Epithelial gene expression in the lung is thought to be regulated by the coordinate activity of several different families of transcription factors including the Fox family of winged-helix/forkhead DNA-binding proteins. In this report, we have identified and characterized two members of this Fox gene family, Foxp1 and Foxp2, and show that they comprise a new subfamily of Fox genes expressed in the lung. Foxp1 and Foxp2 are expressed at high levels in the lung as early as E12.5 of mouse development with Foxp2 expression restricted to the airway epithelium. In addition, Foxp1 and Foxp2 are expressed at lower levels in neural, intestinal, and cardiovascular tissues during development. Upon differentiation of the airway epithelium along the proximal-distal axis, Foxp2 expression becomes restricted to the distal alveolar epithelium whereas Foxp1 expression is observed in the distal epithelium and mesenchyme. Foxp1 and Foxp2 can regulate epithelial lung gene transcription as was demonstrated by their ability to dramatically repress the mouse CC10 promoter and, to a lesser extent, the human surfactant protein C promoter. In addition, GAL4 fusion proteins encoding subdomains of Foxp1 and Foxp2 demonstrate that an independent and homologous transcriptional repression domain lies within the N-terminal end of the proteins. Together, these studies suggest that Foxp1 and Foxp2 are important regulators of lung epithelial gene transcription.

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The mouse lung arises from the laryngo-tracheal groove in the primitive foregut at approximately gestational day 9.5 (E9.5) of mouse development (for review see Refs. 1 and 2). Further development through a process termed branching morphogenesis results in a primitive epithelial lined tubular structure by E12.5. Additional differentiation of this primitive epithelial lining along the proximal-distal axis during the pseudoglandular stage of development results in highly differentiated airway epithelial cells capable of surfactant protein expression and gas exchange essential for postnatal lung function. The molecular mechanisms regulating the process of branching morphogenesis and proximal-distal patterning of the lung epithelium are poorly understood. However, recent reports have indicated that lung-specific gene expression is regulated at the level of transcription (reviewed in Refs. 1 and 2). Several transcription factors have been implicated in this transcriptional program including the homeodomain protein Nkx2.1/TTF-1, the zinc-finger transcription factor GATA-6, and members of the winged-helix/forkhead (Fox) family of transcription factors (3–10).

The Fox family of transcription factors is a large group of proteins that share a common DNA binding domain termed a winged-helix or forkhead domain. Upon the founding member of this group, the fox gene in Drosophila (for review see Ref. 11). Several Fox genes are expressed in the lung and have been implicated as important regulators of lung gene transcription including Foxa1, Foxa2, Foxf1, Foxf2, and Foxj1. These Fox family members are expressed in a variety of different lung cell lineages including ciliated cells of the upper respiratory tract (Foxj1), lung mesenchyme (Foxf1 and Foxf2), and airway epithelium (Foxa1 and Foxa2) (12–15). Several of these Fox genes have been shown to regulate multiple lung-specific promoters including the human surfactant protein B (SP-B) promoter, the mouse and rat CC10 promoters, and the Nkx2.1/TTF-1 promoter (4, 6, 8, 16, 17).

In this report, we describe the isolation and characterization of two cDNAs encoding Foxp1 and Foxp2 that define a new subfamily of winged-helix/forkhead DNA binding domain transcription factors. We show that both Foxp1 and Foxp2 are expressed at high levels in embryonic and adult mouse lung with Foxp2 expression in the lung restricted to the distal epithelium. In addition, Foxp1 and Foxp2 are expressed in defined neural, intestinal, and cardiovascular cell types during embryogenesis. Both Foxp1 and Foxp2 contain a divergent winged-helix domain that is significantly different from that of other Fox transcription factors expressed in the lung. Foxp1 and Foxp2 share a high level of protein similarity within the winged-helix/forkhead DNA binding domain and in regions N- and C-terminal to this domain. Foxp1 and Foxp2 are expressed as at least three different mRNA messages. We show that Foxp1 and Foxp2 act as transcriptional repressors that are able to repress the mouse CC10 and human SP-C promoters to differing degrees. Moreover, we show that Foxp1 and Foxp2 contain an independent and homologous transcriptional repression domain that contains a novel zinc-finger motif that is located N-terminal to the winged-helix/forkhead DNA binding domain. Together, these data suggest that the Foxp1 and Foxp2 subfamily of transcriptional regulators play an important role in spatially restricting the expression of certain target genes such as CC10 in the lung and, in turn, regulate the

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The abbreviations used are: SP, surfactant protein; bp, base pair(s); RACE, rapid amplification of cDNA ends; aa, amino acid(s); kb, kilobase; PCR, polymerase chain reaction.

