Transcriptional Regulation of a Mouse Clara Cell-Specific Protein (mCC10) Gene by the NKx Transcription Factor Family Members Thyroid Transcription Factor 1 and Cardiac Muscle-Specific Homeobox Protein (CSX)

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This report defines the elements between bp –800 and –166 that regulate the quantitative level of mouse CC10 (mCC10) transcription in the lungs. The elements in this promoter domain are the response elements for the NKx 2.1 homeobox protein, thyroid transcription factor 1 (TTF1). DNase I footprint analysis identified five binding sites for TTF1 between bp –800 and –166. These sites are located at bp –344 to –335, –282 to –273, –268 to –263, –258 to –249, and –199 to –190. In addition to these enhancer elements, two TTF1 binding sites were identified in the proximal promoter region (bp –166 to +1), at bp –74 to –69 and –49 to –39. An identical footprint of the mCC10 promoter region was also observed with another member of the NKx family, NKx 2.5, the cardiac muscle-specific homeobox protein (CSX). Deletion and linker-scanner mutational analyses of the TTF1 binding sites in the mCC10 distal promoter region with transient cotransfection into CV1 cells with either TTF1 or CSX identified the site located between bp –282 and –273 as the major regulator of CC10 expression, with minor regulation by sites at bp –344 to –335 and –258 to –249. The importance of the NKx binding site at bp –282 to –273 was verified in vivo. Transgenic mice generated with the human growth hormone gene fused to 800 bp of the mCC10 promoter containing a mutation in the TTF1 binding site at bp –282 to –273 showed a reduction in transgene expression equal to that of the mice generated with only 166 bp of 5'-flanking DNA. This report emphasizes the importance of TTF1 or related factors as major regulators of pulmonary gene expression and demonstrates the potential of NKx proteins to bind and activate heterologous target genes.

The primary function of the pulmonary epithelium is to allow the exchange of gases between the tissues of the body and the external environment (20). In order to accomplish this function, the pulmonary epithelium is composed of a heterogeneous population of cells. This cellular heterogeneity makes the pulmonary epithelium an intriguing model to study the regulation of organ and cellular differentiation. One specific cell type located in the proximal airway epithelia is the Clara cell. The Clara cells are nonciliated secretory cells lining the bronchioles of the lung (43). These cells serve as the progenitor cells of the proximal airways during epithelial repair in response to lung injury (21, 39, 49). The major secretory product of the Clara cell is a Clara cell-specific 10-kDa protein (CC10), which serves as a marker for this cell type (51, 59). The CC10 protein is known to bind polychlorinated biphenyl compounds and inhibits phospholipase A2 (3, 36). However, the physiological function of this protein has not yet been defined.

Although the physiological function of CC10 is unknown, the abundance of this protein and the cellular specificity of its expression makes it an ideal model to investigate the regulation of pulmonary epithelial cell-specific gene expression (10, 24, 25, 36, 42, 52, 54). Transient transfection as well as transgenic analysis has been conducted on the rabbit (15), rat (61), and mouse (53) genes to identify the elements necessary for tissue- and cell-specific expression of this gene. Transient transfection into H441 cells, a human lung adenocarcinoma cell line that displays a Clara cell phenotype, by using deletion

constructs of the rabbit, rat, and mouse promoter sequences fused to the chloramphenicol acetyltransferase (CAT) reporter gene has identified promoter sequences proximal to the transcription start site required for maintaining the tissue specificity (15, 61). The analysis of the rat CC10 promoter has shown that 165 bp of the rat CC10 promoter sequence directs the expression of the reporter gene. DNase I footprint analysis with an H441 nuclear extract demonstrated a protected region between 79 and 128 bp upstream of the transcription start site (61). Analysis of the DNase I footprint sequence and an electrophoretic mobility shift assay with an H441 nuclear extract have identified members of the AP1, hepatocyte nuclear factor 3 (HNF3), and octamer-binding protein transcription factor families as playing the major role in regulation of CC10 gene expression in the lung (5, 56). The sequence of the mouse CC10 (mCC10) promoter is highly homologous to that of the rat CC10 promoter (53). Analysis of the mCC10 promoter demonstrated that the first 166 bp of DNA flanking the mCC10 gene was able to confer maximal expression of a reporter gene in H441 cells. In vivo analyses, using transgenic mice to express various reporter genes, 3.3 kb of the promoter sequence of the rabbit gene, 2.4 kb of the rat promoter, and 166 bp of the mouse promoter have been shown to be sufficient to confer tissue-specific expression of this gene in transgenic mice (15, 53, 61). Deletion analysis of the mCC10 promoter in transgenic mice has shown that although as little as 166 bp of the promoter sequence confers the tissue specificity of this gene, achievement of the full level of CC10 expression requires an additional 634 bp of upstream sequences. Thus, elements outside the proximal promoter elements are required for the full pulmonary expression of this gene in vivo. A similar result

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was found for the rabbit gene when deletion analysis was conducted. In the case of the rabbit, 2.7 kb of 5'-flanking DNA conferred full expression in the lungs, while 600 bp of 5'-flanking DNA conferred tissue-specific but reduced expression in transgenic mice (15). In this paper, we report that the elements required for the full expression of the mCC10 promoter are response elements for the NKx homeobox proteins.

