCTATCAAGCAATG. Oligonucleotides were synthesized using the Sequenase method except that glycerol shock was not used (31). Transfections were done in six-well plates with 0.5 pmol of reporter luciferase construct, 0.2 pmol of transactivator, and 200 μg/ml salmon sperm DNA at 42° C.

Cell Transfection, Luciferase, and β-Galactosidase Assays—Cell transfection was performed by the calcium phosphate coprecipitation method except that glycerol shock was not used (31). Transfections were done in six-well plates with 0.5 pmol of reporter luciferase construct, 0.2 pmol of transactivator (or empty vector), and 0.1 pmol of CMV-β-galactosidase to normalize for transfection efficiency. CMV-rtvf-1 was a gift of Dr. R. Di Lauro (Stazione Zoologica, A. Dohrn, Naples, Italy), a gift of Dr. R. Di Lauro (Stazione Zoologica, A. Dohrn, Naples, Italy), and 200 μg/ml salmon sperm DNA at 42° C.

Identification of Genomic Clones—A 129SvJ mouse liver genomic DNA library (Lambda DASH II, Stratagene, La Jolla, CA) was screened with Marica M. Shull, University of Cincinnati. Approximately 3.4 genome equivalents were plated and lifted onto Hybond N (Amersham Pharmacia Biotech). This was screened with a 1.3-kb rat TTF-1 cDNA provided by D. R. Di Lauro (Naples, Italy). The probe was labeled by random primer labeling and hybridized at a concentration of 106 dpm/ml in 6× SSC, 5× Denhardt’s, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA at 55° C. The final wash conditions were 0.5× SSC, 0.1% SDS at 55° C for 30 min. Four clones contained the TTF-1 gene, the largest clone containing 1.7 kb of 5′-flanking sequence. Sequence of the promoter region was determined by using the dideoxy sequencing reaction product (Sequenase, U.S.). Restriction fragments were used to compare the maps of the clone with mouse genomic DNA.

TTF-1 Luciferase Plasmid Constructions and Mutagenesis—Primers were used to amplify the desired region of the TTF-1 gene. The 3′-end of all constructs included all of the 5′-untranslated region up to, but not including, the ATG and had a HindIII site inserted in to aid in cloning. The DNA fragment was subcloned into pGL2-Basic (Promega, Inc., Madison, WI).

Preparation of Nuclear Extracts—Cell lines were main-
The potential GATA site was also located close to a potential region of the TTF-1 gene contains 142 bp 5’ ligated to the luciferase genes to produce mTTF-1–142. This transcription start site, as well as 5’ and rat type II cells. Northern analysis was performed with 15 μg of total RNA from mouse cell lines MLE-15 (lane 1), MLE-F6 (lane 2), mFLM91 (lane 3), 3T3 (lane 4), human cell lines 9/HTEO (lane 5), H441 (lane 6), H446 (lane 7), Beas2B (lane 8), adult mouse lung (lane 9), and freshly isolated adult rat type II cells (lane 10). The probe used to detect GATA-5 was a 2.1-kb EcoRI-XhoI fragment of mouse GATA-5 cDNA, which contained the entire coding sequence. The probe used to detect GATA-6 was a 2.4-kb XbaI–SalI fragment of mouse GATA-6 cDNA, which contained the entire coding region. The probe used for β-actin was a 400-bp EcoRI fragment of human β-actin cDNA.

Fig. 1. Northern blot analysis of human and mouse cell lines and rat type II cells. Northern analysis was performed with 15 μg of total RNA from mouse cell lines MLE-15 (lane 1), MLE-F6 (lane 2), mFLM91 (lane 3), 3T3 (lane 4), human cell lines 9/HTEO (lane 5), H441 (lane 6), H446 (lane 7), Beas2B (lane 8), adult mouse lung (lane 9), and freshly isolated adult rat type II cells (lane 10). The probe used to detect GATA-5 was a 2.1-kb EcoRI–XhoI fragment of mouse GATA-5 cDNA, which contained the entire coding sequence. The probe used to detect GATA-6 was a 2.4-kb XbaI–SalI fragment of mouse GATA-6 cDNA, which contained the entire coding region. The probe used for β-actin was a 400-bp EcoRI fragment of human β-actin cDNA.