1 The abbreviations used are: SP, surfactant protein; bp, base pair(s); RACE, rapid amplification of cDNA ends; aa, amino acid(s); kb, kilobase; PCR, polymerase chain reaction.
proximal versus distal epithelial cell differentiation process in mid to late lung development.

MATERIALS AND METHODS

Isolation of Foxp1 and Foxp2 cDNAs—An adult mouse lung cDNA library (Stratagene) was screened with a cDNA probe corresponding to the forkhead DNA binding domain of the mouse hepatic nuclear factor-3β (Foxa2) cDNA (bp 590–875; GenBank accession number X74937) under low stringency (2× SSC, 0.1% SDS, 57 °C). Of the 32 clones obtained, all but 5 corresponded to known forkhead DNA-binding proteins. Of these 5 clones, 4 corresponded to Foxp1 and 1 to Foxp2. To obtain full-length Foxp1 and Foxp2 cDNAs, the same lung cDNA library was rescreened with the 5’ ends of the partial cDNAs derived from the low stringency screen. Several clones for both Foxp1 and Foxp2 were obtained from these additional screens. Multiple cDNA clones for Foxp1 and Foxp2 were sequenced in both sense and antisense directions. The full-length open reading frames of Foxp1 and Foxp2 were cloned into the pCMVTag2B vector (Stratagene) at the EcoRI and XhoI sites to produce the pCMV/Foxp1 and pCMV/Foxp2 constructs. 5’ and 3’ rapid amplification of cDNA ends (RACE) was performed on 2.9 and 3.9 primers: sense, 5’-GTCCTAGACCCAGATTCTTG-3’; RACE-nested, 5’-GTCCTGCAGCATGTTGGC-3’; Foxp1 3’ RACE-nested, 5’-CCGAGAACACAGGAGGAGAG-3’; Foxp1 5’ RACE, 5’-GTTAGAAGATGACAGAGAGATTGAG-3’; Foxp2 5’ RACE, 5’-GTTAGAAGATGACAGAGAGATTGAG-3’; Foxp2 3’ RACE-nested, 5’-GAGCCCTTATCTCAGGGAGGTCC-3’.

Northern Blot and in Situ Hybridization Analysis—Northern blot analysis of adult tissue total RNA was performed essentially as described (18). Approximately 20 μg of total RNA from the indicated tissues was resolved on a 1.0% formaldehyde-agarose gel, blotted to a Hybond membrane (Amersham Pharmacia Biotech), and probed with [32P]dCTP-labeled probes for Foxp1 (encoding aa 597–705 of isoform A representing the 3’ coding region called probe A) and Foxp2 (encoding aa 495–583 of isoform A representing the 3’ coding region called probe B). The Northern blot analysis was performed essentially as described using 32P-labeled sense and antisense riboprobes corresponding to the same cDNA sequence as the Northern probes (19). Alternative spliced region-specific riboprobes and Northern blot probes representing the spliced isoforms of Foxp1 were generated by PCR that correspond to the unique 5’ untranslated region of isoform C (probe B, bp 56–459; GenBank accession number AF339105) and the C-terminal region of the winged-helix/forkhead DNA binding domain (probe C, encoding aa 539–602 of isoform A).

Plasmid Construction, Cell Culture, and Co-transfection Assays—The pG2/SP-C construct was generated by ligating the 3.7-kb human SP-C promoter (20) into the HindIII site of pG2basic (Promega). The pGL2/CC10 construct was generated by PCR amplifying the ~804-bp mouse mouse CC10 promoter from mouse genomic DNA using the following primers: sense, 5’-CCGATCCGGTAAGGCCTGGGAATGGCTAAC-3’; antisense, 5’-CCCTGAGGGGTTATGTTGGTGTGGTGCTGCC-3’; and cloning the resulting DNA fragment into the pKpnI and XhoI sites of pG2basic.

NIH-3T3 and H441 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. MLE-15 cells were cultured as described previously (21). MLE-15 cells were transfected with 0.5 μg of the pGL2/SP-C vector, 2.5 μg of pCMV/Foxp1 or Foxp2 expression vectors, and 0.5 μg of the pMSVgal control vector using Fugene (Roche Molecular Biochemicals). H441 cells were transfected with 2.5 μg of pGL2/CC10, 12.5 μg of pCMV/Foxp1 or pCMV/Foxp2, and 1 μg of pMSVgal, along with Lipofectin (Life Technologies, Inc.). 48 h after transfection, cells were harvested and analyzed for luciferase and β-galactosidase activity using commercially available kits (Promega). The β-galactosidase values were used to normalize for transcription efficiency. All experiments were performed in triplicate, and data shown are mean ± S.E.

