The WNT7b Promoter Is Regulated by TTF-1, GATA6, and Foxa2 in Lung Epithelium*

Received for publication, December 7, 2001, and in revised form, March 21, 2002
Published, JBC Papers in Press, March 25, 2002, DOI 10.1074/jbc.M111702200

Joel Weidenfeld§§, Weiguo Shu§§, Lili Zhang¶, Sarah E. Millar¶, and Edward E. Morrisey‡‡
From the ‡Department of Medicine, Molecular Cardiology Research Center, and the ¶Department of Dermatology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

In this study, we find that WNT7b is the only member of the WNT family of autocrine/paracrine signaling molecules whose expression in the lung is restricted to the airway epithelium during embryonic development. To study the transcriptional mechanisms that underlie this restricted pattern of WNT7b expression, we isolated the proximal 1.0-kb mouse WNT7b promoter and mapped the transcriptional start sites. Transfection of the lung epithelial cell line MLE-15, which expresses WNT7b, shows that the 1.0-kb mouse WNT7b promoter is highly active in lung epithelial cells. This region of the WNT7b promoter contains several DNA binding sites for the important lung-restricted transcription factors TTF-1, GATA6, and Foxa2. Electrophoretic mobility shift assays showed that TTF-1, GATA6, and Foxa2 can bind to a specific subset of their consensus DNA binding sites within the WNT7b promoter. Using cotransfection assays, we demonstrate that TTF-1, GATA6, and Foxa2 can trans-activate the WNT7b promoter in NIH-3T3 cells. Truncation of GATA6 or Foxa2 binding sites reduced the ability of these transcriptional regulators to trans-activate the WNT7b promoter. Finally, the minimal 118-bp region of the mouse WNT7b promoter containing only TTF-1 binding sites was synergistically activated by TTF-1 and GATA6, and we show that TTF-1 and GATA6 physically interact in vivo. Together, these results suggest that WNT7b gene expression in the lung epithelium is regulated in a combinatorial fashion by TTF-1, GATA6, and Foxa2.

The development and differentiation of lung epithelial cells requires the proper coordination of autocrine and paracrine signaling events initiated by several families of secreted factors including fibroblast growth factor, transforming growth factor, and the bone morphogenetic protein family members (1). These signaling events regulate cell differentiation and proliferation by modifying gene expression programs via transcriptional activation and/or repression. One group of signaling molecules which plays an important role during embryonic development, but whose role during lung development has not been well characterized, is the WNT family of secreted glycoproteins. WNT proteins bind to cell surface receptors of the Frizzled family. Frizzled receptors are seven membrane-spanning transmembrane proteins that share some similarity to G protein-coupled receptors (2). WNTs signal through several different pathways to regulate cell fate, movement, or adhesion (2). In the best studied of these, the so-called canonical WNT signaling pathway, binding of WNT proteins to Frizzled receptors transmits signals from the cell surface to the nucleus by inhibiting the glycogen synthase kinase 3 beta, resulting in an unphosphorylated and stabilized form of beta-catenin which accumulates and translocates into the nucleus, forming complexes with members of the TCF/LEF family of transcription factors, which regulate gene transcription (2).

Although much is known about the expression patterns and the roles of the various WNT genes in other tissues, the expression patterns of WNT genes and their role during lung morphogenesis are not well defined. In this report, we show that WNT7b is the only WNT gene tested which is expressed exclusively in the airway epithelium during lung organogenesis. This restricted pattern of expression suggests that WNT7b is regulated by transcriptional mechanisms restricted to the lung epithelial cell lineage. Gene expression in lung epithelium is thought to be regulated by members of several transcription factor families including the homeodomain (TTF-1), GATA zinc finger (GATA6), and the Fox family of winged helix transcription factors (Foxa2) (3). Many lung-specific gene promoters including those for surfactant proteins A and B (SP-A and SP-B, respectively) and the Clara cell 10 kDa protein CC10 contain binding sites for one or more of these transcription factors (4–7). However, the mechanisms by which these three different transcription factor families functionally regulate lung-specific gene expression are not fully understood.

To examine the mechanisms behind the restricted pattern of WNT7b expression in the lung, we isolated and analyzed the proximal promoter region of the mouse WNT7b gene. The proximal 1.0-kb mouse WNT7b promoter exhibits high levels of transcriptional activity in the lung epithelial cell line MLE-15 and contains multiple binding sites for the transcription factors TTF-1, GATA6, and Foxa2. We show that TTF-1, GATA6, and Foxa2 proteins bind to some, but not all, of their respective consensus DNA binding sites located within the WNT7b promoter. TTF-1 is able to trans-activate a minimal 118-bp region of this promoter, whereas GATA6 and Foxa2 can trans-activate larger regions containing their respective DNA binding sites. Moreover, we show that TTF-1 and GATA6 physically interact in vivo and synergistically activate transcription of the minimal 118-bp mouse WNT7b promoter, indicating that the mouse WNT7b gene is regulated through protein-protein interactions between these two transcription factors.

