Transcriptional Activity of the 1.3-Kilobase Pair Promoter of T1α, a Lung Alveolar Type I Cell Gene*

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Alveolar type I epithelial cells form the major surface for gas exchange in the lung. To explore how type I cells differ in gene expression from their progenitor alveolar type II cells, we analyzed transcriptional regulation of T1α, a gene expressed by adult type I but not type II cells. In vivo developmental patterns of T1α expression in lung and brain suggest active gene regulation. We cloned and sequenced 1.25 kilobase pairs of the T1α promoter that can drive reporter expression in lung epithelial cell lines. Deletion analyses identified regions important for lung cell expression. The base pair (bp) −100 to −170 fragment conferred differential regulation in lung epithelial cells compared with fibroblasts. Sequence alignment of this fragment with type II-specific surfactant protein B and C promoters shows similar consensus elements arranged in a different order. Gel retardation studies with alveolar epithelial cell line nuclear extracts, thyroid transcription factor I (TTF-1) homedomain, hepatic nuclear factor (HNF)-3β, or Sp1 proteins, and supershift assays were used to characterize TTF-1, HNF-3 (TGT3), and Sp1/Sp3 binding sites. The TGT3 site binds factors with binding properties similar to HNF-3/Fkh (hepatic nuclear factor-3/forkhead) proteins but different from HNF-3α or HNF-3β. Co-transfection with a TFF-1 expression vector moderately trans-activated the −170 bp-reporter construct. Mutational analysis of these three binding sites showed reduced transcriptional activity of the −170 bp promoter. Therefore, several regulatory sequences involved in type II cell gene regulation are also present in the T1α promoter, suggesting that genes of the peripheral lung epithelium may be regulated by similar factors.

The alveolar type I epithelial cell forms the major cellular surface (~70 m², human) for gas exchange in the mammalian lung. Despite this important function, very little is known about its molecular phenotype or regulation of expression of its cell-specific genes (1). We have recently cloned, sequenced, and characterized a gene, T1α, that we believe is the first definitive marker for this cell type in the adult rat lung (2, 3). The gene encodes an apical transmembrane protein that is expressed by type I cells but not by adjacent alveolar epithelial type II cells.

Characterizing the regulation of this new marker for the type I cell phenotype is likely to be important for understanding the general processes by which type I cells differ in gene regulation, structure, and biology from other lung epithelial cells, particularly alveolar type II cells.

Expression of T1α is developmentally regulated (2, 3). Both mRNA and protein are expressed in many fore- and midgut derivatives as early as embryonic day 10.5 (rat) including the primitive lung (day 12.5) and the anterior pituitary anlage (Rathke's pouch), in the early embryonic brain, spinal cord, other neural structures, and several other organs. In most of these tissues, however, expression is rapidly repressed during fetal development (brain) or postnatally (bronchiolar epithelium). In the adult rat, T1α mRNA and protein expression can be detected at high levels only in the alveolar type I cell, in choroid plexus epithelium, in ciliary body of the eye, and in a subset of osteoblasts (4, 5). These complex developmental temporal-spatial patterns suggest that active mechanisms of gene regulation determine the highly specific pattern of T1α expression in the adult.

In situ hybridization, immunocytochemical, biochemical, and molecular analyses (2, 6) show that adult alveolar type II cells do not express T1α in vivo, although these cells reside in the alveolar epithelium and act as stem cells to generate new type I cells in normal and injured lung (7). However, when type II cells from normal lung are cultured under conditions where they do not proliferate, they rapidly (within <24 h) express both T1α mRNA and protein, while down-regulating type II cell genes (8).

These and other similar findings suggest that type II and type I cell genes share certain common regulatory elements and transactivating molecules but not others, allowing for expression of their cell-specific phenotypes. There is now considerable information about the regulation of type II cell genes because of an interest in defining the molecular control of synthesis of pulmonary surfactant, a complex lipid-protein material secreted by type II cells. The promoters for surfactant protein (SP)1-A (9–12), -B (13–17), -C (18, 19), -D (20), and Clara cell-specific protein (CCSP) (21–24) genes have been partially characterized, and some cis-regulatory elements and transactivating proteins have been identified. Most of these genes have in common their transactivation by TTF-1 (12, 14,

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1 The abbreviations used are: SP, surfactant protein; CCSP, Clara cell-specific protein; TTF-1, thyroid transcription factor 1; HNF, hepatocyte nuclear factor; CRE, cAMP-responsive element; CREB, CRE-binding protein; kb, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; HD, homeodomain; hSp1, human recombinant Sp1; SV40TII, SV40T type II cells; FREAC, forkhead-related activator; HFH, HNF-3/forkhead homolog.
Transcriptional Regulation of Ttα Gene Expression

17, 19, 24–26 (thyroid transcription factor 1, also known as thyroid enhancer binding protein or T/EBP) and HNF-3 (hepatic nuclear factor 3) family proteins (14, 23, 27, 28), two families of transcription factors enriched in lung tissues, as well as ATF/CREB family members (9, 29, 30) and Sp1 and Sp3 (24, 31).

