

TGT3, Thyroid Transcription Factor I, and Sp1 Elements Regulate 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) homeodomain, 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 TTF-1 expression vector moderately transactivated 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; hrSp1, human recombinant Sp1; SV40TII, SV40T type II cells; FREAC, forkhead-related activator; HFH, HNF-3/forkhead homolog.

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 *T1α* 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 *T1α*. 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; *Hae*III-partially cut DNA ligated into λ gt10 vector) was amplified into 10 fractions that were tested by PCR for the *T1α* cDNA 5'-end using oligos P1 (5'-CCACTAGCTGCTGAGGCTCCAA-3') and P2 (5'-GAGTCCCAGAACCAACG-3') (2). Fractions 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 reprobated. DNA from five different clones, uncut or *Eco*RI-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 *Bam*HI, 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 *Eco*RI, *Bam*HI 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 *T1α* cDNA was previously obtained by rapid amplification of cDNA ends-PCR (2). The transcription initiation site was determined by primer extension (32). The P5 oligonucleotide complementary to nucleotides +82 to +115 (Fig. 1) was end-labeled with [γ -³²P]ATP and purified through Nuc-Trap 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 3 h under mineral oil. After extending the probe using 5 units of avian myeloblastosis virus 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 *Sac*I and partial digestion with *Eco*RI. The 1352-bp segment (-1251 to +101 bp) was cloned into pBluescript SK. -789bp-Bluescript was prepared by *Sac*I and *Avr*II digestion of -1251bp-Bluescript and insertion of the -789 to +101 bp fragment into the *Sac*I-*Xba*I site of pBluescript SK. This construct was further digested with *Pst*I and *Bam*HI 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, -243 bp, -286 bp, -396 bp, -562 bp, and -661 bp of the promoter were sequenced to determine their exact lengths. All constructs were digested with *Kpn*I-*Sac*I prior to insertion of the fragments into pGL-3 Basic Luciferase reporter vector (Promega). All *T1α* 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 oncogene SV40 large tumor antigen (33). IMR-90 is a human lung fibroblast cell line (ATCC CCL 186). MLE-15 cells (provided by Dr. J. Whitsett, 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-

motor (SP-C) (34). SV40TII cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.), 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc.), 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate. IMR-90 cells were grown as described in the ATCC protocol (Rockville, MD). MLE-15 cells were grown as described by Wikenheiser *et al.* (34).

SV40TII cells were transfected by the DEAE-dextran/chloroquine method (35). Cells were seeded onto 10-cm plates and used at about 60% confluency. 15 μg of total DNA, purified by Qiagen-tip 500, (Qiagen Inc., Chatsworth, CA) was mixed with 450 μg of DEAE-dextran (Pharmacia Biotech Inc.) in 3 ml of phosphate-buffered saline and added to the plates. Cells were incubated for 30 min at 37 °C. DMEM, 10% FBS (4.5 ml) and 135 μl of 8 mM chloroquine (Sigma) were added, and the incubation was continued for 2 h. The transfection mixture was replaced by DMEM without FBS for 2 h followed by DMEM, 10% FBS for 48 h. Cells were cotransfected with 1.5 μg of cytomegalovirus (CMV)-*lacZ* construct to normalize data for transfection efficiency. For TTF1 cotransfection studies, 3 μg of CMV-TTF1 (provided by Dr. R. Di Lauro, Stazione Zoologica A. Dohrn, Napoli, Italy) or 3 μg of control pCMV (provided by Dr. Z. Xiao, Boston University, MA) was added to the transfection mixture. Cells were extracted and assayed for luciferase activity using the Promega Luciferase Assay kit (Promega) and assayed for β-galactosidase activity as described by Sambrook (36). IMR-90 cells were transfected with 15 μg of DNA by the calcium phosphate method as modified by Chen and Okayama (37). Data are expressed as the mean of at least three experiments (duplicate samples) ± S.E. Values are presented relative to the level of expression of promoterless construct 0bp-Luc. Data were analyzed by *t* test with differences *p* ≤ 0.05 considered significant.