To identify putative repression domains in Foxp1 and Foxp2, a series of expression plasmids containing GAL4/Foxp1 or GAL4/Foxp2 chimera proteins were generated by PCR amplifying the open reading frame, which contains the 147-amino acid GALA DNA binding domain (22). Thus, the pGAL4/P1aa1–250, pGAL4/P1aa251–490, pGAL4/P1aa491–602, and pGAL4/P1aa603–705 represent subdomains of the Foxp1 protein whereas pGAL4/P2aa1–259, pGAL4/P2aa260–500, pGAL4/P2aa501–613, and pGAL4/P2aa614–714 represent subdomains of Foxp2 fused to the GAL4 DNA binding domain. NIH-3T3 cells were transfected with 0.5 μg of the pGAL4SV40.luc reporter vector, which contains four copies of the GALA DNA binding sequence upstream of the highly active SV40 viral promoter (23), 2.5 μg of the pGAL expression construct, and 0.5 μg of the pMSVgal reference plasmid using Fugene 6. Luciferase and β-galactosidase assays were performed as described above.

RESULTS

Cloning and Sequence Comparison of Foxp1 and Foxp2—To screen for new Fox family members in the mouse lung, low-stringency hybridization utilizing the Foxa2 forkhead DNA binding domain was performed on a mouse lung cDNA library. 32 different clones were isolated and sequenced. All except five corresponded to previously described winged-helix/forkhead genes including Foxa1, Foxa2, Foxf1, Foxf2, and Foxj1 (data not shown). The other five cDNA clones represented two distinct but highly related cDNAs. Four cDNA clones were identical to the previously described partial Foxp1 cDNA whereas the other clone belonged to a related but distinct gene now designated Foxp2. Analysis of the 5’ ends of the Foxp1 and Foxp2 cDNAs revealed that none of the cDNAs contained a consensus initiation codon suggesting that they were not full-length. Therefore, the lung cDNA library was rescreened with the 5’-most regions of the Foxp1 and Foxp2 cDNAs, and several additional cDNAs for both genes were obtained that contained consensus initiation codons. In addition, 5’ and 3’ rapid amplification of cDNA ends was used to isolate additional sequences in the 5’ and 3’ untranslated regions. A combination of a cDNA library screening and 5’ and 3’ RACE revealed three distinct types of Foxp1 cDNAs, with the longest, encoding an open reading frame of 705 amino acids, which contains a glutamine-rich region (aa 55–230), a putative zinc-finger domain (aa 336–359), and a winged-helix/forkhead DNA binding domain (aa 485–573) (Fig. 1A). The longest Foxp2 cDNA contained an open reading frame of 714 amino acids that also had a glutamine-rich region (aa 102–230), a putative zinc-finger domain (aa 347–370), and a winged-helix/forkhead DNA binding domain at the C terminus (aa 495–583) (Fig. 1A). Comparison of the protein sequences of Foxp1 and Foxp2 demonstrated 62% amino acid identity between the two proteins with the highest level of identity located in the conserved winged-helix/forkhead DNA binding domain (Fig. 1A).

As stated above, three distinct types of Foxp1 cDNA clones were identified (Fig. 1B). The Foxp1A cDNAs contained an open reading frame of 705 amino acids with the long glutamine-rich (Q-rich) region at the N-terminal end (Fig. 1B, A). Of note, glutamine-rich regions such as these have been implicated as transcriptional activation/repression domains in other transcription factors (24–27). The Foxp1B cDNAs contained the glutamine-rich region but lacked the C-terminal portion of the winged-helix/forkhead DNA binding domain between aa 539 and 602 (Fig. 1B, B). Finally, Foxp1C cDNAs contained an alternative but longer 5’ untranslated region and alternative initiation codon resulting in an N-terminally truncated Foxp1 protein (Fig. 1, B and C). Of note, reverse transcriptase PCR analysis of adult lung cDNA confirms the expression of the three different Foxp1 isoforms (data not shown). Interestingly, all of the Foxp2 cDNA clones isolated from the lung library corresponded to the structure of Foxp1A. To determine how similar Foxp1 and Foxp2 were to other Fox genes expressed in the lung, an alignment of the proteins sequences containing the forkhead/winged-helix DNA binding domains of Foxp1 and Foxp2 was performed to those of Fox2, Fox5, and Foxj1 (Fig. 2A). This analysis shows that although Foxp1 and Foxp2 are similar to other Fox genes expressed in the lung, they comprise a distinct subfamily of Fox transcription factors as judged by their amino acid sequence.

To determine whether Foxp1 and Foxp2 were conserved through evolution, we performed a computer-assisted search of
the *Drosophila melanogaster* genomic database for possible orthologues. This search revealed a single orthologue (GenBank™ accession number AE003684), which contains a highly related forkhead DNA binding domain (Fig. 2B). These data suggest that Foxp1 and Foxp2 comprise an ancient subfamily of winged-helix/forkhead DNA binding transcription factors.