MATERIALS AND METHODS

In Situ Hybridization and Northern Blot Analysis—In situ hybridization analysis was performed as described previously (8), and a de-
TTF-1, GATA6, and Foxa2 Regulate the WNT7b promoter

Oligonucleotide sequences corresponding to sites within the mouse WNT7b promoter and used in EMSAs

| Site | Sequence |
|------|----------|
| TTF-1 site 1 (T1) | 5′-GATCCGGACAGCTGCGGCTCAAGTGACCCAAC-3′ |
| TTF-1 site 2 (T2) | 5′-GATCCGACGACACCCCTGCGGCTCAAGtgacCCAAC-3′ |
| TTF-1 site 3 (T3) | 5′-GATCCCGGGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| TTF-1 site 4 (T4) | 5′-GATCCCGGGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| GATA6 DNA binding region (S1/S2) | 5′-GATCCCGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| GATA6 site 1 mutant (mS1) | 5′-GATCCCGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| GATA6 site 2 mutant (mS2) | 5′-GATCCCGGGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| GATA6 site 1/2 mutant (mS1/2) | 5′-GATCCCGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| Foxa2 binding site 1 (F1) | 5′-GATCCCGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| Foxa2 binding site 2 (F2) | 5′-GATCCCGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| Foxa2 binding site 3 (F3) | 5′-GATCCCGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |
| Foxa2 binding site 4 (F4) | 5′-GATCCCGGACAGCTGCGGCTCAAGtgacCCAAC-3′ |

1 The abbreviations used are: RACE, rapid amplification of cDNA Ends; BAC, bacterial artificial chromosome; CMV, cytomegalovirus; EMSA, electrophoretic gel mobility shift assay; βgal, β-galactosidase; SP, surfactant protein.

tailed description of the various protocols can be found at the Molecular Cardiology Research Center Web site (www.med.upenn.edu/mc/molecular/histology/histologyhome.html). Probes for WNT2, 3, 3a, 4, 5, 6, 7a, 7b, 8, 9, 10a, 10b, 11, 12, 13, 14, 15, and 16 have been described previously (9). Micrographs were taken on a Zeiss Axioshot microscope.

Total RNA was extracted from MLE-15 cells and dissected E17.5 mouse lung tissue using Trizol reagent (Invitrogen). 20 μg of RNA was resolved by formaldehyde-agarose gel electrophoresis and blotted to a Hybond membrane (Amersham Biosciences). This membrane was probed using a portion of the mouse WNT7b cDNA which corresponded to bp 1–1050 of the published mouse WNT7b sequence (10).

Primer Extension and 5′-Rapid Amplification of cDNA Ends (RACE)—A mouse 129SV bacterial artificial chromosome (BAC) library (Incyte Genomics, Inc.) was screened using oligonucleotides located in the first exon of the mouse WNT7b gene to obtain two independent mouse WNT7b BAC clones. A Kpn1 subclone from one of the BAC clones which encompassed the region surrounding the translational start site was cloned and sequenced. Primer extension analysis was performed using 5 μg of E17.5 mouse embryonic lung or adult mouse liver poly(A)+ RNA and the end-labeled oligonucleotide 5′-GACAAGGAGCTGCGGCTGAGACC-3′. 50,000 cpm of end-labeled oligonucleotide was annealed to RNA overnight at 60 °C. Primer extension was performed at 42 °C for 1 h using Superscript II reverse transcriptase (Invitrogen). Extended products were purified by phenol/chloroform extraction, precipitated with ethanol, resuspended in formamide loading buffer, and loaded onto a 6% denaturing polyacrylamide gel. To determine the size of the extension products, a DNA sequencing ladder generated from a M13 control plasmid was run next to the extension products, a DNA sequencing ladder (Invitrogen). Extended products were purified by phenol:chloroform extraction, precipitated with ethanol, resuspended in formamide loading buffer, and loaded onto a 6% denaturing polyacrylamide gel. To determine the size of the extension products, a DNA sequencing ladder generated from a M13 control plasmid was run next to the extension products. 5′-RACE was performed using E17.5 mouse embryonic lung poly(A)+ RNA, a commercially available kit (Invitrogen), and the following primary and nested oligonucleotides: primary, 5′-CACCGTGACGCTGCGGCTGAGACC-3′; nested, 5′-GACAAGGAGCTGCGGCTGAGACC-3′. PCR products from the 5′-RACE reaction were cloned into the pCR2.1-TOPO TA cloning vector (Invitrogen) and sequenced.

Reporter and Expression Constructs—The following oligonucleotides were used to amplify by PCR: 1.0-kb mouse WNT7b promoter luciferase reporter construct and shorter deletions from WNT7b BAC DNA: 1.005-bp promoter region sequence, 5′-CACCGTGACGCTGCGGCTGAGACC-3′; 829-bp region sequence, 5′-CACCGTGACGCTGCGGCTGAGACC-3′; 5′-CACCGTGACGCTGCGGCTGAGACC-3′; 444-bp region sequence, 5′-CACCGTGACGCTGCGGCTGAGACC-3′; and the 58-bp region sequence, 5′-CACCGTGACGCTGCGGCTGAGACC-3′. All PCRs used the 3′-antisense oligonucleotide 5′-cactgaggacgctggctaggtg-3′. The resulting PCR fragments were cloned into the Kpn1 and XhoI sites of the pGL3 basic luciferase reporter plasmid (Promega) to generate the luciferase reporter plasmids pGL3/Wnt7b-1.0, pGL3/Wnt7b-0.83, pGL3/Wnt7b-0.44, pGL3/Wnt7b-0.83, pGL3/Wnt7b-0.12, or pGL3/Wnt7b-0.06.