Northern and Western Blotting of Cell Lines—Total RNA was purified using TRIzol Reagent (Life Technologies, Inc.). Northern blots were performed by the glyoxal/(CH₃)₂SO denaturation method (36). Total RNA (20 μg/lane) was electrophoresed on 1.5% agarose gels, blotted, and hybridized by standard procedures (36). Probes were labeled by the hexamer-random prime method (38). Rat *T1α* probe was a 520-bp cDNA fragment containing the coding region, and SP-C probe was 574 bp from the rat coding region (2). A 552-bp β-actin probe was used as a control for loading (2). Hybridizations were done in 50% (v/v) formamide solution containing 1–2 × 10⁶ cpm/ml. Membranes were hybridized overnight at 42 °C, washed twice in 2 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate), 0.2% SDS at 65 °C for 1 h and once in 0.2 × SSC, 0.2% SDS at 65 °C for 30 min and then exposed at -70 °C for 3–12 h. Western blots were done exactly as described previously (3). For detection of TTF-1, nuclear proteins (20 μg/lane), purified as below, or rat lung total protein were analyzed by Western blotting. Anti-TTF-1 monoclonal antibody (a gift from Dr. Whitsett) was used at 1:1000 dilution.

Nuclear Extract Preparation—Nuclear extracts were prepared using a miniextraction procedure (14). Protein concentrations were determined by a modified Bradford method (Bio-Rad) using bovine serum albumin as standard.

Synthetic Oligonucleotides—Complementary oligonucleotides with three or four bases of protruding 5'-ends were synthesized and purified by 8% denaturing polyacrylamide gel electrophoresis. Annealing was performed at oligonucleotide concentrations of 10 μM in 50 μl 10 mM Tris (pH 7.5), 10 mM MgCl₂, 50 mM NaCl. Mixtures were heated to 95 °C for 5 min in a dry block and cooled slowly to room temperature. Annealed oligonucleotides (20 pmol) were labeled using [α -³²P]dCTP and DNA polymerase large Klenow fragment (New England Biolabs, Beverly, MA), purified on Nuc Trap columns (Stratagene), recovered in a final volume of 100 μl at ~200,000 cpm/μl, and diluted 1:10 before use.

Electrophoretic Mobility Shift Assay (EMSA)—Assays were performed as described by Bohinski *et al.* (14). All binding reactions were performed on ice. Nuclear protein extract (10 μg, unless otherwise stated) and 1 μg of poly[d(I-C)] (Boehringer Mannheim) were incubated in 20 μl of buffer (12 mM Hepes, pH 7.9, 4 mM Tris, pH 7.9, 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) for 10 min. Labeled oligonucleotide (15–20 fmol, ~20,000 cpm) was added prior to a 20-min incubation. For competition experiments, unlabeled oligonucleotides were incubated with proteins for 10 min before the addition of labeled oligonucleotide. For supershift analyses, mixtures were incubated with 1–2 μl of anti-TTF-1 monoclonal antibody, nonspecific monoclonal antibody (anti-cyclin B1, Oncogene Science, Cambridge, MA), or anti-Sp1, -Sp3, and -Sp4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). TTF-1 homeodomain (TTF-1 HD) was produced in *Escherichia coli* as a glutathione S-transferase fusion protein (Pharmacia) using a construct from Dr. C. Mendelson (University of Texas Southwestern Medical Center, Dallas, TX).

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-Sp6 coupled kit (Promega); HNF-3β 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' *Kpn*I and 3' *Eco*RI restriction site. The 5'-primer used was a 36-mer containing the 5' wild type sequence. The 3'-primers were mutated oligonucleotides complementary to the 3'-sequence. PCR fragments were purified and digested with *Kpn*I and *Eco*RI restriction enzymes. -170Luc was digested with *Kpn*I and *Eco*RI; wild type fragment was removed by agarose electrophoresis, and mutated fragments were inserted into those sites. For the mutated Sp1 site, the PCR fragment was generated from -104 to +101 flanked by a 5' *Eco*RI and 3' *Sac*I 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 *Eco*RI and *Sac*I, 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 (TAAATT) 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.²

Characterization of the Cell Lines—SV40TII and IMR-90 cells and adult rat lung were analyzed for *T1α* and SP-C mRNAs and *T1α* protein expression. High levels of *T1α* mRNA and protein are detectable in adult lung and SV40TII cells (Fig. 3, A and B). Neither *T1α* mRNA nor protein is detectable in IMR-90 cells. SP-C mRNA is undetectable in both cell lines but abundant in lung. MLE-15 cells express SP-A, -B, and -C mRNA (34), but they do not express *T1α* (data not shown).