**FIG. 1.** Amino acid sequence of Foxp1 and Foxp2 and isoform structure of Foxp1. A, amino acid alignment of Foxp1 and Foxp2 using the Clustal W system (MacVector Software; Oxford Molecular, Inc.). The solid underlined region represents the winged-helix/forkhead DNA binding domain, and the dashed underlined region represents the putative zinc-finger domain. The region between the arrowheads demarcates the coding region missing from isoform B of Foxp1. The asterisk indicates the start methionine of isoform C of Foxp1. B, schematic diagram of Foxp1 isoforms indicating the glutamine-rich (Q-rich) region, the putative zinc-finger domain (Zn), and the winged-helix/forkhead DNA binding domain (forkhead). The different probes used in Northern blot and in situ hybridization analysis are indicated below the diagram. Of note, the combined sequences from the lung cDNA library and 5′ and 3′ RACE procedures for the three different Foxp1 isoform cDNAs generate similar sized transcripts (Foxp1A = 3.0 kb, Foxp1B = 2.8 kb, Foxp1C = 2.8 kb) with all three having identical 3′ untranslated sequences.
Postnatal Expression Analysis of Foxp1 and Foxp2—Northern blot analysis was performed to determine the complexity and pattern of expression of Foxp1 and Foxp2 in adult mouse tissues. The Foxp1 3′ coding region cDNA probe (Fig. 1B, probe A) hybridized to three different transcripts of ~7.5, 3.0, and 1.8 kb (Fig. 3A, arrows). The highest level of expression was observed in the lung, brain, and spleen with lower expression observed in other tissues including heart, skeletal muscle, kidney, small intestine, and liver. All three messages were expressed in similar proportion in all tissues with the exception of heart and brain, which appeared to contain lower levels of the 1.8-kb transcript and higher levels of the 7.5-kb transcript, respectively. A probe specific for the Foxp1C isoform (Fig. 1B, probe B) hybridized to the same three transcripts in all Foxp1-expressing tissues except small intestine (Fig. 3B). However, decreased hybridization to the 1.8-kb transcript was observed with this probe whereas an increase in the intensity of the 7.5-kb band was observed with liver RNA (Fig. 3B). These data show that the Foxp1C isoform is expressed in all Foxp1-expressing adult tissues except the small intestine. In addition, probes A, B, and C, which encompass the C-terminal portion of the winged-helix/forkhead domain, which is deleted in Foxp1B (Fig. 1B, probe C), did not exhibit differential hybridization patterns during embryonic development when analyzed by in situ hybridization analysis (data not shown). Of note, only probe B is specific for a single isolated Foxp1 isoform (Foxp1C) whereas probes A and C can hybridize to at least two Foxp1 isoforms. Hybridization of the same Northern blot with a probe derived from the 3′ coding region of the Foxp2 cDNA demonstrated that the Foxp2 gene is expressed as three transcripts of ~9.0, 3.5, and 2.0 kb (Fig. 3C). As with Foxp1, the highest level of Foxp2 mRNA expression was observed in the lung with lower expression levels observed in spleen, small intestine, skeletal muscle, brain, and kidney. Kidney and small intestine appeared to express lower levels of the 9.0-kb transcript, whereas brain expressed higher levels of this Foxp2 transcript.

The original Foxp1A, B, and C cDNAs isolated from the lung cDNA library were 2.2, 2.0, and 2.0 kb in size, respectively, and they all contained an identical 38-base pair 3′ untranslated sequence (data not shown). Addition of 5′ and 3′ sequences isolated using RACE procedures increased the overall size of the known Foxp1A, B, and C transcripts to 3.0, 2.8, and 2.8 kb (data not shown). The data obtained with both probe A and probe B suggests that these transcripts correlate in size to the 3.0-kb band observed on the adult tissue Northern blot (Fig. 3). The longer 7.5-kb band observed with these probes is likely because of an alternative polyadenylation site. It is also likely that the ~1.8-kb band on the Northern blot corresponds to the same Foxp1A, B, and C coding regions described above but with the much shorter 38-base pair 3′ untranslated sequence that was present in all of the Foxp1 cDNAs isolated in the cDNA library screen. Thus, the similarity in size of the different Foxp1 isoforms may preclude the identification of differential hybridization in Northern blot analysis using probes A and B.

The genomic locus for Foxp1 is highly complex and consists of at least nine exons extending over more than 150 kilobases. Reverse transcriptase PCR analysis of lung cDNA and comparison to the deduced genomic structure of Foxp1 reveal that sequences deleted in Foxp1B correspond to the loss of two exons in the Foxp1 transcript whereas the alternate 5′ untranslated region and translational start site for Foxp1C are the result of a splicing event 27 base pairs upstream of the initiating new start codon in Foxp1C. Taken together, these
data suggest that Foxp1 is spliced in a complex manner and that full characterization of the Foxp1 genomic locus may be required to resolve the complex transcript pattern of Foxp1.