A family of mammalian homeobox proteins with homology to the *Drosophila* NK2 homeobox protein family has recently been identified (7, 31). Currently, six family members, NKx 2.1 through 2.6, have been identified (6, 57). NKx 2.1 was the first mammalian gene identified, when the cDNA for thyroid transcription factor 1 (TTF1) was cloned (22, 46). NKx 2.1 is expressed in the brain, thyroid, and lung (33). The members of the NKx transcription factor family were identified by low-stringency hybridization screening, and the expression patterns of only NKx 2.2 and NKx 2.5 have been described. The expression pattern of NKx 2.2 is in the forebrain (50). NKx 2.5 is expressed in the heart and spleen and has been termed the cardiac muscle-specific homeobox protein (CSX) (31). NKx 2.5 has been identified as the mammalian homolog of the *Drosophila tinman* gene (34, 44). Recently, gene ablation of NKx 2.5 has shown that this protein regulates heart development (38). Identification of the potential DNA target sequences for CSX regulation has demonstrated that the CSX protein can bind the target sequences for the TTF1 protein. Thus, the members of the NKx family of genes have the potential to regulate common target genes depending upon the particular chromatin environment. Since these genes play an important role in mammalian organogenesis, understanding the genes which these proteins regulate and the specificity of their interaction with the *cis*-acting elements in the target genes will allow the identification of the mechanism of organ development.

Recently, it has been shown that NKx 2.1 (TTF1) regulates the transcription of the surfactant protein B (SP-B) and SP-A genes (8, 9). TTF1 has also been shown to activate the transcription of other lung-specific genes, including SP-C and CC10, in nonpulmonary cells. In this study, we have identified seven TTF1 binding sites by DNase I footprint analysis in the 5'-flanking region of the mCC10 gene. Analysis of these sites *in vitro* by transient transfection and *in vivo* by transgenic-mouse analysis has identified the TTF1 site between bp -282 and -273 as the primary regulator of mCC10 expression. This study demonstrates that TTF1 is responsible for 90% of the level of expression of mCC10 in Clara cells, confirming this transcription factor or related factors as a major regulator of pulmonary gene expression. This study also demonstrates that the cardiac member of the NKx family, CSX, can not only bind the regulatory elements for TTF1 but also transactivate the mCC10 promoter in a manner identical to that for the TTF1 gene.

MATERIALS AND METHODS

mCC10 deletion and linker-scanner constructions. The initial 5'-flanking DNA constructions containing 5.1, 2.1, 0.8, 0.166, 0.123, 0.087, and 0.023 kb of the mCC10 promoter were generated by using restriction endonucleases or PCR as previously described (53). By the same procedure, constructions with 0.582 or 0.267 kb of the 5'-flanking DNA were generated by PCR amplification of pKS-mCC10 (2.1), using oligonucleotides corresponding to the appropriate junctions. The 5' upstream oligonucleotide primer sequences for the 0.582- and 0.267-kb fragments were 5'-AGTGCTTGACTTCCAAGAG-3' and 5'-TGCTCTGCGTAGGAACAGC-3', respectively. The reverse primer, 5'-CACTATAGGGCGAATTGG-3', was selected from the Bluescript multiple cloning cassette which covers the *Bam*HI restriction site during amplification. The PCR-amplified fragments were digested with *Xba*I and *Bam*HI and subcloned into pBLCAT3 (35). All clones generated from PCR amplification were sequenced to ensure faithful amplification of the mCC10 flanking DNA.

Linker-scanner mutations of the putative TTF1 regulatory sequences were

introduced into the mCC10 promoter sequence by the procedure described by Kunkel et al. (32). Mutagenic oligonucleotides were generated to replace the putative NKx binding sites with the sequence corresponding to a *Bgl*II-*Bam*HI ligation (replaced sequence, 5'-CGGGATCTTC-3'). The oligonucleotides were 30 bp in length and contained 20 bp of mCC10 sequence corresponding to the sequence flanking the NKx site. The remaining 10 bp of these oligonucleotides encodes either a *Bgl*II restriction sequence (forward primer) or a *Bam*HI restriction sequence (reverse primer). Each point mutation was introduced in two steps. The distal portion of the 800-bp mCC10 promoter (with respect to the transcription start site) was amplified by using a forward primer having a *Hind*III restriction sequence attached to it and a reverse primer having a *Bam*HI restriction sequence. In each case the restriction sequence was followed by 20 bases of the unaltered mCC10 promoter sequence. In a similar way, the proximal portion of the 800-bp promoter sequence was amplified by using a forward primer attached to a *Bgl*II restriction sequence followed by 20 unaltered bases of the mCC10 promoter. The reverse primer was chosen from multiple cloning sites of the Bluescript (pKS⁺) cassette as mentioned previously. PCR was performed by a procedure described previously (53), and the PCR-amplified products were ligated into the TA cloning vector (PCRII; Invitrogen, Inc., San Diego, Calif.). The positive clones were identified by blue-white selection by the procedure supplied by the manufacturer. The inserts were isolated by digesting the TA vector with *Hind*III-*Bam*HI for the distal fragment and with *Bgl*II-*Bam*HI for the proximal fragment of the 800-bp mCC10 promoter. pBLCAT3 was digested with *Hind*III-*Bam*HI and ligated with the distal fragment digested with the same enzymes. The pBLCAT3-mCC10 plasmid was digested with *Bam*HI and treated with alkaline phosphatase (Boehringer Mannheim Corporation, Indianapolis, Ind.) according to the protocol supplied by the manufacturer. The proximal *Bam*HI-*Bgl*II fragment was ligated, and positive clones with the correct orientation were identified by *Hind*III-*Bam*HI digestion. The double mutant 800ΔVI,VI was generated by using the 800ΔVI mutant as the template for 800ΔIV PCR amplification. The sequences of mutagenic oligonucleotides used for PCR amplification, with the forward primer (for the amplification of the proximal sequence) given first and the reverse primer (for the amplification of the distal fragment) given second, were as follows: 800ΔVIII, 5'-GAAGATCTTCGCTCTGCGTAGGAACAGC-3' and 5'-CGGGATCCCCGTAACCTGCCAATTGAAGCA-3'; 800ΔVII, 5'-GAAGATCTTCCTCCGGCCTCTGGTCTTCCA-3' and 5'-CGGGATCCCCGAATCACAGGGAAGGACAAA-3'; 800ΔVI, 5'-GAAGATCTTCCTCCGCTTGACTTCCAAG-3' and 5'-CGGGATCCCCGTTCCAGGAACGAACCTGT-3'; 800ΔV, 5'-GAAGATCTTCTCCAAGAGAGGACACAGTTG-3' and 5'-CGGGATCCCCGTGAGCACTCCAGGTTCAGG-3'; 800ΔIV, 5'-GAAGATCTTACACAGTTGTCTTCTACAGT-3' and 5'-CGGATCCCCGAAGCACTGAGCACTC-3'; 800ΔIII, 5'-GAAGATCTTCCACCAAGTGGCTATTGTGTG-3' and 5'-CGGGATCCCCGAGTGGAGGACCAAGTCAG-3'; and 800ΔVI,IV, 5'-GAAGATCTTACACAGTTGTCTTCTACAGT-3' and 5'-CGGGATCCCCGAAGCACTGAGCACTGAGTGAAGATC-3'.