SV40TII cells therefore appear to be a good model for studying regulation of *T1α*, since they have high levels of its mRNA and do not express the type II SP-C (Fig. 3A), SP-A, or SP-B genes (33). IMR-90 cells, expressing neither *T1α* nor type II cell genes, were used as nonexpressing control cells.

Transcriptional Activity of the *T1α* Promoter in Epithelial and Nonepithelial Lung Cell Lines—Twelve *T1α* promoter-luciferase constructs were used in transient expression studies to define regulatory elements required for promoter activity (Fig. 4). In SV40TII cells, the bp -1251 to +101 promoter (-1251bp-Luc) drives luciferase expression about 85-fold over that from a promoterless control construct (Obp-Luc) (Fig. 4). The expression of luciferase constructs in epithelial cells is about ~1.5–

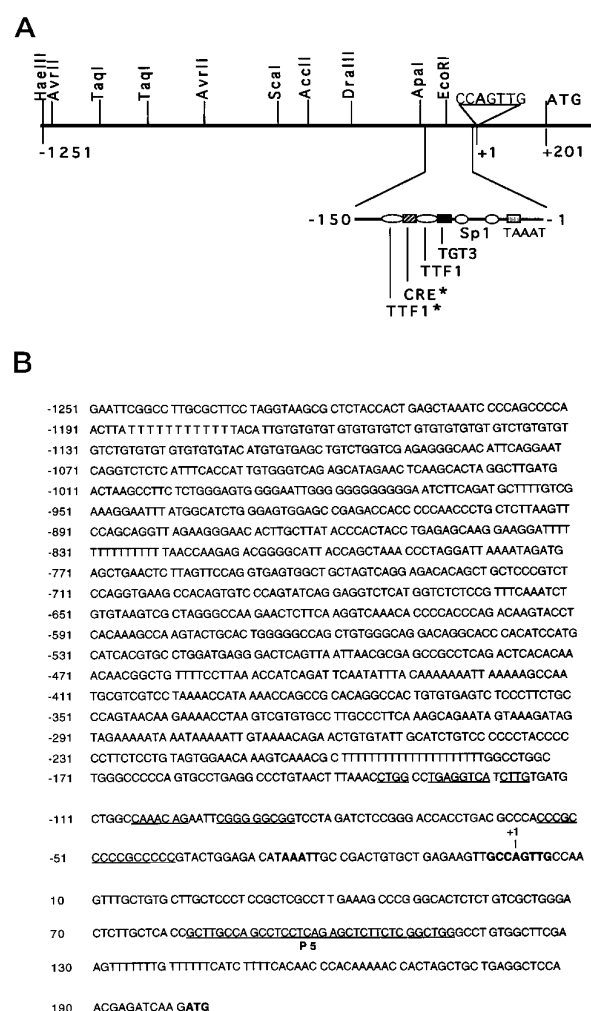


FIG. 1. Analysis of the *T1α* promoter sequence. Shown is a schematic representation of the *T1α* 5'-flanking region (upper panel) showing the restriction map, transcription initiation site (+1), and TATA-like box position relative to the translation initiation site. The bp -1 to -150 fragment contains Sp1, TGT3, and TTF-1 sites analyzed in this work and putative (*) TTF-1 and CRE binding sites. The sequence in the lower panel shows 1.25 kb of the *T1α* promoter and the 5'-untranslated region to the start codon. The TATA-like box and the transcription initiation site are shown in boldface type. Consensus elements analyzed in this study and the oligonucleotide P5 used for mapping the transcription initiation site are underlined.

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 HeLa 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 from 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-

² R. Costa, unpublished data.

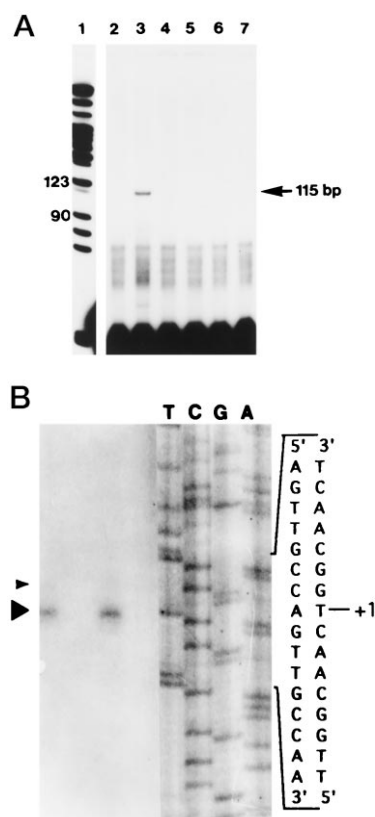


FIG. 2. Transcription initiation site characterized by primer extension. P5 oligonucleotide (Fig. 1) was designed to obtain an extension product of 100–150 bp. **A**, polyacrylamide gel electrophoresis (8%) analysis of the extension reaction at 55 or 65 °C using RNA (15 µg) from SV40TII or IMR-90 cells. 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.