Transfection and Coimmunoprecipitation Assays—To determine relative promoter activities, 1 × 106 MLE-15 and NIH-3T3 cells were transfected as described previously (10). For transactivation assays, 1 × 106 NIH-3T3 cells were transfected with 0.5 μg of the pGL3/Wnt7b-1.0, 0.78 μg of pDNA3G6, or both pCMVTTF-1, pcDNA3G6, or pCMVFoxa2 reporter plasmid. All transfections contained 0.5 μg of the pMSVβgal plasmid to control for transfection efficiency. Cells were harvested 48 h after transfection, and commercially available kits were used to measure luciferase and β-galactosidase activity (Promega). All values are the average of three experiments performed in duplicate ± S.E.

Competition and Coimmunoprecipitation Assays—To determine relative promoter activities, 1 × 106 MLE-15 and NIH-3T3 cells were transfected as described previously (10). For transactivation assays, 1 × 106 NIH-3T3 cells were transfected with 0.5 μg of the pGL3/Wnt7b-1.0, 0.78 μg of pDNA3G6, or both pCMVTTF-1, pcDNA3G6, or pCMVFoxa2 reporter plasmid. All transfections contained 0.5 μg of the pMSVβgal plasmid to control for transfection efficiency. Cells were harvested 48 h after transfection, and commercially available kits were used to measure luciferase and β-galactosidase activity (Promega). All values are the average of three experiments performed in duplicate ± S.E.

For coimmunoprecipitation assays, HEK-293 cells were transfected with pCMVTTF-1, pCMVTag-TTF-1 (FLAG-tagged), pCMVTagGATA6 (Myt-tagged), or both pCMVTTF-1, pCMVTag-TTF-1, and pCMVTagGATA6 using FuGENE 6 according to the manufacturer’s protocol (Roche Molecular Biochemicals). For trans-activation assays, 1 × 106 NIH-3T3 cells were transfected with 0.5 μg of the pGL3/Wnt7b-1.0, 0.78 μg of pDNA3G6, or both pCMVTTF-1, pcDNA3G6, or pCMVFoxa2 reporter plasmid. All transfections contained 0.5 μg of the pMSVβgal plasmid to control for transfection efficiency. Cells were harvested 48 h after transfection, and commercially available kits were used to measure luciferase and β-galactosidase activity (Promega). All values are the average of three experiments performed in duplicate ± S.E.
RESULTS

Expression of WNT Genes during Mouse Embryonic Lung Development—To determine which WNT genes are expressed during mouse lung development, we performed in situ hybridization on E14.5 mouse embryonic lung tissue using radiolabeled riboprobes corresponding to mouse mRNAs for WNT2, 3, 5a, 4, 5, 6, 7a, 7b, 8, 9, 10a, 10b, 11, 12, 13, 14, 15, and 16 (9). The results of this survey indicated that only WNT2, 7b, and 11 were expressed in the lung at this time (Fig. 1). To determine the developmental profile of expression of these WNTs in the lung, in situ hybridization was carried out on E12.5 and E16.5 embryonic sections. Expression of WNT2 throughout the mesenchyme of the developing lung has been demonstrated previously, and our data confirm this expression pattern (Fig. 1, Enchyme of the developing lung has been demonstrated previ-ously, and our data confirm this expression pattern (Fig. 1, A–C) (15). WNT7b expression was observed in the developing airway epithelium from E12.5 through E16.5 both in the distal airways and in the larger mainstem bronchial airways (Fig. 1, D–F). WNT11 expression was observed in both the epithelium and mesenchymal tissue of the developing lung as has been reported previously (Fig. 1, G–I) (16). Sense riboprobes did not exhibit a detectable signal for any of these genes (data not shown). These data show that WNT7b is the only WNT gene analyzed which is expressed exclusively in the lung epithelium during this time of embryonic development.

Cloning and Characterization of the Mouse WNT7b Promoter—The restricted pattern of WNT7b gene expression in the lung airway epithelium suggests that transcription of this gene is regulated by lung epithelium-specific mechanisms. To begin the characterization of the transcriptional mechanisms underlying expression of WNT7b in lung epithelium, we performed primer extension and 5′-RACE analysis on mouse lung poly(A)+ RNA from E17.5 embryos and adult mouse liver poly(A)+ RNA. Primer extension analysis identified three major sites of transcriptional initiation for the mouse WNT7b gene in embryonic lung (Fig. 2A). However, no extension products were obtained with adult liver poly(A)+ RNA, which correlates with an absence of WNT7b transcripts in this tissue (Fig. 2A, lane 2) (10). One of these sites, site B, was consistently produced at higher levels than sites A and C (Fig. 2A). 5′-RACE confirmed sites B and C (Fig. 2B). However, we never obtained 5′-RACE products corresponding to site A. This either suggests that site A is an artifact of the primer extension assay or that analysis of the 5′-RACE products was incomplete. We compared the sequences obtained from the primer extension and 5′-RACE analysis with the proximal upstream genomic DNA sequences in two BACs containing the mouse WNT7b gene. These sequences were found to correspond exactly to those in the WNT7b BAC clones (Figs. 2B and 3). The proximal 1-kb mouse WNT7b proximal promoter region was sequenced and analyzed further.