Northern blot analysis. GATA-6 mRNA was detected in 3T3 cells and in SV40 large T antigen immortalized fetal mouse lung mesenchymal cells (mFLM cells). Neither GATA-6 nor TTF-1 mRNA was detected in HeLa cells (data not shown). GATA-5 mRNA was not detected in H441, MLE pulmonary adenocarcinoma cells or in type II cells. Thus, GATA-6 and TTF-1 were co-expressed in various differentiated pulmonary adenocarcinoma cells and in freshly isolated rat type II cells, consistent with the finding that both mRNAs were detected in subsets of respiratory epithelial cells in the fetal mouse lung (26, 27). In contrast, GATA-5 mRNA was not readily identified in the immortalized respiratory epithelial cells or in type II cells, consistent with its localization in nonepithelial cells in the developing mouse lung as assessed by in situ hybridization (26, 27). The co-localization of GATA-6 and TTF-1 with TTF-1 targets including SP-A, SP-B, and SP-C in various respiratory epithelial cells is consistent with the possibility that GATA-6 may influence transcription of TTF-1 or its target genes.

5’ Regulatory Region of the Mouse TTF-1 Gene Contains a GATA Regulatory Element—Inspection of the 5’ region of the mouse TTF-1 gene revealed the nucleotide sequence TTATCT, located at −101 to −96 relative to the major transcription start site, consistent with its role as a potential GATA cis-acting element. Previous work from this laboratory identified a large DNase protected site by in situ footprint analysis with nuclear extracts from MLE-15 cells in this region (14). The nucleotide sequence of this region of the TTF-1 gene is highly conserved in human rat and mouse TTF-1 genes. The potential GATA site was located near the element TGTCTT, previously demonstrated to bind HNF-3β, activating TTF-1 gene promoter activity (19). The potential GATA site was also located close to a potential TTF-1 site (CAAG), located 5 bp from the GATA site. To test whether GATA family members might regulate TTF-1 promoter activity, the 5’ region of the mouse TTF-1 gene was ligated to the luciferase genes to produce mTTF-1–142. This region of the TTF-1 gene contains 142 bp 5’ of the primary transcription start site, as well as 5’-untranslated sequence derived from the major TTF-1 transcript (Fig. 2). This 5’-untranslated region encodes two alternative transcripts and includes a small potential intron (15).

Transient co-transfection experiments were performed in which mTTF-1–142 was co-transfected with expression vectors encoding mouse GATA-5 (CMV-GATA-5) and GATA-6 (CMV-GATA-6) into HeLa cells. mTTF-1–142 was markedly activated by co-transfection with GATA-6 but not GATA-5 (Fig. 3). Likewise, cotransfection of GATA-6 activated the mTTF-1–142 luciferase construct after transfection into H441 adenocarcinoma cells from 17 ± 2.3 to 28 ± 3.6 luciferase units (mean ± S.E., p < 0.01). Deletion mutations were made producing vectors at −110, −95, and −87 bp relative to the start of transcription of the TTF-1 gene. In HeLa cells, significant stimulation of TTF-1 promoter activity by GATA-6 was observed with 5’ sequences from −110 bp; however, maximal basal and stimulated activities were observed when a larger region (−142 bp) was included. The nucleotide sequences between −110 and −95 contained a region that encodes a consensus GATA binding site TTATCT (Fig. 4). Mutation of the GATA site in mTTF-1–142 (TTATCT to TGAAT) completely inhibited transactivation by GATA-6 after co-transfection in HeLa cells (Fig. 4). Thus, a GATA-6 response element was located at −101 to −96 in the murine TTF-1 promoter. In contrast to the findings in HeLa cells, transfection of MLE-15 cells with CMV-GATA-6 did not further transactivate the wild type TTF-1–142 construct, perhaps related to the high levels of exogenous GATA-6 expression in this cell line. Luciferase activity of the mTTF-1–142 (TGAAT mutant) construct was similar to the wild type construct in MLE-15 cells (data not shown).