ground, identifying potential stimulatory elements in that region (Fig. 4). In contrast, deletion constructs from bp –789 to –396 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 step-wise 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 transactivating 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—

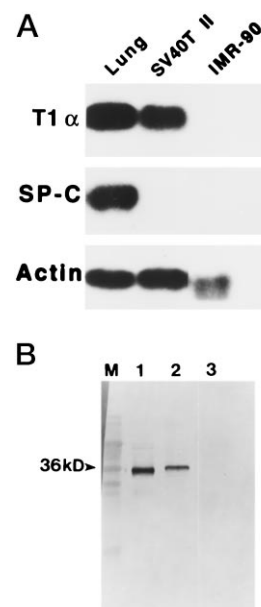


FIG. 3. Northern and Western analyses of SV40TII and IMR-90 cell lines and adult rat lung. **A**, total RNA (20 µg/lane) was hybridized with ³²P-labeled probes for *T1α*, *SP-C*, and *β-actin*. **B**, Western analysis for *T1α* protein expression of cell lines compared with adult rat lung (10 µg of protein/lane) using monoclonal anti-*T1α* antibody detected with an alkaline phosphatase-conjugated second antibody. Lane 1, lung; lane 2, SV40TII cells; lane 3, IMR-90 cells; lane M, markers.

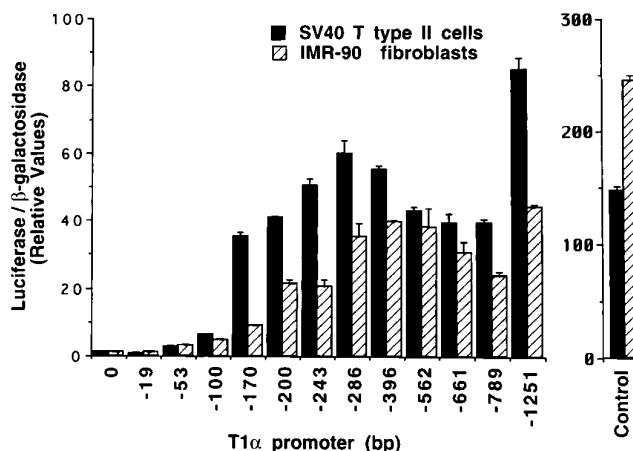


FIG. 4. Deletion studies of the *T1α* promoter. Relative activity of the luciferase reporter gene in transiently transfected SV40TII (black bars) and IMR-90 cells (hatched bars) with the indicated 5'-deletion constructs normalized for *β*-galactosidase activity. Luciferase activity is expressed relative 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

TABLE I

Sequences of the oligonucleotides used in the electrophoretic mobility shift assays

	Oligonucleotides
-56 CCGCCCCCGC CCCGTACTGGAGACATAAATGCCG -20	I
-97 TTCGG GGGCGG TCTAGATCTCCGGGACCACCTGACGCCA -57	II
-146 TAACTTTAAAC CTG CCCT GAGGTCA CTTGTGATGCTGCC CAAA CAGAATT -96	III
GAGGTCATCTTGTGATGCT	IIIA
GAGGTCAT AGTGT GATGCT	IIIA mut
GCTGGCC CAAA CAGAATT	IIIB
GCTGGCC CAACG GAATT	IIIB mut
CGAAAT TAACA ATTACGTGC	HNF-1#3(-)

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.

Lung-enriched Transcription Factors Interact with the bp -100 to -170 Region—Deletion studies show that the bp -100 to -170 fragment confers differential regulation of luciferase expression in SV40TII and IMR-90 cells (Fig. 4). To analyze this region further, we synthesized an oligonucleotide spanning bp -96 to -146 (oligonucleotide III, Table I). This region contains putative binding elements for hepatic nuclear factor-3/forkhead (HNF3/Fkh) and TTF-1 family proteins, both of which have been shown to regulate transcription of lung-specific genes expressed by type II and Clara epithelial cells (12, 14, 17, 19, 23, 24). Alignment of *T1α* oligonucleotide III with proximal regions of the SP-A, SP-B, and SP-C promoters (14, 19) shows that they are strikingly similar and contain TTF-1, TGT3, and CRE-like sites (Fig. 6).