Comparison of the transcriptional start sites obtained from primer extension and 5′-RACE with the 1st kb of WNT7b BAC genomic DNA sequence upstream of these sites reveals a promoter region that lacks a consensus TATA box but contains numerous Sp-1 binding sites (Fig. 3). Interestingly, binding sites for TTF-1, GATA6, and Foxa2, all of which have been shown to play important roles in lung epithelial gene transcription, were found in the proximal 1-kb region (Fig. 3). The GATA6 and Foxa2 putative DNA binding sites were grouped together in a region extending from bp −870 to bp −560 (Fig. 3). In contrast, TTF-1 DNA binding sites were found distributed throughout the WNT7b proximal 1.0-kb promoter (Fig. 3).

Activity of the WNT7b Promoter in MLE-15 Cells—The abundance of DNA binding sites for transcription factors implicated in lung epithelial gene transcription in the proximal 1.0-kb WNT7b promoter suggests that this region may confer transcriptional activity in lung epithelial cell lines. Therefore, the MLE-15 mouse epithelial cell line, which expresses TTF-1, GATA6, and Foxa2, was tested for expression of WNT7b (17). Northern blot analysis showed that MLE-15 cells express the WNT7b gene (Fig. 4A). MLE-15 and NIH-3T3 cells were transfected with a luciferase reporter plasmid containing the WNT7b 1.0-kb promoter (pGL3/Wnt7b-1.0) to test the activity of this promoter region in lung epithelial and non-lung cells. As shown in Fig. 4C, high levels of luciferase activity were obtained with the pGL3/Wnt7b-1.0 construct in MLE-15 cells,
whereas NIH-3T3 cells displayed only low levels of activity (Fig. 4, column A). To analyze further which regions in the WNT7b promoter were essential for in vitro activity in MLE-15 cells, serially truncated fragments of the 1.0-kb region were generated, cloned into the pGL3basic luciferase reporter plasmid, and transfected into MLE-15 and NIH-3T3 cells (Fig. 4B).

Truncation to bp −444 resulted in a net increase in transcriptional activity of the WNT7b promoter in MLE-15 cells of 73% (Fig. 4C, column C). Further truncation to bp −118 resulted in more than 75% reduction in luciferase activity in these cells (compared with the 1.0-kb WNT7b promoter fragment) (Fig. 4C, column D). However, truncation to bp −58 brought the luciferase activity down to almost background levels (Fig. 4C, column E). Of note, none of the WNT7b promoter fragments exhibited high levels of activity in NIH-3T3 cells (Fig. 4C, column C). These data show that the 444-bp proximal region of the WNT7b promoter is sufficient to confer high levels of transcriptional activity in lung epithelial cells in vitro.

**Electrophoretic Mobility Analysis of TTF-1 Binding Sites in the WNT7b Promoter**—The data from MLE-15 cell transfection experiments show that the 444-bp WNT7b promoter is capable of driving high levels of luciferase expression in lung epithelial cells in vitro. This region of the promoter lacks GATA6 and Foxa2 DNA binding sites but contains four putative TTF-1 DNA binding sites (Fig. 4A). TTF-1 has been shown to regulate the transcription of several lung-specific genes including SP-A, SP-B, and SP-C as well as the Clara cell 10 kDa (CC10) protein (6, 7, 18, 19). To determine whether TTF-1 is able to bind to any or all of these four putative sites, EMSAs were performed using double stranded oligonucleotides analogous to these sites and nuclear extracts from HEK-293 cells transfected with an expression plasmid containing the mouse TTF-1 cDNA (Table I).

In addition, a TTF-1-specific monoclonal antibody, along with non-immune antibody, was used to confirm the specificity of this binding. EMSAs of these sites show that sites T1, T2, and T3 were capable of strong binding to TTF-1 (Fig. 5, lanes 1–12). The TTF-1-specific antiserum supershifted the corresponding bands in each of these assays, whereas the non-immune sera had no effect (Fig. 5, lanes 1–16). Site T4 did bind a protein that was unique to the 293/TTF-1 extracts and comigrated with the TTF-1-specific bands in EMSAs performed with site T1-T3 oligonucleotides (Fig. 5, lanes 13–16). However, treatment of the site T4 EMSA reaction with the TTF-1 monoclonal antibody produced only a weak supershift/ablation of this band (Fig. 5, lane 16). This result suggests that TTF-1 binds only weakly to the site T4 oligonucleotide. To determine whether TTF-1 expressed in lung epithelial cells could bind to TTF-1 sites in the WNT7b promoter, MLE-15 nuclear extracts were used in EMSA. EMSAs with oligonucleotides T2 and T3 show a TTF-1-specific shift that was competed with an oligonucleotide corresponding to the well characterized TTF-1 sites located in the human SP-B promoter but not by the Foxa2 binding site oligo-
average of transfections performed in duplicate from the reporter plasmids was measured after 48 h. All values are the NIH-3T3 cells (1.0-kb mouse WNT7b promoter (bar A black ovals sites (Foxa2 DNA binding sites (F1

To determine which site(s) GATA6 was able to bind in the GATA6 wild-type oligonucleotide, GATA6-containing nuclear extracts were used with radiolabeled GATA6 wild-type oligonucleotides with mutations in either site 1 (mS1) or 2 (mS2) (Table I). As expected, increasing concentrations of S1/S2 oligonucleotide competed GATA6-specific shifted band down to background levels (Fig. 6B). Unlabeled mS1 oligonucleotide was not able to compete the GATA6-shifted band (Fig. 6B, lanes 3–6) whereas unlabeled mS2 oligonucleotide competed the GATA6-shifted band as efficiently as the GATA6 wild-type oligonucleotide (Fig. 6B, lanes 10–12). The mS1/S2 oligonucleotide, containing a mutation in both of the putative GATA6 DNA binding sites, did not compete for GATA6 binding to the wild-type oligonucleotide at any of the concentrations tested (Fig. 6B, lanes 13–15). These data show that GATA6 can bind to the S1 but not S2 site of the GATA6 DNA binding region located between –871 bp and –845 bp in the WNT7b promoter.