Cell cultures, transient transfection, and assay of CAT expression. CV1 cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum. All media contained 100 U of penicillin and 0.1 mg of streptomycin per ml. The transient-transfection analysis was conducted with 0.4 pmol of the above-described mCC10-CAT constructs (deletion as well as mutant constructs) or pBLCAT3 alone, along with 0.2 pmol of pCMV-βGAL and 0.4 pmol of either pCGN (expression vector), TTF1-pCGN, or CSX-pCGN. The plasmids were mixed with 25 μl of Lipofectamine (GIBCO-BRL, Gaithersburg, Md.) in 1 ml of DMEM (without serum). Cells growing in the logarithmic phase (50 to 70% confluent) on 100-mm-diameter dishes were washed twice with phosphate-buffered saline (PBS), and 5 ml of DMEM (without serum) was added. After the washing, 1 ml of plasmid-Lipofectamine mixture was added to the cells. The cells were then incubated for 24 h at 37°C and washed with PBS. DMEM plus 10% fetal calf serum was added, and the cells were incubated at 37°C for 36 h.

The cells were harvested and lysed in 0.25 M Tris-HCl (pH 7.5) by three cycles of freezing and thawing in liquid N₂. The protein concentration in the cell lysate was measured with the Bradford assay (Bio-Rad, Richmond, Calif.). The CAT activity of the lysate was assayed as described by Seed and Sheen (58). The CAT assay was conducted with 50 μg of protein for each construct. The reaction mixture was incubated at 37°C for 5 h, and the reaction was stopped by extraction with 200 μl of 2,6,10,14-tetramethylpentadecane (TMPD)-xylene (2:1). The CAT activity was measured by counting the radioactivity of ¹⁴C-acetylated derivatives with a Beckman LS-8000 liquid scintillation counter. The assay for β-galactosidase was done according to the protocol described by Sambrook et al. (55). Variability in the transfection efficiency was corrected by normalizing CAT activity to β-galactosidase activity. The relative strengths of the promoters are expressed as CAT activity (in counts per minute)/β-galactosidase activity (in units).

Expression and purification of TTF1. The plasmid for the rat TTF1 cDNA sequence was a generous gift from Roberto Di Lauro, Stazione Zoologica "Anton Dohrn," Naples, Italy, and was placed in frame in the pMAL-C2 expression vector (New England Biolabs, Beverly, Mass.). The product of this expression vector is a fusion protein of TTF1 with maltose-binding protein. Purification of the protein was done essentially according to the procedure supplied by the manufacturer (40). Briefly, the pMAL-C2-TTF1 plasmid was used to transform competent *Escherichia coli* DH5α cells (GIBCO-BRL). The transformants were grown in rich medium (10 g of tryptone per liter, 5 g of yeast extract per liter, 5

g of NaCl per liter, 2 g of glucose per liter, and sterile ampicillin at a final concentration of 100 µg/ml) until the optical density at 600 nm reached approximately 0.5. At that point, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.3 mM, and the cells were allowed to grow for another 1.5 h. The cells were harvested, centrifuged, suspended in column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA) and left at -20°C overnight. The cells were lysed by short-pulse sonication and centrifuged. The crude extract was passed through an amylose resin affinity column. After the column was washed several times with column buffer, the fusion protein was eluted with column buffer plus 10 mM maltose. Recombinant TTF1 protein was freed from the maltose-binding protein by enzymatic digestion. This was accomplished by digesting 50 µg of fusion protein in column buffer containing 10 mM maltose in the presence of a final concentration of 200 ng of Xa factor (New England Biolabs) per ml. The recombinant proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to verify the molecular weight. Finally, the recombinant protein was verified as TTF1 by Western blot (immunoblot) analysis with the antibody kindly provided by Roberto Di Lauro (33) (data not shown).