We report here our initial studies on the regulation of Ttα using the 1.25-kb promoter that we cloned and sequenced. Using promoter deletion analyses, electrophoretic mobility assays, and mutational analysis, we have identified the minimal promoter and regions that account for differential expression between epithelial cells and fibroblasts. These studies demonstrate that cis-elements known to be involved in type II cell gene expression are also present in the proximal promoter of Ttα. Activation of these elements is unlikely to differentiate between type I and type II phenotypes.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of the 5′-Flanking Region—A rat genomic library (NIH; HaeIII-partially cut DNA ligated into a g10 vector) was amplified into 10 fractions that were tested by PCR for the Ttα cDNA sequence (5′-GAGCTTACAAGGAC-3′) and P2 (5′-GAGTCCGAAGAACAGG-3′) (2). Frctions showing the expected 87-bp PCR fragment were plated at different dilutions and screened on filters with probe P3 spanning the coding region of the cDNA (2). Eleven clones were analyzed by PCR using oligos P1 and P2; five yielded an amplicon of the expected size. For secondary screening, the clones were amplified and analyzed for P3 hybridization. Two clones from each positive plate were selected, replated, and reprobed. DNA from five different clones, uncot or EcoRI-cut, was analyzed on Southern blots using P4 (a probe made by PCR using oligos P1 and P2). DNA from a positive clone was digested with BamHI, and fragments were subcloned into pBluescript (Stratagene, La Jolla, CA). Transformed bacterial colonies were screened with P4. Inserts from six positive clones were analyzed by restriction mapping with EcoRI, BamHI or both, and two clones were sequenced. They contained 60 bp of intron 1, the first exon, and ~1.3 kb of 5′-flanking region. To verify the promoter sequence, genomic DNA from a rat SV40T immortalized type II cell line was amplified by PCR, cloned with the TA cloning kit (Invitrogen, San Diego, CA) in a pCR vector, and sequenced.

Analysis of the Transcription Initiation Site—The 5′-end of the Ttα cDNA was rapidly obtained by rapid amplification of cDNA ends (RACE) (2). The 5′ oligonucleotide complementary to nucleotides +82 to +115 (Fig. 1) was end-labeled with [γ-32P]ATP and purified through NucTrap column (Stratagene, La Jolla, CA). 15 μg of total RNA from SV40T type II (SV40TII) cells or IMR-90 fibroblasts were annealed to oligonucleotide P5 at 55 or 65 °C for 5 h under mineral oil. After hybridization, probe using 5 units of reverse transcriptase (Promega, Madison WI), the products were extracted with phenol/chloroform, precipitated, and electrophoresed on a 8% denaturing polyacrylamide gel for analysis of extension products.

Plasmid Constructions—The 1251bp-Bluescript plasmid was constructed by digestion of a genomic clone with SacI and partial digestion with EcoRI. The 1352-bp segment (~1251 to +101 bp) was cloned into pBluescript SK; 789bp-Bluescript was prepared by SacI and AvrII digestion of ~1251bp-Bluescript and insertion of the ~789 to ~101 bp fragment into the XbaI-SalI site of pBluescript SK. This construct was further digested with PstI and BamHII for construction of plasmids containing progressive unidirectional deletions using the Erase-a-Base System (Promega). Deletion constructs containing ~19 bp, ~53 bp, ~100 bp, ~170 bp, ~200 bp, ~245 bp, ~286 bp, ~390 bp, ~562 bp, and ~661 bp of the promoter were sequenced to determine their exact lengths. All constructs were digested with KpnI-SacI prior to insertion of the fragments into pGL-3 Basic Luciferase reporter vector (Promega). All Ttα promoter constructs contain ~101 bp of the untranslated region.

Cell Cultures, Transfection Methods, and Reporter Activity Assay—SV40TII cells were derived from neonatal rat alveolar type II cells immortalized in vitro with the viral oncone SV40 large tumor antigen (33). IMR-90 is a human lung fibroblast cell line (ATCC CCL 186). MLE-15 cells (provided by Dr. J. Whittsett, Children's Hospital Medical Center, Cincinnati, OH) are murine lung epithelial cells produced from tumors in transgenic mice expressing the SV40 large tumor antigen under transcriptional control of the human surfactant protein C pro-
After purification and cleavage of the glutathione S-transferase region with thrombin protease, the TTF-1 HD (−1 ng) was used in EMSA. Incubation with TTF-1 HD was done for 1 h. HNF-3β protein was obtained by in vitro transcription-translation using a construct provided by Dr. R. Costa (University of Illinois, Chicago, IL) and the TNT-Sep coupled kit (Promega); HNF-3α protein (−3 ng) was incubated with oligonucleotides in the same conditions as for nuclear proteins. HNF-3α and HNF-3β polyclonal antibodies used in supershift assays (data not shown) were provided by Dr. R. Costa. Gels (5%) were dried and exposed at −70 °C for 16−36 h unless otherwise stated.  

**Mutation of the T1α Promoter**—Mutated TGT3, TTF-1 sites, and double mutant fragments were generated by PCR under standard conditions (annealing temperature 42 °C, 30 cycles) using −170Luc as template. The mutated fragments (from bp −170 to −89) were flanked by a 5′ Kpn1 and 3′ EcoRI restriction site. The 5′-primer used was a 36-mer containing the 5′ wild type sequence. The 3′-primer was mutated oligonucleotides complementary to the 3′-sequence. PCR fragments were purified and digested with Kpn1 and EcoRI restriction enzymes. −170Luc was digested with Kpn1 and EcoRI; wild type fragment was removed by agarose electrophoresis, and mutated fragments were inserted into these sites. For the mutated Sp1 site, the PCR fragment was generated from −104 to +101 flanked by a 5′ EcoRI and 3′ SacI site. The 5′-primer was a 30-mer mutated in the Sp1 site. The 3′-primer was complementary to the wild type sequence. −170Luc construct was digested with EcoRI and SacI, and the mutated fragment was inserted as described before. Mutated −170Luc constructs were sequenced and transfected in SV40TII cells as described above.  