GATA-6 Binds to the mTTF-1 Gene—Electromobility shift assays were performed using nuclear extracts from MLE-15, MLE-F6, 3T3, and H441 and HeLa cells (Fig. 5). A gel shift was readily detected with an oligonucleotide −110 to −87 containing the GATA-6 site with nuclear extracts from MLE-15, MLE-F6, and 3T3 cells consistent with the detection of GATA-6 mRNA as determined by Northern blot analysis. This DNA complex was lacking in HeLa and H441 cells, also consistent with the lack of GATA-6 mRNA in these cells. The protein-DNA complex was inhibited by puavity or absence of the unlabeled self-oligonucleotide. The same oligonucleotide containing the mutated GATA site (−110 to −87) (TTATCT → TGAAT) did not compete for binding to MLE-15 cell nuclear extracts (Fig. 5, lane 3). An oligonucleotide containing the GATA-6 site identified in the histidine decarboxylase gene (33) bound to MLE-15 nuclear extracts. Binding of the histidine carboxylase GATA-6 site was competed by self oligonucleotide and by the oligonucleotide containing the GATA-6 site from the TTF-1 gene. Formation of the complex with the histidine decarboxylase GATA-6 site was not inhibited by the oligonucleotide containing the mutant GATA site (Fig. 6), further demonstrating the specificity of the interaction.

Supershift Analysis Identifies GATA-6 Binding—Antiserum to human GATA-6 caused a supershift when co-incubated with MLE-15 nuclear extract and the TTF-1 oligonucleotide −110 to −87 (Fig. 5B). Binding of GATA-6 and the supershift were markedly decreased when the mutant GATA oligonucleotide was utilized in the EMSA (Fig. 5B, lanes 4–6).

GATA-6 Enhances TTF-1 Transcription Independently of TTF-1 or HNF-3β—A canonical TTF-1 binding site, CAAG, is located in close apposition to the GATA-6 binding site in the murine TTF-1 gene. Since GATA-4 and Nkx2.5 are known to interact in a synergistic manner to activate myosin heavy chain gene expression in cardiac cells (28), we tested whether GATA-6 or TTF-1 might interact synergistically on the TTF-1 promoter. Expression vectors encoding rat TTF-1 (CMV-TTF-1)
and GATA-6 (CMV-GATA-6) were co-transfected with the parental TTF-1-luciferase reporter construct (TTF-1–142) into HeLa cells. TTF-1 did not enhance, but slightly inhibited, GATA-6-dependent activity (Fig. 7). Thus, GATA-6 stimulation of TTF-1 transcription neither requires nor is synergized by TTF-1. Consistent with this observation, recombinant TTF-1 homeodomain did not bind to the CAAG element in the GATA site-containing oligonucleotide from the TTF-1 gene. Likewise, MLE-15 nuclear extracts did not bind to the potential TTF-1 site as assessed by supershift analysis (data not shown). While HNF-3\(^b\) enhanced activity of the TTF-1–142 construct and HeLa cells, co-transfection of CMV-GATA-6 with an expression vector expressing rat HNF-3\(^b\) (CMV-HNF-3\(^b\)) did not further influence the effects of GATA-6 on TTF-1 promoter activity.

**DISCUSSION**

TTF-1 and GATA-6 mRNAs were co-expressed in freshly isolated type II epithelial cells and immortalized respiratory epithelial adenocarcinoma cells in vitro. GATA-6, but not GATA-5, activated transcriptional activity of the murine TTF-1 promoter construct, GATA-6 binding to a cis-active element identified at \(-142\) to \(-95\) in the mTTF-1 promoter; however, maximal GATA-6-dependent activity was observed with the larger construct \(-142\). A consensus site (TTACTC) for GATA binding was located at \(-96\) to \(-101\) in this region. This site was mutated to TGAATT and tested for GATA-6 transactivation in HeLa cells. Relative luciferase values were determined as described under "Experimental Procedures." The deletion and mutant constructs are further described under "Experimental Procedures" and in Fig. 1. Values represent mean ± S.E. of three separate experiments. * and **, \(p < 0.001\) and \(p < 0.005\), respectively, versus pCDNA1.1 control.

**Fig. 2.** Nucleotide sequence of the 5' region of the mouse gene. Nucleotide sequence was determined as described under "Experimental Procedures." The downward arrows represent the 5'-end of the mTTF-1 luciferase constructs. The upward arrow represents the 3'-end, which stops just short of the ATG codon. The 5'-end of the fragments are located at \(-142\), \(-110\), \(-95\), and \(-87\) bp upstream of the major start site. The boldface asterisk denotes the major transcriptional start site, and the other two asterisks denote the other minor start sites as previously published (14, 15). The previously identified HNF-3\(^b\) and GATA-6 binding sites and the TTF-1 consensus site are illustrated. The alternatively spliced intron in the 5'-untranslated region is underlined. The mutant base pair changes for transfections and EMSA are shown above the GATA binding site.