Expression Pattern of Foxp1 and Foxp2 during Embryonic Development—To characterize the expression pattern of Foxp1 and Foxp2 during embryonic development, in situ hybridization was performed on staged mouse embryos at days E12.5, E14.5, and E16.5 using radiolabeled riboprobes derived from the Foxp1 or Foxp2 cDNA sequences, respectively. During gestation in the mouse, the highest levels of Foxp1 and Foxp2 expression are observed in the developing lung. Additional sites of expression include neural, gastrointestinal, and cardiovascular tissues. In the lung, Foxp1 and Foxp2 are both expressed in the airway epithelium starting at E12.5 (Fig. 4, A and D, arrowheads). Expression of Foxp1 in the lung continues through E16.5 both in the epithelium and in the surrounding mesenchyme (Fig. 4, B and C). In contrast, Foxp2 expression is restricted to the airway epithelium from E12.5 through E16.5 (Fig. 4, D–F). Coincident with the differentiation of the pulmonary epithelium along the proximal-distal axis, which produces distinct epithelial cell lineages capable of gas exchange and surfactant protein expression, Foxp2 expression becomes restricted to the distal alveolar epithelium and is no longer expressed in the proximal airways (Fig. 4F, arrowhead). Of note, this pattern of expression is similar to the zinc-finger transcription factor GATA-6 and of surfactant proteins A and C (14, 19, 28). To our knowledge, this is the first report of a Fox gene that is expressed exclusively within the distal epithelium of the lung during pulmonary development. Together, these data suggest that Foxp1 and/or Foxp2 may play important roles in developing neural, gastrointestinal, and cardiovascular tissues.

Expression of Foxp1 and Foxp2 is also observed within the developing nervous system. In the spinal cord at E12.5, Foxp1 expression is observed in the developing motor neurons in a pattern reminiscent of Hoxa-2 and islet-1 (Fig. 5A, yellow arrowhead) (29–31). Foxp1 and Foxp2 are also expressed in a subset of interneurons dorsal to the motor neurons in a pattern reminiscent of Hoxa-2 and islet-1 (Fig. 5, A and C, red arrowhead) (32). At E16.5, expression of Foxp1 and Foxp2 in the brain is observed in the neopallial cortex where the future cerebral cortex develops. Foxp1 is expressed in the outer cortical plate (Fig. 5B, black arrow) whereas Foxp2 expression is observed in the inner intermediate zone of the neopallial cortex (Fig. 5D, white arrow). Foxp1 and Foxp2 expression is also observed in the developing cerebral hemispheres (Fig. 5, B and D, asterisk). In the gastrointestinal system, Foxp1 is expressed in the mesodermal layer (Fig. 6, A and B, white arrowhead), as well as the epithelial layer of the developing intestine (Fig. 6, A and B, yellow arrowhead). Foxp2 expression is observed in the outer mesodermal layer but is absent from the developing epithelium of the intestine (Fig. 6, C and D). Although Foxp1 is expressed in the adult heart (Fig. 3A), only Foxp2 was expressed in the developing cardiovascular system during embryogenesis. At E14.5, expression of Foxp2 is observed in the outflow tract region of the developing heart (Fig. 7A). By E16.5, Foxp2 expression is observed in the outflow tract and the atrium of the heart but not the ventricles (Fig. 7B, bracket). These data suggest that Foxp1 and Foxp2 may play important roles in developing neural, gastrointestinal, and cardiovascular tissues.

**Fig. 3. Northern blot analysis of Foxp1 and Foxp2 expression in adult tissues.** Northern blot analysis was performed on ~20 μg of total RNA extracted from adult mouse tissues. Lane 1, heart; lane 2, lung; lane 3, kidney; lane 4, skeletal muscle; lane 5, spleen; lane 6, small intestine; lane 7, brain; lane 8, liver. A, Foxp1 3' coding region probe showing hybridization to 7.5-, 3.0-, and 1.8-kb transcripts. B, Northern blot showing hybridization of the unique 5' untranslated region from Foxp1C to the 7.5-, 3.0-, and 1.8-kb transcripts. Notice the decreased hybridization to the 1.8-kb transcript and the lack of hybridization to small intestine RNA. C, Foxp2 3' coding region probe showing hybridization to 9.0-, 3.5-, and 2.0-kb transcripts. D, ethidium bromide stain of agarose gel prior to transfer showing equal loading of RNA. Molecular mass markers, in kilobases, are indicated to the left of each blot. Arrows indicate the position of the three transcripts for Foxp1 and Foxp2.