**RESULTS**  

**Isolation and Sequencing of the Rat T1α Gene Promoter**—A fragment of the 5′-flanking region of the T1α gene was isolated by PCR screening of a rat genomic library using two primers in the 5′ end of the cDNA (2). Positive clones, characterized by Southern blotting and restriction enzyme analysis (data not shown), were shown to contain −1.45 kb of the 5′-flanking region. The sequence of the proximal promoter is shown Fig. 1. A major transcription initiation site was determined by primer extension using SV40TII cell mRNA as template (Fig. 2). The T1α 5′-flanking region contains a 201-bp 5′- untranslated region and 1251-bp promoter. A TATA-like box (TAAAT) is located at position −25 bp (Fig. 1). The sequence around the transcription initiation site (CCAGTTG) is characteristic of a transcriptional initiator identified in many TATA-less promoters (39, 40). A number of putative binding sites for ubiquitous and lung-enriched transcription factors were identified by computer analysis comparing the 1.25-kb promoter sequence to known consensus sequences.  

**Characterization of the Cell Lines**—SV40TII and IMR-90 cells and adult rat lung were analyzed for transcriptional activity of the T1α promoter-luciferase constructs in epithelial cells is about 1.5−4-fold higher than that in fibroblasts, although the expression patterns are similar in epithelial cells and fibroblasts. This indicates that the 1.25-kb promoter is sufficient to confer at least partial specificity between the two types of cells. The modest expression of luciferase driven by T1α promoter in IMR-90 cells is similar to recent findings that show low levels of reporter expression driven by the SP-C promoter in HEK293 cells (19) that do not express endogenous SP-C and to studies with other promoters that drive low levels of expression of reporter genes in nonexpressing cell lines (41). The endogenous gene in IMR-90 cells could be silenced by methylation (42, 43), but cells may contain elements, presumably transcription factors, that allow expression of the unmethylated promoter-reporter constructs. It is also possible that the endogenous gene is silenced because of its chromosomal location (44). Again the presence of appropriate transcription factors in the IMR-90 cells would allow transcription of the transfected promoter, because it is not integrated into the genome.

Deleting the T1α promoter from bp −1251 to −789 decreases expression in SV40TII cells from −85- to −40-fold over back-
ground, identifying potential stimulatory elements in that region (Fig. 4). In contrast, deletion constructs from bp −789 to −398 have lower activity than either 5'- or 3'-constructs, suggesting the presence of negative regulatory elements therein. The activity of the −286bp-Luc construct is about 60-fold over background, while the −286 to −170 constructs show a stepwise reduction of the activity to −35-fold over background.

Additional cell-specific differences in expression are observed with the −170 bp promoter. Although the −100bp-Luc construct yields about the same expression levels in SV40TII and IMR-90 cells (−6-fold over 0bp-Luc) (Fig. 4), the −170 bp promoter drives about a 4-fold higher expression level in SV40TII cells compared with IMR-90 cells (SV40TII cells, −35-fold over background; IMR-90 cells, −9-fold over background). This observation provides the rationale for selecting the −100 to −170 region for detailed analysis of cis-elements and trans-activating proteins expressed in SV40TII cells. Several putative binding sites for lung-enriched factors are present in this fragment (Fig. 1).

The bp −1 to −100 region contains the TATA-like box as well as several GC-rich regions (Fig. 1). Expression patterns indicate that this fragment constitutes the minimal promoter required for expression.

**Sp1 Family Proteins Bind to the T1α Proximal Promoter**—

**FIG. 2.** Transcription initiation site characterized by primer extension. P5 oligonucleotide (Fig. 1) was designed to obtain an extension product of 100–150 bp. A, polyacrylamide gel electrophoresis (8%) analysis of the extension reaction at 55 or 65 °C using RNA (15 μg) from SV40TII or IMR-90 cells. Lane 1, 32P-labeled markers, Lane 2, SV40TII cell RNA at 55 °C; lane 3, at 65 °C. Lane 4, IMR-90 cell RNA at 55 °C, lane 5, at 65 °C. Lane 6, control with no RNA at 55 °C; lane 7, at 65 °C. A product of ~115 bp is shown in lane 2. B, the extension reaction was run in a 8% sequencing gel (left) along with a sequencing reaction performed with P5 oligonucleotide and the original DNA clone (pBluescript containing the 1.45-kb 5'-flanking region) as a template (right). A major (large arrow) and a minor (small arrow) transcription initiation site are shown. The sequence of this region is shown on the right.

**FIG. 3.** Northern and Western analyses of SV40TII and IMR-90 cell lines and adult rat lung. A, total RNA (20 μg/lane) was hybridized with 32P-labeled probes for T1α, SP-C, and β-actin. B, Western analysis for T1α protein expression of cell lines compared with adult rat lung (10 μg of protein/lane) using monoclonal anti-T1α antibody detected with an alkaline phosphatase-conjugated second antibody. Lane I, lung; lane 2, SV40TII cells; lane 3, IMR-90 cells; lane M, markers.