To verify that GATA6 from lung epithelial cells could bind to the WNT7b GATA6 wild-type oligonucleotide, MLE-15 cell nuclear extracts were used in EMSAs with the presence of preimmune or immune GATA6 antiserum. As shown in Fig. 6C, the GATA6 immune antiserum generated supershifted bands, whereas the preimmune antiserum did not (Fig. 6C, lanes 2 and 3). These data suggest that GATA6 expressed in lung epithelial cells can bind to the defined GATA binding site located in the WNT7b promoter.

Electrophoretic Mobility Analysis of Foxa2 Binding Sites in the WNT7b Promoter—Foxa2 is another transcriptional regulator expressed in the lung epithelium and thought to play an important role in lung epithelial gene transcription (4, 7, 21). To test whether Foxa2 was capable of binding to any of the four putative DNA binding sites located in the WNT7b promoter, EMSAs were performed with double stranded oligonucleotides F1, F2, F3, and F4 and nuclear extracts from HEK-293 cells transfected with an expression plasmid containing the mouse Foxa2 cDNA. In addition, specific antiserum and non-immune serum were used to confirm the specificity of Foxa2 binding to these sites. Foxa2 binding was detected with the F1, F2, and F4 oligonucleotides (Fig. 7). Furthermore, the addition of the specific antiserum for Foxa2 produced a supershift in reactions with the F2 and F4 oligonucleotides (Fig. 7, lanes 10 and 20). The Foxa2 antiserum did not produce a detectable supershift using the F1 oligonucleotide, which means that either the Foxa2-shifted band is nonspecific using this oligonucleotide or the supershifted band is obscured by other background bands in the EMSA reaction (Fig. 7A, lane 5). Of note, non-immune serum did not affect the Foxa2-shifted bands with any of the oligonucleotides tested. These results show that Foxa2 strongly

nucleotide F1 located in the mouse WNT7b promoter (Fig. 5, lanes 17–22, and Ref. 31). Together, these data corroborate well the MLE-15 transfection data showing high levels of transcriptional activity in the proximal 444-bp WNT7b promoter region, suggesting that a significant proportion of this transcriptional activity is because of the binding of TTF-1 to sites T1–T4.  

**Figure 4.** Expression and activity of the WNT7b promoter in MLE-15 cells. A, Northern blot analysis of E17.5 mouse lung total RNA (lane 1) and total RNA from the mouse lung epithelial cell line MLE-15 (lane 2). The WNT7b transcript is denoted by the black arrow on the right of the blot. B, schematic representation of the 1.0-kb mouse WNT7b promoter, showing the TTF-1, GATA6, and Foxa2 DNA binding sites along with the serial truncations used in transfection assays. The pGL3/Wnt7b-1.0, pGL3/Wnt7b-0.83, pGL3/Wnt7b-0.44, pGL3/Wnt7b-0.12, and pGL3/Wnt7b-0.06 plasmids correspond to arrows A, B, C, D, and E, respectively. The TTF-1 binding sites (T1–T4, white rectangles), Foxa2 DNA binding sites (F1–F4, gray ovals), and GATA6 DNA binding sites (black ovals) used in EMSAs are indicated. C, the full-length 1.0-kb mouse WNT7b promoter (bar A), in addition to the truncated regions (bars B–E), was transfected into MLE-15 cells (black bars) and NIH-3T3 cells (white bars), and the resulting relative luciferase activity from the reporter plasmids was measured after 48 h. All values are the average of transfections performed in duplicate ± S.E.
Trans-activation of the WNT7b Promoter with TTF-1, GATA6, and Foxa2—The data described in Figs. 5–7 show that TTF-1, GATA6, and Foxa2 bind to regions within the WNT7b promoter which confer high level transcriptional activity in lung epithelial cells. Our results suggest that these factors may regulate expression of WNT7b in lung epithelial cells. To test this hypothesis, we asked whether forced expression of TTF-1, GATA6, or Foxa2 could trans-activate the WNT7b promoter in non-lung cells. NIH-3T3 cells were transfected with luciferase reporter plasmids corresponding to different regions of the mouse WNT7b 1.0-kb promoter which contain or lack DNA binding sites for TTF-1, GATA6, or Foxa2, along with expression constructs for each of these transcription factors.