**Fig. 4. Expression of Foxp1 and Foxp2 in the mouse lung at E12.5, E14.5, and E16.5.** Radioactive in situ hybridization was performed on E12.5 (A and D), E14.5 (B and E), and E16.5 (C and F) mouse embryos using gene-specific riboprobes for Foxp1 (A–C) and Foxp2 (D–F). Expression in the airway epithelium of the lung is observed for both Foxp1 and Foxp2 at E12.5 (A and D, arrowheads). Expression of Foxp1 continues in the epithelium but is also observed in the surrounding mesenchyme (B and C). Foxp2 expression is restricted to the airway epithelium and by E16.5, and expression is further restricted to the distal epithelium and is absent from the proximal epithelium (F, arrowhead). Magnification is as follows: × 400 (A and D), × 200 (B and E), and × 100 (C and F).
Foxp1 and Foxp2 Repress Epithelial Gene Expression in the Lung—Because both Foxp1 and Foxp2 are expressed at high levels within the developing airway epithelium of the lung, we tested whether each factor could regulate expression of the lung epithelial-specific promoters from the human SP-C gene and the mouse CC10 gene. The surfactant protein C gene is expressed in distal type II pneumocytes in the lung, and the 3.7-kb human SP-C promoter directs expression of transgenes in these cells in the mouse (20). The mouse CC10 gene is expressed exclusively in non-ciliated Clara epithelial cells in the upper airways of the lung, and the 804-bp mouse promoter has been shown to confer expression in these same cells in transgenic mice (33). Thus, these two promoters represent distinct cell populations in the lung that differentiate from the early pluripotential airway epithelium in the late pseudoglandular stage of lung development. Of note, the 804-bp mouse CC10 promoter contains two well characterized Fox binding sequences whereas the 1.2-kb proximal region of the 3.7-kb human SP-C promoter contains at least two consensus Fox binding sequences (see Ref. 34, and data not shown). Both promoters were cloned into the pgL2basic luciferase reporter vector and co-transfected along with expression constructs for Foxp1 and Foxp2 into MLE-15 cells (SP-C promoter) or H441 cells (CC10 promoter). MLE-15 cells are a mouse lung epithelial cell line representative of type II pneumocytes and are known to express SP-C whereas H441 cells are a human lung epithelial cell line representative of Clara epithelial cells, which express the CC10 gene (4, 21). Co-transfection of the pgL2/CC10 reporter construct, along with the pCMV vectors harboring the Foxp1 and Foxp2 cDNAs into H441 cells, results in a dramatic (greater than 80%) decrease in luciferase activity (Fig. 8A). Interestingly, co-transfection of either Foxp1 or Foxp2 results in a more moderate decrease in SP-C promoter activity in H441 cells of 40–50% (Fig. 8B). Of note, expression of Foxp1 or Foxp2 did not affect the expression of β-galactosidase from the pMSVβgal reference plasmid (data not shown), suggesting that the observed repression of the CC10 and SP-C promoters is specific. These results show that Foxp1 and Foxp2 repress the mouse CC10 and human SP-C promoters suggesting that these Fox family members may restrict expression of certain genes in the epithelium of the lung during development.

Identification of an Independent Repression Domain in the N Terminus of Foxp1 and Foxp2—To characterize the region within Foxp1 and Foxp2 that is responsible for the transcriptional repression activity observed on the CC10 and SP-C promoters, a series of expression plasmids encoding chimeric proteins containing the 147-amino acid yeast GAL4 DNA binding domain fused to regions of the Foxp1 and Foxp2 proteins were generated. These subdomains of Foxp1 and Foxp2 contained several of the identified motifs found in these proteins including the glutamine-rich region (pGAL4/P1aa1–250 and pGAL4/P2aa1–250), the zinc-finger-containing region (pGAL4/P1aa251–490 and pGAL4/P2aa260–500), the winged-helix/forkhead DNA binding domain (pGAL4/P1aa491–602 and pGAL4/P2aa501–613), and the carboxyl-terminal coding region (pGAL4/P1aa603–705 and pGAL4/P2aa614–714). These plasmids were co-transfected into NIH-3T3 cells with the pSV40GAL4.luc reporter plasmid, which contains four copies of the GAL4 DNA binding sequence upstream of the highly active SV40 viral promoter (23). Therefore, GAL4 chimeric proteins that encode independent repressor function tethered to the GAL4 DNA binding domain should decrease the activity of the...
quences in transfection efficiencies were corrected using a commercial 

obtained following transfection with the pCMVTag2B plasmid. Differ-
relative luciferase activity was measured and normalized to the activity 

reference plasmid. 48 h after transfection, cells were harvested, and 

B

expression plasmids, the 
pGL2/CC10 (A) or pGL2/SP-C (B) reporter plasmid, and the pMSVβgal 

reference plasmid. 48 h after transfection, cells were harvested, and 

relative luciferase activity was measured and normalized to the activity 

obtained following transfection with the pCMVTag2B plasmid. Differences 
in transfection efficiencies were corrected using a commercial 

β-galactosidase assay. The data are presented as % maximum of rela-
tive luciferase activity obtained upon co-transfection of the pGL2 re-
porter plasmid with the pCMVTag2B plasmid ± S.E. All experiments 

were performed in triplicate.