**FIG. 4.** Deletion studies of the T1α promoter. Relative activity of the luciferase reporter gene in transiently transfected SV40TII (black bars) and IMR-90 cells (hatched bars) with the indicated 5'-deletion constructs normalized for β-galactosidase activity. Luciferase activity is expressed relative to the promoterless plasmid 0bp-Luc (pGL3) in each cell line. Data are expressed as the mean of three or more transfections with duplicate assays ± S.E. The relative value of SV40 promoter-Luc construct (right) used as control shows the maximum expression level in this system.

The first 100-bp region of the T1α promoter drives reporter expression −6-fold over background and contains two GC-rich regions near the TATA-like element that are putative binding sites for Sp1-like proteins. Two oligonucleotides from this proximal region (Table I; oligonucleotide I, bp −20 to −56; oligonucleotide II, bp −57 to −95) containing the GC-rich sequences were analyzed by competition EMSA using SV40TII nuclear proteins or human recombinant Sp1 (hrSp1) (Promega) (Fig. 5A). SV40TII nuclear extract forms a complex of identical mobility to that formed by hrSp1 protein. With oligonucleotide I, a single complex is present that can be competed with excess unlabeled oligonucleotide I or oligonucleotide II but not by oligonucleotide III (Table I). Oligonucleotide II binds hrSp1
protein and can be competed by unlabeled oligonucleotide II or oligonucleotide I but not by oligonucleotide III. That each fragment competes with the other for Sp1 binding supports the identity of these regions as Sp1-binding elements. Using oligonucleotide II and hrSp1 protein, a second complex of lower mobility is detected that is not present with SV40TII cell nuclear extract. This complex could represent multimeric forms of Sp1 protein (45) that interact with the oligonucleotide in the absence of other nuclear proteins. Supershift analysis with anti-Sp1, -Sp3, and -Sp4 monoclonal antibodies (Fig. 5B) showed that the Sp1 site in oligonucleotide I, next to the TATA box, and oligonucleotide II bind Sp1 and Sp3 proteins that are present in SV40TII nuclear extracts. These findings suggest that GC regions between bp −42 and −56 and between bp −87 and −95 bp recognize Sp1 family proteins and are likely to be involved in basal and/or specific expression of the T1α gene.

**Table I**

| Oligonucleotides | Probe I | Probe II |
|------------------|---------|---------|
| Oligonucleotide I (bp 95 to 20) | SV40TII | Sp1 |
| Oligonucleotide II (bp 95 to 20) | SV40TII | Sp1 |

**Fig. 5.** The −95 bp region of the T1α promoter contains two Sp1-like binding elements. A. EMSAs were performed using 32P-labeled oligonucleotide I (from bp −20 to −56) and 32P-labeled oligonucleotide II (from bp −57 to −95). Binding of SV40TII nuclear extract (10 µg) to both oligonucleotides was competed by a 1000-fold excess of unlabeled specific oligonucleotide (a) or oligonucleotide II (b) but not by a 100-fold excess of nonspecific oligonucleotide (ns). B. Monoclonal antibodies block formation of a protein-nucleic acid complex. B. Monoclonal antibodies block formation of a protein-nucleic acid complex. Band I is competed by a 100-500-fold excess of unlabeled specific oligonucleotide and is not competed by a 100-500-fold excess of nonspecific or mutated oligonucleotide, respectively. With oligonucleotide IIIA, IMR-90 nuclear extracts form two complexes of similar mobility to those formed by SV40TII extracts. The lower intensity of the bands suggests that fibroblasts contain fewer binding proteins or proteins with lower affinity. The lower band (IMR-90) can be equally competed by specific, nonspecific, or mutated oligonucleotides (Fig. 8). The upper band (IMR-90) is similar in mobility and binding characteristics to band I (SV40TII) but is less intense.

The addition of monoclonal anti-TTF-1 interferes with the formation of band II (Fig. 9A) and increases the intensity of band I; a control nonspecific monoclonal antibody does not change the binding pattern. No supershifted complex was detected in the presence of anti-TTF-1 although tested under various electrophoretic conditions. This is similar to many other studies in which antibodies block formation of a protein-DNA complex rather than binding and supershifting the complex. The TTF-1 homeodomain peptide (TTF-1 HD), known to reproduce the binding specificity of the entire protein (25), binds to oligonucleotide III (Fig. 9B). The addition of monoclonal anti-TTF-1 interferes with the formation of band II (Fig. 9A) and increases the intensity of band I; a control nonspecific monoclonal antibody does not change the binding pattern. No supershifted complex was detected in the presence of anti-TTF-1 although tested under various electrophoretic conditions. This is similar to many other studies in which antibodies block formation of a protein-DNA complex rather than binding and supershifting the complex. The TTF-1 homeodomain peptide (TTF-1 HD), known to reproduce the binding specificity of the entire protein (25), binds to oligonucleotide III (Fig. 9B).
**Transcriptional Regulation of tTα Gene Expression**

**FIG. 6.** Alignment of the proximal regulatory regions of lung specific promoters. These proximal regions of lung-specific promoters are involved in the transcriptional regulation of alveolar epithelial genes and bind lung-enriched transcription factors. Shown are the murine surfactant proteins SP-A (19), SP-B (14, 19), and SP-C (19) and rat Tα. These fragments show a cluster of similar binding sites in a different arrangement. Highlighted are TTF-1 sites (*), TGF3 sites (**), and a CRE-like element (underlined). The vertical lines show similarities in the TTF-1 binding site used to align the promoter fragments.