**Fig. 3.** GATA-6, but not GATA-5, transactivates the TTF-1 promoter. Luciferase assays were performed in HeLa cells after transient transfection of the mTTF-1–142 luciferase construct with pCDNA1.1 (vector without transactivator), CMV-GATA-5, and CMV-GATA-6. Transfections were normalized to \(\beta\)-galactosidase activity. Luciferase activity of the TTF-1 promoter construct was divided by the luciferase activity from the promoterless luciferase vector (pGL2-Basic) transfected with the appropriate transactivator. Values are mean ± S.E. \(n = 3\), \(p < 0.0005\) as assessed by unpaired \(t\) test versus pCDNA1.1.

**Fig. 4.** A site of GATA-6 activation is located between \(-142\) and \(-95\). Deletion analysis was used to identify the site of GATA-6 activation on the mTTF-1 promoter. GATA-6-dependent activity was lost in deletions between \(-110\) and \(-95\) in the mTTF-1 promoter; however, maximal GATA-6-dependent activity was observed with the larger construct \(-142\). A consensus site (TTACTC) for GATA binding was located at \(-96\) to \(-101\) in this region. This site was mutated to TGAATT and tested for GATA-6 transactivation in HeLa cells. Relative luciferase values were determined as described under "Experimental Procedures." The deletion and mutant constructs are further described under "Experimental Procedures" and in Fig. 1. Values represent mean ± S.E. of three separate experiments. * and **, \(p < 0.001\) and \(p < 0.005\), respectively, versus pCDNA1.1 control.

**GATA-6 Activates Transcription of TTF-1**
role in pulmonary morphogenesis and in the regulation of a number of gene products involved in pulmonary function and host defense. TTF-1 is expressed in the developing lung buds of the rat, mouse, human, and chick, the level of expression decreasing in the perinatal and postnatal period of lung development (2, 4, 5, 35). TTF-1 is increasingly restricted in the postnatal lung, being detected primarily in type II epithelial cells of the fetal lung overlaps with that of the SP-A, SP-B, and SP-C genes. Recent findings from this laboratory demonstrated that GATA-6 activated transcriptional activity of SP-A and SP-C genes by binding to cis-active elements located in the 5’ regulatory regions of each of the genes.2 The present findings that GATA-6 activates TTF-1, taken together with observations that GATA-6 directly influences expression of the surfactant protein genes by binding to their promoters support a potential regulatory role for GATA-6 in surfactant protein expression.

The finding that GATA-6, but not GATA-5, activated TTF-1 gene transcription is consistent with the similar distribution of GATA-6 and TTF-1 in fetal lung in vivo and in MLE-15 cells in vitro. Expression of TTF-1, HNF-3β, and GATA-6 are expressed in a similar distribution in respiratory epithelial cells in the fetal lung in vivo and in MLE-15 cells in vitro. Of interest, the selectivity observed for GATA-6 in mediating pharyngeal and gut development in the worm C. elegans. Forkhead homologue pha-4, GATA homologue elt-2, and Nkx homologue ceh-22 interact in a transcriptional pathway mediating pharyngeal and gut development in the worm (39). In the present study, GATA-6 activation of the TTF-1 gene transcription did not require expression of TTF-1 or HNF-3β, neither being expressed in HeLa cells (14).2 TTF-1, HNF-3β, and GATA-6 are activated in a similar distribution in respiratory epithelial cells in the fetal lung in vivo and in MLE-15 cells in vitro. Expression of TTF-1, HNF-3β, and GATA-6 in fetal lung overlaps with that of the SP-A, SP-B, and SP-C genes. Recent findings from this laboratory demonstrated that GATA-6 activated transcriptional activity of SP-A and SP-C genes by binding to cis-active elements located in the 5’ regulatory regions of each of the genes.2 The present findings that GATA-6 activates TTF-1, taken together with observations that GATA-6 directly influences expression of the surfactant protein genes by binding to their promoters support a potential regulatory role for GATA-6 in surfactant protein expression.