SV40 promoter. A greater than 60% reduction in luciferase 
activity was demonstrated when the pGAL4/P1aa251–490 and 
pGAL4/P2aa260–500 plasmids were co-transfected with the 
pGAL4SV40.luc plasmid into NIH-3T3 cells (Fig. 9). In con-
trast, expression plasmids encoding other Foxp1 and Foxp2 
domains failed to specifically repress the pSV40GAL4.luc re-
porter. Of note, the amino acid sequence of Foxp1 and Foxp2 
encoded by the pGAL4/P1aa251–490 and pGAL4/P2aa260–
500 plasmids are highly related, further suggesting an important 

conserved repressor function (Fig. 1). Taken together, 

data demonstrate that Foxp1 and Foxp2 contain a func-
tionally and structurally conserved independent transcriptional 

repression domain, which lies in the N-terminal end of the protein 

encompassing the zinc-finger motif.

**DISCUSSION**

The transcriptional mechanisms underlying lung development 
have only recently begun to be elucidated. The Fox gene 
family of winged-helix/forkhead transcription factors has been 
implicated in regulating lung gene expression. In this report, 
we describe the isolation and characterization of two novel 
cDNAs, Foxp1 and Foxp2, which comprise a unique subfamily 
within the Fox family of transcriptional regulators. Transcripts 
from these genes are detected at high levels in the airway 
epithelium of the lung as early as E12.5 of mouse development, 
and expression continues in the lung into postnatal life. In 
addition, Foxp2 is the first Fox gene characterized that is 
expressed exclusively in the distal epithelium of the lung dur-
ing pulmonary development. Foxp1 and Foxp2 are also ex-
pressed in neural, intestinal, and cardiovascular tissues during 
development. We show that both Foxp1 and Foxp2 can repress 
the 804-bp mouse CC10 promoter and the 3.7-kb human SP-C 

promoter. Finally, we show that Foxp1 and Foxp2 contain 
similar independent transcriptional repression domains lo- 
cated in the N terminus of the protein, which encompasses a 
unique zinc-finger motif. These data serve to identify Foxp1 
and Foxp2 as candidate regulators of lung gene expression, and 
to our knowledge, this is the first report of Fox genes expressed 
in the lung that act as transcriptional repressors.

Several other Fox genes are expressed in the lung and have 
been shown to regulate lung-specific gene expression. In addi-
tion, overexpression of certain Fox genes in the distal lung 
epithelium has resulted in disruption of lung development and 
the respecification of certain lung epithelial cell lineages. 
Foxa2 has been shown to regulate lung-specific genes including 
the human SP-B and rat CC10 promoters (4, 16). Overexpres-
sion of Foxa2 in the distal epithelium of the lung using the 
3.7-kb human SP-C promoter results in a dramatic disruption 
of branching morphogenesis and arrest of lung development at 
the pseudoglandular stage (35). Because Foxa2 is expressed in 
both the proximal and distal epithelium of the lung, the results 
from the SP-C/Foxa2 transgenic mice suggest that the expres-
sion level of Foxa2 in the proximal versus distal epithelial 

must be carefully balanced for proper lung epithelial cell dif-
erentiation and development. Additional evidence that Fox 
genes play an important role in epithelial cell specification in 
the lung comes from studies where Foxj1 was overexpressed in 
the lung using the human SP-C promoter, which resulted in the 
extopic appearance of ciliated epithelial cells in the distal air-
ways of the lung (36). Because ciliated epithelium is normally 

present only in the proximal airways, these data indicate a 
direct role for Foxj1 in the specification of the ciliated epithelial 
cell lineage in the lung. This observation is further supported 
by the lack of ciliated cells in all tissues of Foxj1 null mice (37). 
Together, these data provide strong evidence that Fox genes 
play an integral role in lung epithelial cell lineage differen-
tiation and specification.