**FIG. 7.** EMSA indicating differential binding of SV40TI and IMR-90 cell nuclear proteins to the bp -95 to -146 region. SV40TI and IMR-90 nuclear extracts (10 μg of total protein) were incubated with oligonucleotide III (bp -95 to -146). Competition assays (Comp) were done with excess unlabeled specific (s) or nonspecific (ns) oligonucleotides at 10-, 100-, and 1000-fold ratios. Fewer and less intense complexes are formed with IMR-90 nuclear extracts compared with SV40TI cells, in agreement with the differences in the level of intense complexes are formed with IMR-90 nuclear extracts compared with SV40TI cells, in agreement with the differences in the level of

**FIG. 8.** A TTF-1 minimal consensus sequence specifically binds alveolar epithelial cell line nuclear proteins. SV40TI and IMR-90 nuclear extracts (20 μg) were incubated with oligonucleotide IIIA spanning bp -110 to -128. Two complexes (I and II) were formed with SV40TI nuclear extract. Competition assays were performed with a 100- and 500-fold excess of unlabeled specific (s), nonspecific (ns), or mutated (mut) oligonucleotides. Band II can be competed by a 100–500-fold excess of unlabeled oligonucleotide but not by nonspecific oligonucleotide. The same excess of an oligonucleotide mutated in two bases of the TTF-1 putative site does not compete for this complex. Band I is competed by a 500-fold excess of unlabeled oligonucleotide and is not competed by a 500-fold excess of nonspecific oligonucleotide or a 500-fold excess of the mutated oligonucleotide, respectively. The formation of both complexes depends on the same nucleotides because mutation of two bases of the TTF-1 site reduces the ability of the oligonucleotide to compete. IMR-90 nuclear extracts form two complexes of lower intensity. The lower band (IMR-90) can be equally competed by specific, nonspecific, or mutated oligonucleotides (Fig. 8). The upper band (IMR-90) is similar in mobility and binding characteristics to band I (SV40TI) but is less intense. The new band (*) seen in IMR-90 competition experiments is believed to be nonspecific.

**FIG. 9.** The TTF-1 minimal consensus sequence binds both TTF-1 protein in the SV40TI nuclear extract and recombinant TTF-1 HD. A, monoclonal anti-TTF-1 antibody (1 μl (+)) or 2 μl (++) interferes with the formation of complex II, while an excess (++) of nonspecific antibody (ns Ab) does not change the binding pattern. Band II formation is impaired by TTF-1 monoclonal antibody, while band I complex is slightly increased. B, recombinant TTF-1 HD specifically binds to oligonucleotide IIIA. TTF-1 homeodomain (TTF-1 HD) recognizes the CTGAG site in the Tα promoter. Competition assays (Comp) were performed with a 100- and 500-fold excess of specific (s) or nonspecific (ns) oligonucleotides.

500-fold excess of nonspecific oligonucleotide IIIB.

Together these results indicate the presence of a TTF-1 binding site(s) in the -128 to -110 DNA region. Band II behaves like a TTF-1-DNA complex, but under the conditions tested the affinity of this site appears to be weak, since it can be specifically competed with a 100-fold excess of cold oligonucleotide. The protein(s) associated with band I (upper band) are uncertain. The failure to compete band I and band II with a mutated oligonucleotide suggests that a TTF-1 family protein may be part of a protein complex in band I or that the bands are formed by different protein(s) that recognize an overlapping consensus sequence. Such an overlap (TTF-1 and Pax8) has been demonstrated in some thyroid-specific promoters (46).

To see if the cell lines express endogenous TTF-1 protein, we analyzed SV40TI, IMR-90, and MLE-15 cell nuclear extracts and total adult rat lung protein by Western blotting (Fig. 10). MLE-15 nuclear extract shows bands at ~40 kDa, similar to the molecular mass described for TTF-1 in thyroid cell lines (47), and at ~55 kDa, similar to the molecular masses detected in rat lung total proteins (doubt at about 55 kDa). In SV40TI, only the ~55-kDa band is detected; no specific band is detected in IMR-90 nuclear extracts. Three TTF-1 mRNAs have been described in lung (25), and the protein can also have different phosphorylation states (47). Assuming that both bands are TTF-1-related proteins, the ratio between the two bands differs among MLE-15 cells, the other epithelial cell lines, and lung. No endogenous TTF-1 was detected in IMR-90 cells.

Transactivation of Tα Promoter by TTF-1—To determine if the TTF-1 binding site can activate the Tα promoter in vivo, co-transfection experiments were performed in SV40TI cells.
Transcriptional Regulation of t1α Gene Expression

Fig. 10. Characterization of TTF-1 protein expression in SV40TII, IMR-90, and MLE-15 cell lines. Western analysis was performed with nuclear protein (20 µg) from cell lines or total adult lung protein and blotted with anti-TTF-1 detected with an alkaline phosphatase-labeled secondary antibody. Lane M, molecular markers; lane 1, lung; lane 2, SV40TII cells; lane 3, IMR-90 cells; lane 4, MLE-15 cells. MLE-15 nuclear extract shows bands at ~40 kDa, similar to the molecular mass described for TTF-1 in thyroid cell lines (47), and at ~55 kDa, similar to the molecular mass detected in rat lung total proteins (doublet at about 55 kDa). In SV40TII, only the ~55-kDa band is detected; no specific band is detected in IMR-90 nuclear extracts. The lower band in lane 1 (~20 kDa) is thought to be degraded TTF-1 (25).