DNase I footprint analysis. The mCC10 promoter sequence, spanning bp -417 to +7, was PCR amplified by using a forward primer containing a *Bgl*II restriction site attached to it (5'-GAAGATCTTCCTTGTGGCTTGAAGTCTA-3'). The reverse primer was chosen from the polylinker sequence of Bluescript (pKS⁺) mentioned above. The PCR-amplified product was ligated into the TA cloning vector (PCRII; Invitrogen), and the insert was liberated from the vector by digestion with *Bgl*II-*Sma*I. The 5' end of the isolated fragment was labeled with [α -³²P]dGTP by using reverse transcriptase (Superscript III; GIBCO-BRL) according to the procedure supplied by the manufacturer. The unincorporated nucleotides were removed by passing the reaction mixture through a Sephadex G-50 quick-spin column (Boehringer Mannheim Corporation). In order to identify the TTF1 binding sites within the first 166 bp of the flanking sequence of the mCC10 gene, this fragment of mCC10 promoter was liberated from pKS-mCC10 (2.1) by digestion with *Sac*I-*Bam*HI. The 3' end of the isolated fragment was labeled by using [α -³²P]dGTP and reverse transcriptase (Superscript III; GIBCO-BRL). The DNase I reaction was performed by the procedure described previously by Hennighausen and Lubon (26). Briefly, either purified recombinant TTF1 or CSX was incubated in 50 µl of binding buffer (50 mM NaCl, 0.1 mM EDTA, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.5], 0.5 mM dithiothreitol [DTT], 10% glycerol) with 1 µg of poly(dI-dC) for 15 min at room temperature. The end-labeled probe (40,000 cpm; specific activity, approximately 10⁸ cpm/µg) was added to the protein and the mixture was incubated at room temperature for 10 min. MgCl₂ and CaCl₂ were added to final concentrations of 5 and 1 mM, respectively. Pancreatic DNase I at a concentration of 4 µg/µl was diluted with 25 mM NaCl-10 mM HEPES-KOH (pH 7.5)-0.5 mM DTT. The reaction mixture without protein contained 2 or 4 ng of DNase I, and the probes incubated with protein contained 240 ng of DNase I. After 30 s of digestion with DNase I, the reaction was stopped by adding 100 µl of stop buffer (0.375% SDS, 15 mM EDTA, 100 mM NaCl, 100 mM Tris-HCl [pH 7.6], 50 µg of sonicated salmon sperm DNA per ml, and 100 µg of pronase per ml). The samples were incubated at 37°C for 15 min and at 90°C for 2 min and then subjected to phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. The reaction products were analyzed on a polyacrylamide-8 M urea sequencing gel. A nucleotide-sequencing reaction (44) for the region being footprinted was run in parallel in the same gel for accurate identification of the sizes and nucleotide sequences of the footprinted regions. After electrophoresis, the gels were fixed, dried, and exposed to X-ray film at -80°C.

Preparation of nuclear extracts. H441 nuclear extracts were prepared by the procedure described by Attardi and Tjian (2) with the following modifications. Cells growing logarithmically (70 to 90% confluent) on 100-mm-diameter dishes were harvested. The cells were washed with PBS, resuspended, and incubated for 5 min on ice in 0.25 ml of buffer A (10 mM HEPES-KOH [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1 mM EGTA [ethylene glycol-bis(β-aminocaprylate)-N,N,N',N'-tetraacetic acid], 0.5 mM phenylmethylsulfonyl fluoride, and 2 µg each of the protease inhibitors antipain, pepstatin A, and aprotinin per ml). Then, 1.25 µl of 10% Nonidet P-40 was added, and the cells were incubated for another 2 min on ice. The cells were centrifuged at low speed (1,700 rpm), and the supernatant was removed (cytosolic fraction). To the pellet, 0.125 ml of buffer B (0.4 M NaCl, 10 mM HEPES-KOH [pH 7.9], 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) was added. The mixture was vortexed at 4°C and left on ice for 5 min. The extracts were then centrifuged, and the supernatant was dialyzed against 50 volumes of buffer C (20 mM HEPES-KOH [pH 7.9], 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) for 4 h at 4°C with one change of buffer C. After 4 h, the materials which precipitated during dialysis were removed by centrifugation, and the supernatant was aliquoted, flash frozen in liquid N₂, and stored at -80°C until further use. The protein concentration was estimated with the Bradford protein assay kit (Bio-Rad).

EMS assay. The probe used for the electrophoretic mobility shift (EMS) assay was the nucleotide sequence spanning bp -282 to -273. Both ends of this fragment were labeled by filling in the 5' protruding ends (by use of *Bam*HI at the 5' end and *Bgl*II at the 3' end of the oligonucleotide, after annealing of the

two strands) on both sides by using [α -³²P]dATP and [α -³²P]dGTP with reverse transcriptase (Superscript III; GIBCO-BRL). Purified recombinant TTF1 protein or H441 nuclear extract was incubated with 40,000 cpm of the labeled oligonucleotide probe (specific activity, 10⁹ cpm/µg) in buffer B (50 mM NaCl, 0.1 mM EDTA, 20 mM HEPES-KOH [pH 7.5], 0.5 mM DTT, 10% glycerol) in a total volume of 15 µl. The reaction was allowed to proceed for 10 min at room temperature. The competition reaction was performed by adding the competing oligonucleotides to the reaction mixture during incubation of the protein with the probe. The supershift reaction was performed by adding either the anti-TTF1 or anti-mCC10 rabbit antiserum to the reaction mixture after the binding reaction and incubating the mixture for an additional 10 min. The reaction mixtures were then subjected to electrophoresis through a 6% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide ratio, 19:1) in 0.5× TBE buffer (0.001 M EDTA [pH 8.0] and 0.045 M Tris-borate).

Generation and identification of transgenic mice. The 800 bp of 5'-flanking DNA of the mCC10 promoter containing a linker-scanner mutation at bp -282 to -273 was placed 5' to the human growth hormone (hGH) reporter gene as previously described (53). The 800ΔVI-hGH transgene construction was isolated from plasmid sequences by digestion with the appropriate restriction enzymes. The fragments were separated by electrophoresis through low-melting-point agarose (SeaPlaque; FMC Bioproducts, Rockland, Maine). The 800ΔVI-hGH transgene was excised, and the DNA was isolated with the QIAEX gel extraction kit (Qiagen, Inc., Chatsworth, Calif.). The isolated fragment was diluted to a concentration of 2 µg/ml in TE buffer (10 mM Tris [pH 7.5] and 0.25 mM EDTA). Transgenic mice were produced as described by Hogan et al. (27). DNA fragments were microinjected into (ICR × B6D2)F₁ embryos. The transgenic founder mice were identified by Southern blot analysis (60) of *Pvu*II-digested mouse tail DNA. The Southern blots were probed with a randomly primed [α -³²P]dCTP-labeled hGH probe (18).