NIH-3T3 cells transfected with the pGL3/Wnt7b-1.0 plasmid and the pCMVTTF-1 expression plasmid resulted in ~20-fold trans-activation compared with the pCMVTag vector alone (Fig. 8A). Interestingly, cells cotransfected with TTF-1 and the pGL3/Wnt7b-0.12 plasmid resulted in greater trans-activation (~50-fold) (Fig. 8A). These data suggest that TTF-1 regulates the WNT7b promoter and that the short minimal 118-bp promoter sequence can be trans-activated by TTF-1. Cotransfection of NIH-3T3 cells with the pcDNA3G6 expression plasmid along with the pGL3/Wnt7b-1.0 reporter plasmid resulted in an 8.5-fold trans-activation of the 1.0-kb mouse WNT7b promoter (Fig. 8B). Cotransfection of the pGL3/Wnt7b-0.44 reporter (which lacks GATA6 DNA binding sites) and pcDNA3G6 expression plasmids showed a significant decrease in this trans-activation (Fig. 8B). Foxa2 trans-activated the pGL3/Wnt7b1.0 reporter plasmid by 14.8-fold, but truncation of the four putative Foxa2 DNA binding sites in the pGL3/Wnt7b-0.44 reporter plasmid abrogated most of this trans-activation (Fig. 8C). Together, these data suggest that TTF-1, GATA6, and Foxa2 regulate WNT7b expression by binding and activating the WNT7b promoter.

Synergistic Activation of the Minimal WNT7b Promoter by TTF-1 and GATA6—The data presented above suggest that TTF-1, GATA6, and Foxa2 can individually regulate the WNT7b promoter. Previous studies have shown that Nkx2.5, a homeodomain transcription factor related to TTF-1, can synergistically regulate cardiac gene transcription via interaction with GATA4 (22, 23). Because lung airway epithelium expresses both TTF-1 and GATA6, we hypothesized that the WNT7b promoter could be regulated by synergistic interactions between these two proteins. To test this hypothesis, NIH-3T3 cells were transfected with TTF-1, GATA6, or a TTF-1/GATA6 combination along with the pGL3/Wnt7b-0.12 reporter plasmid. This reporter plasmid does not contain GATA6 DNA binding sites but does have two TTF-1 binding sites. Therefore, any trans-activation by GATA6 would be mediated through other DNA-binding proteins. To measure synergistic trans-activation, suboptimal amounts of the TTF-1 and GATA6 expression plasmids were used. NIH-3T3 cells transfected with these levels of TTF-1 exhibited only 2.8-fold induction of activity, whereas GATA6-transfected cells exhibited background activity (Fig. 9). However, upon transfection with both TTF-1 and GATA6, the pGL3/Wnt7b-0.12 luciferase reporter construct exhibited 7.8-fold induction in luciferase levels. This synergy was not observed using the pGL3/Wnt7b-0.06 reporter plasmid, which lacks the TTF-1 DNA binding sites (Fig. 9). Furthermore, transcriptional synergy between TTF-1 and Foxa2 was not observed using the pGL3/Wnt7b-0.12 luciferase reporter construct (data not shown). These data suggest that TTF-1 and GATA6 synergistically activate the mouse WNT7b promoter.

FIG. 5. EMSAs of TTF-1 binding to the mouse WNT7b promoter. A, EMSAs were performed with the TTF-1 oligonucleotides corresponding to the four proximal TTF-1 DNA binding sites (indicated as T1–T4) in the WNT7b promoter and nuclear extracts from either untransfected HEK-293 cells (293 ext.) or HEK-293 cells transfected with a TTF-1 expression plasmid (293/TTF-1 ext.) and analyzed on 4% polyacrylamide gels. The brackets on the right of each panel indicate the TTF-1-specific shifted bands; arrows on the right indicate the supershifted bands specific to the TTF-1 antiserum (TTF-1 Ab) and not observed with non-immune antiserum (non-immune Ab). B, EMSAs were performed with MLE-15 cell nuclear extracts and either the T2 or T3 oligonucleotides and analyzed on 5% polyacrylamide gels. Cold competitor oligonucleotides corresponding to either the Foxa2 F1 site (lanes 18 and 21) or the human SP-B TTF-1 binding sites (lanes 19 and 22) were added at a 100-fold molar excess to the labeled oligonucleotides. The arrow denotes the TTF-1-specific band that is competed by the hSP-B/TTF-1 but not the Foxa2 F1 oligonucleotide.

binds to sites F2 and F4, but only weak binding is observed with F1. MLE-15 cell nuclear extracts did not produce noticeable band shifts with any of the above Foxa2 oligonucleotides, which correlates with the lack of detectable Foxa2 protein as determined by Western blot analysis (data not shown).
GATA6 and TTF-1 Physically Interact in Vivo—Synergistic activation of the 118-bp WNT7b promoter suggests that GATA6 and TTF-1 interact in vivo to regulate transcription. To determine whether GATA6 and TTF-1 could physically interact in vivo, coimmunoprecipitation assays were performed using HEK-293 cells transfected with Myc-tagged GATA6 and FLAG-