Fig. 11. TTF-1 protein transactivates T1α promoter deletion constructs when co-transfected in SV40TII cells. Normalized luciferase activity in SV40TII cells transiently transfected with the indicated 5′ deletion constructs in the presence of coexpressed pCMV-TTF-1 (hatched bars) or pCMV alone (black bars). Data, relative to the promoterless plasmid 0bp-Luc (pGL3) in each condition, are expressed as the mean ± S.E. of two different transfections analyzed in duplicate. Asterisks indicate statistically significant differences determined by the t test; p ≤ 0.05.

(Fig. 11). T1α deletion-luciferase constructs were co-transfected with either a pCMV-TTF-1 expression construct or the control pCMV vector. Normalized luciferase activity from the −170bp-Luc is increased 1.6-fold by overexpression of TTF-1 protein. No significant difference is detected with the −100bp-Luc construct lacking TTF-1 sites. Larger constructs showed no additional increase from the −170bp-Luc construct, suggesting that TTF-1 functional binding site(s) was present only in the bp −100 to −170 region.

A TGT3 Site in the bp −96 to −112 Fragment Binds SV40TII Cell Nuclear Extracts and Pure HNF-3β Protein—A putative TGT3 site was identified by computer analysis at bp −106 to −101 (Fig. 1). It differs in the 5′ and 3′ bases from the HNF-3 consensus sequences determined by alignment of protein-selected DNA binding sites (48). Subtle nucleotide changes, 5′ or 3′ to the core binding sequence, appear to dictate differential HNF-3/Fkh protein recognition and determine the relative affinity of HNF-3 protein-DNA interactions (48). EMSAs using oligonucleotide IIIB from −96 to −112 (Table I) were therefore performed to test whether the −106 to −101 bp site binds HNF-3 family members. SV40TII nuclear proteins specifically bind to this region, as does recombinant HNF-3β protein (Fig. 12A). HNF-3β protein can be displaced by a 500-fold excess of specific oligonucleotide but not by a 500-fold excess of either nonspecific oligonucleotide or oligonucleotide mutated in two of six bases of the HNF-3 core sequence (CGTTGG for TGTTTG, Table I). Binding of SV40TII nuclear proteins is competed with a 100-fold excess of specific oligonucleotide but not by a 500-fold excess of nonspecific oligonucleotide or 100-fold excess of mutated oligonucleotide. IMR-90 nuclear extracts show a different binding pattern, and the specific complex of lower mobility formed by SV40TII cells (Fig. 12C, arrow) is not formed by IMR-90 nuclear extracts. IMR-90 cells have been shown to express FREAC2 (49). This could account for one of the complexes that is formed by SV40TII as well as IMR-90 nuclear extracts.

The addition of polyclonal anti-HNF-3α or anti-HNF-3β fails to disrupt the SV40TII protein-DNA complexes, although anti-HNF-3β supershifts the complex formed by the recombinant protein (data not shown). Anti-HNF-3α and anti-HNF-3β also impared complex formation with MLE-15 nuclear extracts (data not shown) that have HNF-3α and HNF-3β proteins (14). This suggests that SV40TII cells contain a protein that recognizes the TGT3 site but that the protein is not the lung-enriched HNF-3α or HNF-3β protein. Moreover, competition assays using an HNF-3 high affinity site oligonucleotide (HFH-1#3 oligonucleotide (see Ref. 48 and Table I) effectively compete the binding of HNF-3β protein to oligonucleotide IIIB but not binding of SV40TII cell nuclear extract (Fig. 12B). SV40TII nuclear extract forms a complex with HFH-1#3 oligonucleotide (Fig. 12D) of higher mobility than the complex formed by HNF-3β recombinant protein. The HFH-1#3 complex is competed by oligonucleotide IIIB but not by a nonspecific oligonucleotide. No changes in the binding pattern were obtained by adding anti-HNF-3α or anti-HNF-3β antibodies (data not shown).

Mutagenesis Analysis of the −170 bp Proximal Promoter—Sp1, TGT3, and TTF-1 sites identified by binding assays were mutated in the −170 bp proximal sequence to evaluate the importance of each site in the transcriptional activity of the promoter. Single and double mutations were performed by PCR and mutated constructs were transiently transfected in SV40TII cells (Fig. 13A).

Mutation of TGT3 diminishes the transcriptional activity of the −170 bp promoter by 40% (Fig. 13B). TTF-1 mutation moderately reduces the activity of the promoter by 20%, but combination of these two mutations reduces the activity by 70%, similar to the absence of the bp −100 to −170 region, indicating that the integrity of both binding sites is essential for maximal transcriptional activity of the −170 bp promoter. Mutation of the Sp1/Sp3 site close to the TGT3 and TTF-1 sites reduces the activity of the −170 bp promoter by 76%, suggesting that this site is important for the optimal transcriptional activity of the nearby sites in the bp −100 to −170 fragment.