RNA isolation and Northern (RNA) blot analysis. In order to increase the speed of Northern blot analysis, the founder mice were sacrificed for expression analysis. The tissues were removed from euthanized transgenic mice and were homogenized in RNazol B (Cinna-Biotech Lab, Inc., Houston, Tex.). The RNA was isolated by the procedure described by Chomczynski and Sacchi (12). The mRNA for the hGH transgene and the endogenous mCC10 were identified by Northern blot analysis (62). The isolated RNA was denatured in formaldehyde-containing buffer and separated in a denaturing 1.5% agarose gel containing 2.2 M formaldehyde. RNA was then transferred to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, Ill.) by capillary blotting in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) overnight. The filters were hybridized with 20 million counts of either the [α -³²P]dCTP-labeled mCC10 cDNA or the [α -³²P]dCTP-labeled hGH cDNA probe. The probes were labeled to equivalent specific activities (2 × 10⁹ cpm/µg of DNA). Hybridization, washing of the membrane, and autoradiography were performed as described previously (18). The mRNA levels for both mCC10 and hGH in Northern blots were quantitated with a Betascope 603 blot analyzer (Betagen, Waltham, Mass.).

RESULTS

TTF1 activation of mCC10 deletion constructions. Bohinski and coworkers (8), using transient-transfection, DNase I footprint, and EMS analyses, have identified the consensus sequence of GCNCTNNAG as the response element for TTF1 regulation of the SP-B gene. A computer search of the 800 bp of the 5'-flanking DNA of the mCC10 promoter identified this sequence at bp -589 to -580 and -282 to -273. By using a strategy similar to that of Bohinski and coworkers, i.e., TTF1 transactivation of reporter constructions containing pulmonary epithelium-specific promoter elements in nonpulmonary cells (8), the functional TTF1 binding sites in the mCC10 promoter were mapped. CV1 cells were transfected with deletion constructions of the mCC10 promoter with either an expression vector containing TTF1 or a control expression vector with no TTF1 inserted. The CMV-β plasmid was also cotransfected with these constructions, and expression of β-galactosidase was used as an internal control to monitor the transfection efficiency. Figure 1 shows the results of TTF1 activation of the mCC10 promoter with various lengths of 5'-flanking DNA. TTF1 dramatically increased the activity of the 800-bp promoter. The activation observed with 582 bp of 5'-flanking DNA was not significantly less than that with the 800-bp promoter. However, when only 267 bp of mCC10 flanking DNA was used to drive the expression of the reporter gene, CAT activity was significantly reduced compared with that with the 800-bp promoter. Further deletions to bp -166 of 5'-flanking

FIG. 1. Transactivation of the mCC10 promoter with successive deletions at the 5'-flanking region by TTF1 or CSX in CV1 cells. Various lengths of mCC10 promoter sequence (800 to 23 bp) attached to the CAT reporter gene were transiently cotransfected with internal control CMV- β GAL and either an empty expression vector (pCGN) or the expression vector containing the full-length cDNA for TTF1 or CSX in CV1 cells. The data are means from several independent transfections normalized for β -galactosidase activity, and the error bars represent the standard error for each construction.

DNA showed another significant reduction in activation of the mCC10 promoter, indicating regulatory elements within this fragment. No further significant decrease in TTF1 activation was observed until 23 bp of 5'-flanking DNA of the mCC10 promoter was used to drive CAT expression. However, activation of the mCC10 promoter by TTF1 was observed in all deletion constructions up to and including 87 bp of the promoter sequence. These results indicate that there are potential TTF1 binding sites within the proximal promoter elements that were not identified in the computer search. Also, the consensus site at bp -589 to -580 was not a critical site in the regulation of mCC10 by TTF1.

DNase I footprint analysis. The sequences in the mCC10 promoter which could bind TTF1 were identified by DNase I footprint analysis. Figure 2 shows the pattern of DNase I footprinting of recombinant TTF1 of bp -417 to +7 (Fig. 2A) and bp -166 to +7 (Fig. 2B) of the mCC10 promoter. In the region spanning bp -417 to +7, five protected regions were identified, and their locations and sequences are listed in Table 1. By using the -166 to +7 promoter sequence, another two protected regions were identified, and the site and sequence are shown in Table 1. The identification of the proximal TTF1 binding sites explains the transactivation observed in the deletion constructions. Analysis of the region spanning bp -803 to -344, which includes the site at bp -589 to -580, a potential TTF1 binding site initially identified by a computer search, showed no protected regions (data not shown). In order to identify the relative affinities of these sites for TTF1 and their functional significance, EMS analysis and linker-scanner mutational analysis were conducted with the sequences identified by the footprint analysis.

EMS assay. Prior to the affinity and functional analyses of

the TTF1 binding sites in the proximal promoter region of the mCC10 promoter, supershift analysis, using antibodies to TTF1, was conducted to confirm the role of TTF1 in the binding to the mCC10 promoter region. EMS analysis was conducted with an oligonucleotide probe corresponding to region VI and recombinant TTF1 protein or H441 cell nuclear extract. This probe was used because it was shown by deletion analysis to mediate TTF1 transactivation of the mCC10 promoter. Figure 3A shows the result of the EMS and supershift analyses. The antibody used in the supershift was raised to TTF1 peptides outside the homeodomain in order to ensure specificity for TTF1 (33). The TTF1 antibody was able to upshift the reaction complex corresponding to recombinant TTF1. An identical upshift was observed when a nuclear extract of H441 cells was incubated with TTF1 antibody. There was no upshift of the complex when the mCC10 antibody was used. The antibody upshift observed with the TTF1 antibody did not completely shift all of the complexes because of the limiting amount of antibody used. This result suggests that TTF1 is the factor that binds to the mCC10 promoter in pulmonary cells.