**FIG. 6.** EMSA of GATA6 binding to the mouse WNT7b promoter. A. EMSAs were performed with the GATA6 wild-type oligonucleotide spanning both putative GATA6 DNA binding sites (S1/S2) in the WNT7b promoter and nuclear extracts from HEK-293 cells transfected with a GATA6 expression plasmid (293/GATA6 ext.). The arrow on the left indicates the GATA6-specific shifted band; white arrows on the right indicate the supershifted bands specific to the GATA6 antisemum (anti-GATA6 Ab) and not observed with preimmune antiserum (preimmune Ab). B, competition of the GATA6 binding activity with mutant oligonucleotides to determine which site(s) GATA6 binds in the GATA6 wild-type oligonucleotide. Nuclear extracts from either untransfected HEK-293 cells (293 ext.) or cells transfected with a GATA6 expression plasmid (293/GATA6 ext.) were used as indicated. Competition was performed with the unlabeled GATA6 wild-type oligonucleotide (S1/S2), GATA6 oligonucleotides with mutations in either site 1 (mS1) or site 2 (mS2), or mutations in both site 1 and 2 (mS1/2). The arrow on the right of the gel indicates the GATA6-specific band. The asterisk indicates the comigrating background band in panels A and B which is present in the untransfected HEK-293 nuclear extracts and is likely caused by an endogenous GATA factor expressed in HEK-293 cells. C, EMSAs performed with MLE-15 cell nuclear extracts and the S1/S2 oligonucleotide. The asterisk marks the GATA6-specific band that is supershifted by the GATA6 antisemum (arrow) but not by the preimmune serum.

**FIG. 7.** EMSAs of Foxa2 binding to the WNT7b promoter. EMSAs were performed with the Foxa2 oligonucleotide corresponding to the four putative Foxa2 DNA binding sites (indicated as F1–F4) in the WNT7b promoter and nuclear extracts from either untransfected HEK-293 cells (293 ext.) or HEK-293 cells transfected with a Foxa2 expression plasmid (293/Foxa2 ext.). Solid arrows on the right of each panel indicate the Foxa2-specific shifted bands; dashed arrows on the right indicate the supershifted bands specific to the Foxa2 antisemum (Foxa2 Ab) and not observed with non-immune antiserum (non-immune Ab). Note that oligonucleotide F3 did not bind Foxa2. The faint band observed using the F1 oligonucleotide and the 293/Foxa2 nuclear extracts is denoted by the black arrow on the right of the first panel.
tagged TTF-1. As shown in Fig. 10, TTF-1 was coimmunoprecipitated with GATA6. These data are the first to show an in vivo physical interaction between GATA6 and TTF-1 and suggest a protein-protein interaction mechanism for GATA6/TTF-1 regulation of the WNT7b promoter.

**DISCUSSION**

WNT7b is expressed in the lung throughout embryonic development and is the only WNT gene described whose expression is restricted to the pulmonary epithelium (Ref. 24 and this report). In this paper, we have characterized the proximal mouse 1.0-kb WNT7b promoter to understand how its precise pattern of expression is regulated in lung epithelium. This promoter region is highly active in the lung epithelial cell line MLE-15. We show that the proximal 1.0 kb of the WNT7b promoter contains binding sites for TTF-1, GATA6, and Foxa2, three important transcription factors implicated in the regulation of lung epithelium-specific gene expression. Finally, we provide data suggesting that the mouse WNT7b gene is regulated in a synergistic manner by TTF-1 and GATA6.

WNT proteins have been shown to regulate various develop-
mental and cellular differentiation processes (2). To understand what role these signaling molecules play during lung development, we surveyed the expression pattern of the 18 known mouse WNT genes in the embryonic lung. We found that WNT7b is the only WNT gene of those tested which is expressed exclusively in airway epithelium during lung development. This result suggests that expression of WNT7b in lung epithelium is regulated in a cell lineage-specific manner. The proximal 1.0 kb of the mouse WNT7b promoter is highly active in the lung epithelial cell line MLE-15, and the proximal 444 bp of the WNT7b promoter was sufficient to produce maximal gene transcription in MLE-15 cells. Analysis of the sequence of the mouse 1.0-kb WNT7b promoter revealed binding sites for several transcription factors known to be important regulators of gene transcription in the lung including TFF-1, GATA6, and Foxa2. TFF-1 is expressed throughout the epithelium from the initiation of lung development through late gestational development where expression is observed in both conducting airway epithelium (Clara cells) and distal epithelial type 2 epithelial (AEC-2) cells (25). TFF-1 DNA binding sites are found in the promoter regions of most lung-specific genes, and TFF-1 has been shown to trans-activate several of these including SP-A, SP-B, and SP-C and the Clara cell 10 kDa protein (CC10) (4, 6, 18, 19, 26). Mice homozygous for a null allele of TFF-1 exhibit defects in lung epithelial development including attenuated airway branching resulting in severely hypoplastic lungs (27). In addition, TFF-1 null mice lack expression of the AEC-2-specific marker gene surfactant protein C, suggesting defects in epithelial cell differentiation (28). Our finding of nine putative TFF-1 DNA binding sites within the proximal 1.0-kb WNT7b promoter suggested that TFF-1 plays a key role in the regulation of WNT7b gene expression in airway epithelium. Indeed, the minimal 118-bp mouse WNT7b promoter, which still retains significant activity in the mouse lung epithelial cell line MLE-15, contains two TFF-1 consensus DNA binding sites, and our results show that this minimal promoter can be trans-activated by forced expression of TFF-1 in NIH-3T3 cells. Thus, TFF-1 likely plays an important role in regulating the gene expression of WNT7b in lung epithelium.