DISCUSSION

We believe that this is the first study to describe the transcriptional regulation of an alveolar type I cell gene and to begin to characterize the molecular regulation of the type I cell phenotype. Although a number of other genes expressed by type I cells are known (e.g., intercellular adhesion molecule 1, carboxypeptidase M) (50, 51), none has been studied at the level of transcriptional regulation in the context of the type I cell. We have previously shown that the pattern of expression of T1α mRNA and protein in the developing rat is complex and changes, during development, from a widespread pattern of expression in brain, gut, and elsewhere to expression in highly...
Transcriptional Regulation of \( T1\alpha \) Gene Expression

Fig. 12. A TGT3 site in the bp \(-96 \) to \(-112 \) fragment binds SV40TII cell nuclear extracts and HNF-3\( \beta \) protein. A, oligonucleotide IIIIB was incubated with \( 10 \mu \)g of SV40TII nuclear extract or \(-3 \)ng of recombinant HNF-3\( \beta \) protein. Competition assays were performed in the presence of a 100- and 500-fold excess of specific (s), nonspecific (ns), or mutated (mut) oligonucleotides. B, same as A but with competition assays with a 100- and 500-fold excess of HFH-1#3 oligonucleotide. C, SV40TII and IMR-90 nuclear extracts were incubated with oligonucleotide IIIIB. Competition assays were performed in the presence of a 100-fold excess of specific (s) or nonspecific (ns) oligonucleotides. The arrows (in A, B, and C) indicate the specific complex formed by SV40TII cells but not by IMR-90 nuclear extracts. D, binding of SV40TII nuclear extract to HFH-1#3 oligonucleotide. Competition was performed with a 500-fold excess of specific oligonucleotide (s), oligonucleotide IIIIB, or nonspecific oligonucleotide (ns). Gel was exposed 5 h.

Fig. 13. Mutational analysis of the bp \(-84 \) to \(-137 \) region of the \( T1\alpha \) promoter. A, TTF-1, TGT3, and Spi sites are highlighted by bars, and the bases mutated in each site are indicated below by vertical lines. B, scheme of the mutated promoters used to drive expression of the luciferase reporter gene. All constructs contain the bp \(-170 \) to +101 promoter mutated on the indicated sites (open boxes). Transcriptional activity of the mutated promoters transiently transfected in SV40TII cells is shown as luciferase activity normalized by \( \beta \)-galactosidase activity. C, transcriptional activity with fibroblasts, contains sites for TTF-1 (25, 53, 54) and HNF-3/Fkh (27, 28) as well as a TGAGGCTCA region similar to the CREB or the steroid receptor superfamily binding sites (29).

Using the 1.25-kb \( T1\alpha \) promoter, we now provide evidence that this promoter fragment drives expression of luciferase reporter constructs in a lung epithelial cell line and is differentially regulated in epithelial cells versus fibroblasts.

Sequence analysis of the \( T1\alpha \) promoter shows the presence of binding sites for a number of ubiquitous and lung-enriched transcription factors. The bp \(-100 \) to \(-170 \) fragment, conferring differential regulation in lung epithelial cells compared with fibroblasts, contains sites for TTF-1 (25, 53, 54) and HNF-3/Fkh (27, 28) as well as a TGAGGCTCA region similar to the CREB or the steroid receptor superfamily binding sites (29). These sites are of interest because certain of these transcription factors appear to regulate expression of alveolar type II cell genes; alignment of the \(-100 \) to \(-170 \) fragment with the proximal regions of the lung-specific SP-B and SP-C promoters shows the presence of similar binding sites but in a different arrangement. These similarities in 5\'-sequence suggest that there are common regulatory properties of genes expressed by peripheral lung epithelial cells (type I, type II, and Clara cells) and that these elements in the proximal promoter are unlikely to direct gene expression that distinguishes type II from type I cells.

Of particular interest is the putative TTF-1 cis-element in the \( T1\alpha \) proximal promoter because TTF-1, a homeodomain transcription factor, has been shown to regulate several other lung-specific promoters including SP-A, -B, and -C and CCSP, all of which are expressed by alveolar type II and/or bronchiolar Clara cells (14, 19). We now show that TTF-1 binds specifically to at least one cis-element in the \( T1\alpha \) promoter and that there is a moderate increase in expression of a \(-170 \) bp \( T1\alpha \)-Luc construct resulting from increased TTF-1 protein expression. The magnitude of this increase (1.6-fold) is similar to the levels shown for the 0.23-kb SP-C promoter, where more than one TTF-1 binding site is present (19). The specific binding of the purified TTF-1 homeodomain to this fragment and the ability of a monoclonal TTF-1 antibody to interfere with the formation of the complex between and SV40TII nuclear protein and the bp \(-110 \) to \(-128 \) fragment support our conclusion that this TTF-1 binding site can influence \( T1\alpha \) expression. Mutation of

\(^8\) M. I. Ramirez, Y. X. Cao, and M. C. Williams, unpublished observations.
the TTF-1 site moderately reduced the activity of the –170 bp promoter. However, simultaneous mutation of TTF-1 and TGT3 sites notably reduced the activity of the –170 bp promoter to the level of expression of the –100 bp fragment alone, suggesting that integrity of these two sites is essential for maximal expression of the –170 bp promoter.

TTF-1 mRNA is detected at the onset of embryonic thyroid and lung development (rat) as well as in restricted areas of the embryonic brain, in a pattern that overlaps T1α expression (2, 3). It has been proposed to be a key regulator of early lung development (14, 25, 55–57). This concept is supported by studies of mice with null mutations in the TTF-1 (T/EBP) gene in which organogenesis of the lung, thyroid, ventral forebrain, and pituitary is blocked (56). In lung tissues, TTF-1 expression has been detected in type II cells (human) but not T1α-expressing type I cells (53), although this may be an artifact due to the attenuation of type I cells and the infrequent visualization of their nuclei.