The finding that GATA-6, but not GATA-5, activated TTF-1 gene transcription is consistent with the similar distribution of GATA-6 and TTF-1 in subsets of developing respiratory epithelial cells in the mouse lung in vivo (5, 26, 27). GATA-5 mRNA was detected in mesenchymal but not epithelial cells of the developing lung. The finding that GATA-6, but not GATA-5, activated TTF-1 gene expression was somewhat surprising, given that both proteins bind to similar consensus elements in target genes. Of interest, the selectivity observed for GATA-6 in activation of the TTF-1 gene was not observed in the studies presented herein.

2 J. R. Shaw-White, M. D. Bruno, and J. A. Whitsett, unpublished observations.
with the SP-C promoter, wherein both GATA-5 and GATA-6 were active in enhancing SP-C gene transcription. Thus, the effects of GATA-6 on TTF-1 gene transcription are probably determined by mechanisms distinct from that involved in activation of surfactant protein C gene transcription by GATA-6.

GATA-6 bound to a site in a consensus element located near the start of transcription of the TTF-1 gene. The cis-active element and flanking regions are highly conserved in the TTF-1 gene sequences available for comparison (human, mouse, and rat), supporting the potential importance of this region in gene regulation (14, 15, 18). The DNA binding site identified in the murine TTF-1 gene is consistent with binding sites for many other GATA family members. Therefore, the selectivity of the response to GATA-6 is likely to be dependent upon interactions with this region of the TTF-1 gene and TTF-1 itself. This finding is distinct from recent studies in which Nkx2.5 (a TTF-1 family member) and GATA-4 enhanced myosin heavy chain gene expression in the heart (28). Co-transfection with TTF-1 modestly inhibited transcriptional activity of the mTTF-1 construct in the presence of GATA-6 in HeLa cells; however, the significance of this observation is unclear. Activation of the TTF-1 promoter by GATA-6 was not influenced by co-transfection with HNF-3β, which is known to bind to two sites in the region −135 to −3 in the human TTF-1 gene. Thus, GATA-6 binds and activates the cis-acting element in the TTF-1 gene independently of either TTF-1 or HNF-3β.

The finding that GATA-6 activates the TTF-1 promoter adds to our understanding of the potential interactions among various families of transcription factors in the developing respiratory epithelium. Present data support a model in which the transcription factor(s) bind to and activate target genes, including the surfactant proteins and CCSP, but may also interact at the level of regulation of the transcription factors themselves, HNF-3β and GATA-6 modulating TTF-1 gene expression. The co-expression of HNF-3β, GATA-6, and TTF-1 in the developing lung is consistent with the concept that respiratory epithelial differentiation and gene expression may be modulated by interactions among these distinct families of transcription factors (9).

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FIG. 6. The GATA-6 binding site in the mouse TTF-1 gene competes with a GATA-6 binding site from the histidine decarboxylase gene. EMSA analysis was performed using a labeled histidine decarboxylase (HDC) oligonucleotide (33) with nuclear extract from MLE-15 cells (lane 1). The complex was inhibited by 100× self-competitor and labeled self (lane 2). The addition of an unlabeled oligonucleotide consisting of −110 to −87 in the mTTF-1 gene resulted in a loss of this complex (lane 3, TTF-1). Oligonucleotides with mutations in the GATA-6 site of TTF-1 did not compete with the histidine decarboxylase probe, labeled MUT (lane 4).

FIG. 7. GATA-6 activation of the TTF-1 promoter does not require TTF-1. To test whether TTF-1 and GATA-6 cooperatively enhanced TTF-1 gene transcription, CMV-TTF-1 (marked TTF-1) and CMV-GATA-6 (GATA-6) were co-transfected with mTTF-1–142 luciferase in HeLa cells and compared with the activity observed with the control vector pCDNA1.1. GATA-6-dependent activity was not increased by the addition of CMV-TTF-1. Relative luciferase activity (mean ± S.E., n = 3) was determined as described under “Experimental Procedures.” * and **, p < 0.0009 and p < 0.0008, respectively, versus cDNA1.1 control as assessed by unpaired t test. S.E. for TTF-1-transfected cells was ±2.7.
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