GATA6 has also been demonstrated to play an important role in gene expression and development in lung epithelium. GATA6 expression is observed primarily in cardiac, lung, and intestinal tissues during development (8, 29). In lung epithelium, GATA6 is expressed from as early as E10.5 through gestational development where expression is observed in the pulmonary epithelium under certain experimental conditions (13). Morrisey et al. (1998) Genes Dev. 12, 3579–3590. They found that WNT7b gene expression may similarly require the contribution of GATA6 in vivo to regulate the WNT7b promoter provides important clues toward the understanding of this regulation.

REFERENCES
1. Warburton, D., Schwarzen, M., Telft, D., Flores-Delgado, G., Anderson, K. D., and Cardoso, W. V. (2000) Mech. Dev. 92, 55–81.
2. Wodaar, A., and Nasse, R. (1998) Annu. Rev. Cell Dev. Biol. 14, 59–88.
3. Mendelson, C. R. (2000) Annu. Rev. Physiol. 62, 875–915.
4. Marzana, R. K., and Boggaram, V. (1997) J. Biol. Chem. 272, 3083–3090.
5. Sawaya, P. L., Stripp, B. R., Whitsett, J. A., and Luse, D. S. (1999) Mol. Cell. Biol. 13, 3860–3871.
6. Bohinski, R. J., Di Lauro, R., and Whitsett, J. A. (1994) Mol. Biol. Cell. 14, 5671–5681.
7. Braun, H., and Suske, G. (1998) J. Biol. Chem. 273, 9821–9828.
8. Morrisey, E. E., Ip, H. S., Lu, M. M., and Parmacek, M. S. (1996) Dev. Biol. 177, 309–322.
9. Reddy, S. Andl, T., Bagasa, A., Lu, M. M., Epstein, D. J., Morrisey, E. E., and Millar, S. E. (2001) Proc. Natl. Acad. Sci. USA 98, 13249–13254.
10. Gavin, B. J., McMahon, J. A., and McMahon, A. P. (1990) Genes Dev. 4, 2319–2332.
11. Kaestner, K. H., Friemisch, H., Luckow, B., and Schutz, G. (1994) Genomics 20, 377–385.
12. Ip, H. S., Wilson, D. B., Heikinheimo, M., Zhang, T., Ting, C. N., Simon, M. C., Leiden, J. M., and Parmacek, M. S. (1994) Mol. Biol. Cell. 14, 7517–7526.
13. Morrisey, E. E., Tang, Z., Sigrist, K. L., Lu, M. M., Jiang, F., Ip, H. S., and Parmacek, M. S. (1998) Genes Dev. 12, 3579–3590.
14. Shu, W., Yang, H., Zheng, L., Lu, M. M., and Morrisey, E. K. (2001) J. Biol. Chem. 276, 27488–27497.
15. Monkley, S. J., Delaney, S. J., Pennisi, D. J., Christiansen, J. H., and Wainwright, B. J. (1996) Development 122, 3343–3353.
16. Lake, M., Strachan, R., Mall, K., Wilson, D. I., Rohson, S. C., and Lindsay, S. (1998) Gene (Amst.) 219, 101–110.
17. Bruno, M. D., Kerbagen, T. R., Liu, C., Morrisey, E. E., and Whitsett, J. A. (2000) J. Biol. Chem. 275, 1043–1049.
18. Bruno, M. D., Bohinski, R. J., Hueslman, K. M., Whitsett, J. A., and Kerbagen, T. R. (1995) J. Biol. Chem. 270, 6531–6536.
19. Glasser, S. W., Burzhus, M. S., Eszterhas, S. K., Bruno, M. D., and Kerbagen, T. R. (2000) Am. J. Physiol. 278, L933–L945.
20. Shaw-White, J. R., Bruno, M. D., and Whitsett, J. A. (1999) J. Biol. Chem. 274, 2658–2664.
21. Ikeda, K., Shaw-White, J. R., Wett, S. E., and Whitsett, J. A. (1996) Mol. Cell. Biol. 16, 3626–3636.
22. Durocher, D., Charron, F., Warren, R., Schwartz, R. J., and Nemer, M. (1997) EMBO J. 16, 5687–5696.
23. Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., and...
24. Pepicelli, C. V., Lewis, P. M., and McMahon, A. P. (1998) *Curr. Biol.* 8, 1083–1086
25. Zhou, L., Lim, L., Costa, R. H., and Whitsett, J. A. (1996) *J. Histochem. Cytochem.* 44, 1183–1193
26. Kelly, S. E., Bachurski, C. J., Burhans, M. S., and Glasser, S. W. (1996) *J. Biol. Chem.* 271, 6881–6888
27. Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M., and Gonzalez, F. J. (1996) *Genes Dev.* 10, 60–69
28. Minoo, P., Su, G., Drum, H., Bringas, P., and Kimura, S. (1999) *Dev. Biol.* 209, 60–71
29. Morrissey, E. E., Ip, H. S., Tang, Z., Lu, M. M., and Parmacek, M. S. (1997) *Dev. Biol.* 183, 21–36
30. Keijzer, R., van Tuyl, M., Meijers, C., Post, M., Tibboel, D., Grosveld, F., and Koutsourakis, M. (2001) *Development* 128, 503–511
31. Yan, C., Sever, Z., and Whitsett, J. A. (1995) *J. Biol. Chem.* 270, 24852–24857

TTF-1, GATA6, and Foxa2 Regulate the WNT7b promoter