The affinity of each of the TTF1 binding sites for TTF1 was assayed by EMS competition analysis. An end-labeled oligonucleotide sequence corresponding to the site at bp -282 to -273 was used as a probe for the EMS competition assay. The competition reaction was performed with 20- and 40-fold molar excesses of the unlabeled oligonucleotide sequence for the other four TTF1 binding sites. Figure 3B shows the results of the competition analysis. Each oligonucleotide sequence competed, with various degrees of affinity, except the oligonucleotide for the sequence from bp -589 to -580. The two sites flanking the sequence used as the probe located at bp -258 to -249 (site IV) and bp -344 to -335 (site VII) showed the highest degree of competition. Linker-scanner mutation of these sites was conducted to identify the role that these sites played in the transactivation of mCC10 by TTF1.

Linker-scanner mutation of the putative TTF1 binding sites.

In order to identify the functional importance of TTF1 binding to the sites in the mCC10 promoter, linker-scanner mutational analysis was conducted with each of the five TTF1 binding sites identified between bp -800 and -166. Figure 4 shows the results of the linker-scanner mutational analysis. Each individual linker-scanner mutant showed only a minor reduction in the ability of TTF1 to transactivate the mCC10 promoter. Mutant 800 Δ VI (bp -282 to -273) showed the greatest reduction in activity when mutated in the context of the 800-bp promoter. A smaller but significant reduction was observed with mutant 800 Δ VII (bp -344 to -335) and mutant 800 Δ IV (bp -258 to -249). The activity of the TTF1 on each of the linker-scanner mutants correlated well with the observed affinity of each for the TTF1 protein as observed in the EMS competition assay. Mutant 800 Δ VI, however, did not reduce the activation by TTF1 to the levels observed in the construction with only 166 bp of the mCC10 promoter. However, as shown in Fig. 4, a double mutant, mutant 800 Δ VI,IV (bp -282 to -273 and -258 to -249) showed a reduction of the reporter gene expression to levels close to those with the 166-bp construction. From this analysis, it can be concluded that TTF1 acts at multiple sites in the mCC10 promoter. Although TTF1 shows differential affinities for these sites, there is redundancy in these sites mediating TTF1 transactivation of the mCC10 promoter in this transient-transfection assay.

Transgene expression of hGH under the control of the mCC10 promoter. The role of TTF1 binding site VI, at bp -282 to -273, was evaluated in vivo by transgenic analysis. The 800-bp fragment containing the linker-scanner mutation

FIG. 2. DNase I footprinting of the mCC10 promoter with recombinant TTF1 or CSX. DNase I footprinting was performed as described in the text in the absence of CSX and TTF1 (lanes 5 and 6) or in the presence of 10 μ g of CSX (lanes 1 and 2) or 15 μ g of TTF1 (lanes 3 and 4) by using end-labeled DNA probes corresponding to bp -417 to +7 (A) and bp -166 to +7 (B) of the 5'-flanking region of the mCC10 promoter, labeled on the noncoding strand. After DNase I digestion, the reaction products were resolved in an 8% (wt/vol) acrylamide sequencing gel, fixed, dried, and analyzed by autoradiography. The locations of the regions protected from DNase I digestion are indicated at the left of each panel. Lanes G+A and G, Maxam-Gilbert chemical sequencing ladders for the corresponding DNA fragments of the mCC10 promoter used as a probe.

at site VI was placed 5' to the hGH reporter gene. The transgene construction was called 800 Δ VI-hGH. Eight transgenic founder mice with this construction were made. The level of hGH mRNA in the lungs of these mice was compared with the level of endogenous mCC10 expression and the level of expression observed in mice generated with the wild-type 800-bp

promoter or the 166-bp promoter (53). Figure 5 shows the results of this analysis. Mice generated with the 800 Δ VI-hGH construction showed a level of expression comparable to that of those generated with the 166-bp promoter. This analysis indicates that the disruption of the TTF1 binding site at bp -282 to -273 significantly reduces mCC10 expression *in vivo*. Figure 5C shows a graphic presentation of the levels of transgene expression from different constructions.

Activation by other members of the NKx family. TTF1 is a member of the NKx homeobox protein family. Recently, it was shown that another member of this family, NKx 2.5, also known as the cardiac muscle-specific homeobox protein (CSX), can bind with high affinity to the response element of TTF1 (11). The ability of CSX to bind and activate the TTF1 sites in the mCC10 promoter was evaluated to verify the binding of CSX to the TTF1 binding sites in the context of a pulmonary epithelium-specific promoter and to evaluate the functional ability of CSX to transactivate a pulmonary epithelium-specific gene. Figure 1 shows that the CSX protein was

TABLE 1. Sequences protected in DNase I footprinting analysis and their relative positions

Site	Protected sequence (5'→3')	Location (bp)
I	TCTACCTCTTG	-49 to -39
II	CCTGAA	-74 to -69
III	CCTGAATACT	-199 to -190
IV	CCAAGAGAGG	-258 to -249
V	GCTTGA	-268 to -263
VI	CTGGAGTGCT	-282 to -273
VII	CCTGAAGGGT	-344 to -335