The lung appears to express three TTF-1 mRNAs (25). By Western analysis, we detected two immunoreactive bands in MELE-15 cells (type II-like cells) and only one in SV40TII cells (type I-like cells). This observation suggests that expression of TTF-1 or TTF-1-like proteins may differ between lung epithelial cell types, depending on which mRNA species is produced (25). There may also be cell-specific differences in the state of phosphorylation of TTF-1 protein (47). Either of these could influence the rapid change in phenotype in cultured adult rat type II cells as they begin to express type I cell mRNAs and proteins. There are also other TTF-1 family members that recognize the same consensus sequence (58).

We also characterized a TGT3 element in the proximal promoter in this study, and our data indicate the presence of a HNF-3/Fkh site, but antibodies for HNF-3α and HNF-3β do not interfere with the complex formation. Furthermore, we found that HNF-3α and HNF-3β mRNA and protein were marginally detectable or not detectable in SV40TII nuclear extracts, although they were readily detected in MELE-15 cells. As a positive control for the conditions of DNA-protein binding and electrophoresis, we determined that HNF-3β protein and MELE-15 nuclear proteins bind this TGT3 element and that complex formation can be impaired with specific antibodies.

We believe, therefore, that proteins with no immunological identity to HNF-3α or HNF-3β but having similar DNA binding specificities to the HNF-3/Fkh family are expressed in SV40TII cells and interact with the T1α promoter. This interaction could not be competed for by an oligonucleotide known to bind HFH-1, HFH-2, and HNF-3 proteins (48), indicating that the SV40TII cell protein, currently unidentified, has other binding sequence requirements. This is reminiscent of the human lipoprotein lipase promoter that contains HNF-3-like sites that appear not to bind HNF-3 proteins (59).

The HNF/Fkh family is an extensive group of transcription factors that are thought to play important roles in tissue-specific and developmental gene regulation, and a number of family members are known to be expressed in lung. Of several HNF-3/Fkh homologues (HFHs 1–8 and 11), all except HFH-3 are expressed in lung (28, 60, 61). Also, six new Fkh family members (fkh-1 to fkh-6) (62) have been recently cloned, and four (fkh-3, fkh-6, fkh-1, and fkh-2) are expressed in the lung.

Two other forkhead proteins, FREAC1/HFHI-8 and FREAC2 (forkhead-gelated activator) have been described only in lung and placenta (49). These factors recognize sites similar to HNF-3 that are present in certain lung epithelial promoters (CCSP proximal promoter activated by FREAC1; SP-B promoter activated by FREAC1 and -2). HNF-3α and HNF-3β have been reported to bind to FREAC recognition sites in the CCSP promoter but appear to result in no transactivation or low transactivation, as shown by cotransfection studies (63, 64). These and other studies (14) raise the possibility that a FREAC or another Fkh protein binds to and transactivates T1α via this TGT3 site, a possibility that would explain some level of expression of T1α in IMR-90 cells that have been shown to express FREAC2 (49).

A detailed analysis of temporal and spatial expression patterns is required to understand the regulation of pulmonary genes, including T1α, by forkhead proteins, but this information is currently incomplete. HNF-3α and HNF-3β mRNA expression patterns have been studied (65, 66). They are expressed in the primitive foregut at the laryngotracheal groove (day 9.5) and are later found in embryonic pulmonary and tracheal epithelia with patterns that change during development. In adult lung, the HNF-3/Fkh family is expressed in a cell-specific pattern, but there is not clear information about which members of this family are expressed in type II and/or type I cells. Thus, an important goal of future studies will be to identify the specific cellular sites of expression of members of this large transcription factor family.

By aligning the proximal regions of the T1α and SP-B promoters, we detected a 9 bp sequence identical to the canonical binding site for CREB/ATF family members and some orphan members of the steroid receptor superfamily (29). In the T1α promoter, this putative binding site is juxtaposed to the TTF-1 binding region and, in the SP-B promoter, to the HNF-3 site adjacent to the TTF-1 sites. Synergistic transcriptional activation of several promoters by CREB and other cell-specific transcription factors has been reported (67). For example, cooperativity of TTF-1 and CREB is important for activation of the thyrotropin receptor promoter, enhancing expression about 20-fold. The T1α promoter is moderately transactivated by TTF-1, suggesting the possibility that maximal transcriptional activation may require other transcription factors.

Our studies indicate that the proximal 100 bp 5′ to the transcription initiation site act as a minimal promoter and that two GC-rich areas in this fragment bind Sp1 and Sp3 proteins. These proteins have been shown to mediate basal transcriptional activation (45, 68) and also to regulate some cell-specific promoters, such as the lung epithelial CCSP (24), SP-B (31), and MUC1 (69) genes. They may also act cooperatively with other transcription factors to regulate promoter activity (45, 70). The proximity in the T1α promoter of an active Sp1/Sp3 site to the TGT3 and TTF-1 sites and the reduced transcriptional activity of the mutated Sp1/Sp3 site in the context of the –170 bp promoter suggest that such an interaction may be functionally important.

We believe that the identification of T1α as a molecular marker for type I cells provides an important new tool for studies of lung development and lung cell differentiation, particularly when used in conjunction with SP-C, a definitive marker for the type II cell phenotype. We have recently cloned 10 kb of the T1α 5′-flanking sequence. Additional studies of the cis- and trans-acting elements that regulate the T1α promoter will be directed toward identifying molecular mechanisms that differentiate between these two cells.

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