The importance of Fox transcription factors in regulating 
lung gene regulation and cell specification led us to investigate 
whether additional family members were expressed in the 
lung. From these studies we identified Foxp1 and Foxp2 as two 
new members of the Fox family that are expressed in the lung 
epithelium and act as transcriptional repressors. Several other 
Fox proteins, such as Foxe1 and Foxd3, have been character-
ized as transcriptional repressors (38, 39). However, until the 
identification of Foxp1 and Foxp2, all the known Fox genes 
that were implicated in regulating lung gene expression were 
characterized as transcriptional activators. The fact that 
neither Foxp1 nor Foxp2 significantly affected expression of β-galacto-
sidase from the MSV viral promoter in the co-transfection 
assays further supports the idea that these proteins act as 
sequence-specific transcriptional repressors. Confirmation of 
this hypothesis is provided by the identification of a function-
ally and structurally conserved independent transcriptional 
repressor domain located in the N-terminal region of the Foxp1
and Foxp2 proteins using the GAL4 heterologous DNA binding domain fusion protein system. Database and protein sequence analysis of this region shows that it contains a single zinc-finger motif that is most similar to the first zinc-finger found in the Gli 1, 2, and 3 transcription factors (Fig. 10). Interestingly, zinc-finger motifs have been implicated in transcriptional repression, most notably in the case of YY1 and AEBP2 (40, 41). In addition, Gli 2 and Gli 3 are known to regulate lung development as shown by a complete lack of lung tissue in Gli $2^{-/-}$/Gli $3^{-/-}$ double homozygous null mice (42). Future studies will be directed toward characterizing the role of this zinc-finger motif in Foxp1 and Foxp2 function and determining whether their transcriptional repression activity is because of their ability to interact with other co-repressor molecules.

The expression patterns of Foxp1 and Foxp1 in neural, intestinal, and cardiovascular tissues of the developing mouse embryo suggest that these transcription factors may perform important roles in the differentiation and development of these tissues. Several Fox genes are expressed in neural tissue including Foxg1, which has been shown to be essential for normal...
Foxp1 and Foxp2 Regress Lung-specific Gene Transcription

The expression of Foxp2 in the outflow tract and atrium of the heart is reminiscent of that observed with the Foxc1 and Foxc2 genes that are expressed in the outflow tract of the developing heart. Inactivation of Foxc1 or Foxc2 results in severe cardiovascular defects including an interrupted aortic arch and ventricular septal defects (46, 47). Interestingly, mice that are doubly heterozygous for the Foxc1 and Foxc2 alleles also exhibit similar cardiovascular defects suggesting a gene-dosage effect (47). These studies clearly point to a role for Fox genes in the regulation of cardiac development. Future studies, including the generation of a null allele for Foxp3, should help to elucidate the function of this gene during cardiovascular development.

Because cell-specific gene expression can be regulated at the level of gene activation and/or repression, it is noteworthy that both Foxp1 and Foxp2 repress the mouse CC10 and human SP-C promoters, albeit to slightly different degrees. In addition, the finding that Foxp2 is the first Fox gene identified that is expressed solely in the distal alveolar epithelium during lung development suggests that Foxp1 and/or Foxp2 participate in the balance of transcriptional activation and repression that is involved in regulating epithelial cell identity and development along the proximal-distal axis of the lung. These developmental processes are crucial for the differentiation of distal epithelial cells such as alveolar type I and II epithelial cells, which are responsible for gas exchange and surfactant protein expression essential for postnatal lung function. It is therefore possible that Foxp1 and/or Foxp2 restrict expression of proximal epithelial genes from the distal epithelium, which in turn contributes to the specification of epithelial cell lineages during the pseudoglandular and canalicul/saccular stages of lung development. The quantitative differences observed in the repression activity of Foxp1 and Foxp2 on the mouse CC10 and human SP-C promoters suggests that certain lung genes may be more regulated than others. These results could be because of species differences between the Foxp1 and Foxp2 cDNAs and promoters (i.e., human SP-C promoter) or fine differences in DNA binding preferences in the Fox gene binding sites located in the mouse CC10 and human SP-C promoters. The identification of additional transcriptional targets of Foxp1 and Foxp2 should provide answers as to their role in regulating gene expression along the epithelial proximal-distal axis of the lung during embryonic development.

From previous studies, it is clear that Fox genes play an important role in the specification and differentiation of lung epithelium. Foxp1 and Foxp2 are newly identified candidates that may regulate these processes during lung development and in the development of other tissues including the brain, intestine, and cardiovascular system. Future studies characterizing the down-stream transcriptional targets of Foxp1 and Foxp2, as well as generation of tissue-specific null alleles for Foxp1 and Foxp2, should help in clarifying the role of this Fox subfamily in regulating gene transcription and development in the lung and other tissues.

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Characterization of a New Subfamily of Winged-helix/Forkhead (Fox) Genes That Are Expressed in the Lung and Act as Transcriptional Repressors
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