FIG. 3. Gel mobility shift assay of purified recombinant TTF1 and an H441 nuclear extract. The probe used for the mobility shift assay was the DNA sequence of site VI, located at bp -282 to -273, with protruding ends for *Bam*HI and *Bgl*II at the 5' and 3' ends, respectively. The oligonucleotide was 17 bp long, and both ends of the oligonucleotide were labeled as described in the text. (A) The reaction was performed with 1.5 μ g of purified TTF1 (lanes 2 to 5) or 6 μ g of H441 nuclear extract (lanes 6 and 7) with the 32 P-labeled site VI oligonucleotide as a probe alone (lane 1), in the absence of a competitor (lanes 2 and 6), in the presence of a 100-fold molar excess of specific unlabeled competitor (lane 3), in the presence of 4 μ l of diluted TTF1 antibody (lanes 4 and 7), or in the presence of a control antibody against mCC10 (lane 5). (B) Relative affinities of TTF1 for different sites within bp -800 to -166 of the mCC10 promoter sequence. The reaction was performed with 1.5 μ g of purified recombinant TTF1 with the 32 P-labeled site VI oligonucleotide sequence as a probe alone (lane 1), in the absence of a competitor (lane 2), or with a 20- or 40-fold molar excess, respectively, of unlabeled specific competitors corresponding to the different TTF1 binding sites as follows: lanes 3 and 4, site VI; lanes 5 and 6, site III; lanes 7 and 8, site VII; lanes 9 and 10, site IV; lanes 11 and 12, site V; lanes 13 and 14, site VIII. In the case of site VII, lane 7 represents a 40-fold and lane 8 represents a 20-fold molar excess of the unlabeled specific competitor. The specific complexes in the reaction are designated by arrows marked a (super-shifted complex) and b.

able to activate the deletion mutants of mCC10 in a pattern similar to that for the TTF1 gene. Also, as shown in Fig. 2, CSX bound the mCC10 promoter in a pattern identical to that for TTF1. Thus, the binding site of TTF1 in the mCC10 promoter

can be recognized by other NKx family members. The ability of the members of the NKx family to bind to common response elements may imply the existence of common target genes within the respective organs.

DISCUSSION

TTF1 was originally identified as a transcriptionally active DNA-binding protein involved in the regulation of the thyroid-specific genes for thyroglobulin and thyroid peroxidase (19, 46). In addition to the thyroid, TTF1 is also expressed in the lung and forebrain (33). Cloning of TTF1 has identified this protein as a member of the NKx homeobox protein family. Other members of this family include the product of the *Drosophila tinman* gene, NKx 2.5 (the mammalian homolog of the *tinman* product) (11), a cardiac muscle-specific homeobox protein (CSX), and a brain-specific protein (NKx 2.2). Analysis of the function of the *Drosophila tinman* gene and the CSX gene has products identified the NKx gene products as transcription factors which are important for organogenesis. CSX is expressed in the early heart progenitor cells and may function in commitment to the myocardial lineage. Evidence for this lies in the fact that CSX is also expressed in primitive pharyngeal endoderm, the tissue that is thought to secrete the heart inducer (29, 34). In addition, a recent knockout of the endogenous murine CSX gene caused embryonic lethality due to the failure of folding the heart tube into a chambered heart and to the lack of myocardial cell expansion associated with ventricular trabeculation (38). It has been proposed that in the forebrain, TTF1 in combination with NKx 2.2 may cooperate in the delimitation of the forebrain structure (50). TTF1 also functions to regulate pulmonary gene expression (8). In this report we have demonstrated that TTF1 binding elements in the distal promoter region of the mCC10 promoter are responsible for 90% of the level of expression of the mCC10 gene.

In this report, we have identified seven TTF1 binding sites in the 800 bp of DNA flanking the mCC10 gene. Using the sequence protected by DNase I footprinting within the 800 bp of the mCC10 promoter sequence, we have generalized a consensus sequence, CCTGA(g)AG(t), that is very similar to the sequence reported by Bohinski et al. (8) for the lung-specific SP-B gene (GCNCTNNAG). The organization of the TTF1 binding sites in the mCC10 promoter is similar to what has been observed for the TTF1 binding sites in the SP-B and SP-A genes and in thyroid-specific genes such as those for thyroglobulin and thyroid peroxidase (8, 9, 14, 17, 47). In these genes there are at least five TTF1 binding sites distal to the promoter and two binding sites proximal to the promoter. This report has identified at least three functional TTF1 binding sites distal to the promoter, located between bp -344 and -249, as the elements regulating the expression of mCC10 in transient transfection. The functionality of these sites has been identified by using linker-scanner mutation and transgenic mouse analyses. Mutation of the center binding site in the distal promoter region has shown that this site is essential for the full quantitative level of mCC10 gene expression in transgenic mice. Thus, in the case of mCC10 expression, the TTF1 elements at this distal locus act as elements responsible for the pulmonary expression of this gene. However, the cellular specificity of mCC10 is determined by more proximal elements. On the basis of this information, we have proposed a model in which the elements regulating the full expression of mCC10 reside in two domains, one proximal and one distal to the start of transcription. Deletion analysis with transgenic mice has shown that the proximal domain determines the Clara cell specificity of the expression of this gene (53). The elements

FIG. 4. The TTF1 binding site VI at bp -282 to -273 is the most critical site for transactivation of the mCC10 promoter. The positions and sequences of TTF1 binding sites are listed in Table 1. Six single mutants and one double mutant located within bp -800 to -166 bp were transiently cotransfected with internal control CMV β -GAL and either an empty expression vector (pCGN) or the expression vector containing the full-length cDNA for TTF1 to CV1 cells. 800 wt, unaltered 800 bp of the mCC10 promoter; 800 Δ , site or sites that have been mutated within the context of 800 bp of the promoter sequence. The relative positions of the TTF1 binding sites that were mutated are indicated. The 166-bp mCC10 promoter sequence (166 wt) was used to compare the levels of transactivation with different mutant constructs having single or double mutations, and 23 wt represents the minimal promoter TATA attached to pBLCAT3. The data are means from several independent transfections normalized to β -galactosidase activity, and the error bars represent the standard error for each construction.

within this domain include members of the AP1 and HNF families of transcription factors and may also include response elements for the TTF1 transcription family. This report shows that the TTF1 binding sites in the distal promoter are responsible for elevating the level of expression of the mCC10 gene in the lung.