We therefore analyzed binding activities of SV40TII and IMR-90 cell nuclear extracts using oligonucleotide III. These data show DNA-protein interactions with complex patterns (Fig. 7). Both extracts contain proteins that bind specifically to this region, although SV40TII proteins form more complexes than IMR-90 nuclear proteins and the complexes differ in their mobility (Fig. 7). This suggests the likelihood that the up-regulation of luciferase expression observed with the -170bp-Luc construct may be due to specific binding of nuclear proteins expressed by the lung epithelial cells. To characterize these binding elements further, we prepared two overlapping oligonucleotides (oligonucleotides IIIA and IIIB, Table I) that contain some of the putative binding sites in this region.

The Lung-enriched Transcription Factor TTF-1 Binds to the *T1α* Promoter—Oligonucleotide IIIA (bp -128 to -110) contains a putative TTF-1 site and part of a putative CRE site. This oligonucleotide forms two complexes (Fig. 8, bands I and II) with SV40TII nuclear proteins under the conditions tested. Band II can be competed by a 100–500-fold excess of unlabeled oligonucleotide (Fig. 8). A nonspecific oligonucleotide (*oligo IIIB* in Fig. 8) or a oligonucleotide mutated in two bases of the TTF-1 putative site (oligonucleotide IIIA mut, Table I) (Fig. 8)

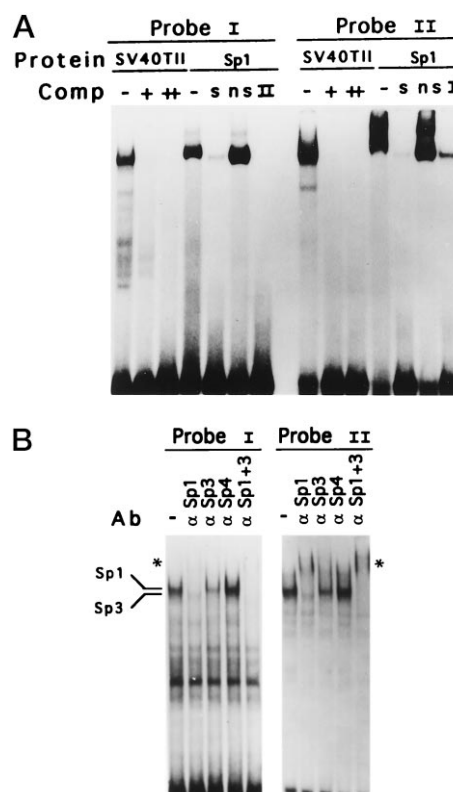


FIG. 5. The -95 bp region of the *T1α* promoter contains two Sp1-like binding elements. A, EMSAs were performed using ³²P-labeled oligonucleotide I (from bp -20 to -56) and ³²P-labeled oligonucleotide II (from bp -57 to -95). Binding of SV40TII nuclear extract (10 μg) to both oligonucleotides was competed by a 100- (+) and 1000-fold (++) excess of unlabeled specific oligonucleotide. Binding of human recombinant Sp1 protein (1 footprint unit) to oligonucleotide I was competed by a 1000-fold excess of specific oligonucleotide (s) or oligonucleotide II (II) but not by a 1000-fold excess of nonspecific oligonucleotide (ns). Similar competition results are shown with oligonucleotide II except for incomplete competition by a 1000-fold excess of oligonucleotide I (I). B, supershift analyses of the complexes formed by SV40TII nuclear extract and oligonucleotides I and II. Monoclonal anti-Sp1, -Sp3, and -Sp4 (2 μl) were incubated for 15 min with the nuclear extracts before the labeled probe was added. Oligonucleotides I and II bind Sp1 and Sp3 proteins. *, supershifted complexes.

does not interfere with complex formation. Band I is competed by a 100–500-fold excess of unlabeled 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 IIIA (Fig. 9B). Binding is competed by a 500-fold excess of unlabeled oligonucleotide but not by a