TTF1 may play a role in the cellular specificity of the mCC10 gene. Two TTF1 binding sites were identified in 166 bp of the proximal promoter region. Previous experiments using deletion mutants of the mCC10 promoter transfected into H441 cells have shown that a construction containing the two proximal TTF1 sites and none of the other putative regulatory elements of mCC10 gene expression was expressed at levels above those of a minimal TATA promoter (53). In this study, the same construction, containing 87 bp of 5'-flanking DNA, was transactivated in CV1 cells by TTF1. Thus, TTF1 may play a regulatory role in the proximal element of mCC10. However, when footprint analysis of the mCC10 promoter was conducted with an H441 nuclear extract, no protection of the proximal TTF1 binding sites was observed (61). The functional significance of TTF1 in the proximal promoter needs to be determined.

H441 cells have been used to map the promoter of the mCC10 gene (5, 53, 56, 61). Elements located within the first 166 bp of 5'-flanking DNA have been shown to direct the cell-specific expression of this gene in H441 cells and transgenic mice. Deletion analysis showed that the 800 bp of flanking DNA showed a lower level of expression of reporter genes than the 166 bp of flanking DNA. Also, when the 800-bp mCC10-CAT construct was transfected to H441 cells along with the mutant clones for TTF1 binding sites, there was practically no difference in transactivation of the reporter gene (data not shown). Thus, transient-transfection analysis with

H441 cells has not been successful in identifying TTF1 as important for expression of this gene. This is puzzling, since H441 cells contain TTF1 and were capable of defining the role of TTF1 in the regulation of the SP-B gene (8, 28). One explanation of this phenomenon is that the multiple binding sites in the promoter of mCC10 display a functional redundancy in transient transfection. The TTF1 sites in the proximal promoter may be all that is necessary to give maximal expression of the mCC10 gene in transient transfection. Evidence for this redundancy is the linker-scanner mutations. Mutation of single TTF1 binding sites showed small reductions in the transactivation of the mCC10 promoter in the cotransfection assay in CV1 cells and no reduction in mCC10 expression in transfection of these mutants alone in H441 cells. An alternative explanation of this phenomenon may be posttranslational regulation of TTF1 gene involvement in the regulation of its specificity. Avvedimento et al. have shown that the activity of TTF1 could be regulated by phosphorylation (4). Dephosphorylation of TTF1 abolished the DNA binding ability and subsequently the transactivation of the thyroglobulin promoter. In a very recent report, Arnone et al. (1) demonstrated that the binding activity of TTF1 could be regulated by redox potential by showing that in vitro binding activity decreases in an oxidative state and can be fully restored upon exposure to reducing agents. Thus, the phosphorylation status or redox status of the TTF1 protein may be altered in the H441 cells and may not enable this protein to interact with the TTF1 sites in the distal promoter. This report highlights the limitations of transient transfection in determining the elements regulating the expression of the mCC10 gene. In this study, the in vivo transgenic analysis defined the functional significance of the TTF1 binding sites in the analysis of the regulation of the expression of the mCC10 promoter.

FIG. 5. Effect of mutation of TTF1 binding site VI at bp -282 to -273 on pulmonary expression of the hGH transgene and comparison with hGH transgene expression driven by 800-bp (800wt) and 166-bp (166wt) wild-type mCC10 promoters. Ten micrograms of total lung RNA was electrophoresed and transferred to nitrocellulose paper as described in the text. Blots were probed with 32 P-labeled hGH cDNA (A) or 32 P-labeled mCC10 cDNA (B). The arrows indicate the expression of hGH (A) or endogenous mCC10 (B) from each line of mice for a particular mCC10-hGH construction. (C) Quantitation of the levels of transgene expression from different constructions. Bars, mean level of expression of the transgene from each construction; open circles, datum points for individual mice.

One interesting observation from this report is the ability of the CSX protein to bind and activate the lung-specific mCC10 promoter in a manner identical to that of the TTF1 protein. This observation may explain the report of the cardiac expression of the hGH transgene under the control of the rat CC10 promoter (23). Although CC10 is not expressed in the heart, given the appropriate location in the genome, the TTF1 binding sites may be recognized by CSX, allowing the cardiac expression of the transgene. This rationale might also explain the report of thyroid tumors in transgenic mice expressing the simian virus 40 large-T-antigen gene under the control of the rabbit uteroglobin (CC10) promoter (16). In this case, the T-antigen transgene may have integrated into a chromatin environment, allowing TTF1, present in the thyroid, to activate transgene expression. This report demonstrates that the tight tissue specificity of the NKx target genes must be regulated by elements other than the NKx genes. This could involve the interaction of a particular combination of factors bound to DNA (45, 48), an interaction between a DNA-bound factor and a non-DNA-bound cofactor (37, 41), or covalent modification of a factor resulting in a change of affinities for other

proteins (30). Finally, the tissue specificity of gene expression may be dependent on the accessibilities of these *cis*-acting DNA elements to the transcription factors, which may depend on the location of the gene in the chromatin.

The specificities of the NKx2 genes for their tissue-specific targets may be determined by the above-described factors. However, the ability of CSX and TTF1 to activate gene expression at the same response elements may indicate that these factors can regulate a set of common target genes within their respective tissues. These common genes may function in regulating processes universal to the regulation of the development of these tissues. This raises an important question as to the function of TTF1 in lung development. Current analysis of the function of TTF1 has been limited to the investigation of how TTF1 regulates the expression of the tissue-specific protein products (19, 22). The fact that CSX regulates heart morphogenesis (38) may indicate that TTF1 may regulate genes involved in lung morphogenesis. The identification of the other genes controlled by TTF1 may lead to an understanding of the role that TTF1 plays in regulating pulmonary development.

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