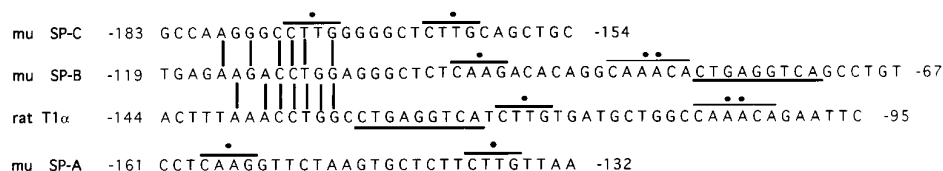


FIG. 6. **Alignment of the proximal regulatory regions of lung specific promoters.** These proximal regions of lung-specific promoters are involved in the transcriptional regulation of alveolar epithelial genes and bind lung-enriched transcription factors. Shown are the murine surfactant proteins SP-A (19), SP-B (14, 19), and SP-C (19) and rat *T1α*. These fragments show a cluster of similar binding sites in a different arrangement. Highlighted are TTF-1 sites (*), TGT3 sites (**), and a CRE-like element (underlined). The vertical lines show similarities in the TTF-1 binding site used to align the promoter fragments.

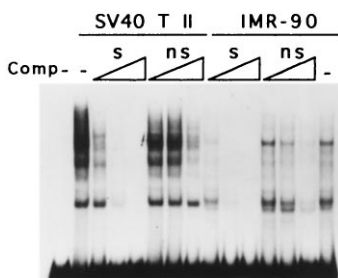


FIG. 7. **EMSA indicating differential binding of SV40TII and IMR-90 cell nuclear proteins to the bp -95 to -146 region.** SV40TII and IMR-90 nuclear extracts (10 μ g of total protein) were incubated with oligonucleotide III (bp -95 to -146). Competition assays (Comp) were done with excess unlabeled 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 SV40TII cells, in agreement with the differences in the level of expression of the -170bp-*T1α* deletion construct in the cell lines.

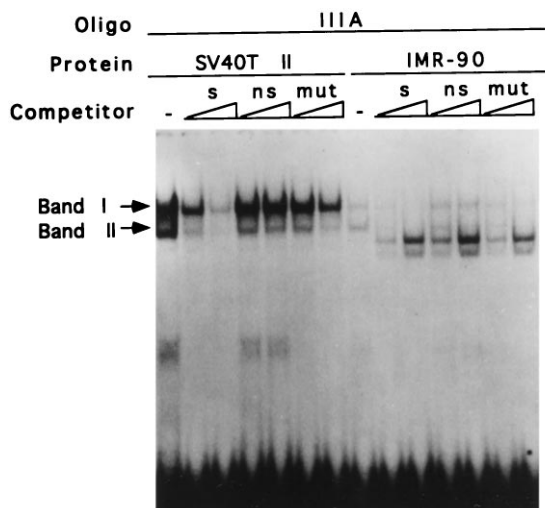


FIG. 8. **A TTF-1 minimal consensus sequence in the *T1α* promoter specifically binds alveolar epithelial cell line nuclear proteins.** SV40TII and IMR-90 nuclear extracts (20 μ g) were incubated with oligonucleotide IIIA spanning bp -110 to -128. Two complexes (I and II) were formed with SV40TII nuclear 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 (SV40TII) but is less intense. The new band (*) seen in IMR-90 competition experiments is believed to be nonspecific.

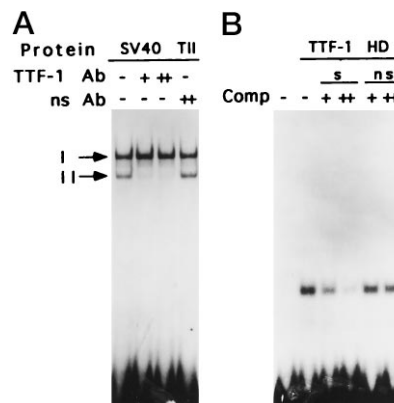


FIG. 9. **The TTF-1 minimal consensus sequence binds both TTF-1 protein in the SV40TII nuclear extract and recombinant TTF-1 HD.** A, monoclonal anti-TTF-1 antibody (1 μ l (+) or 2 μ l (++)) interferes with the formation of complex II, while an excess (++) of nonspecific antibody (ns Ab) does not change 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 CTTG site in the *T1α* 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 SV40TII, 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 (doublet at about 55 kDa). In SV40TII, 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 *T1α* Promoter by TTF-1—To determine if the TTF-1 binding site can activate the *T1α* promoter *in vivo*, co-transfection experiments were performed in SV40TII cells

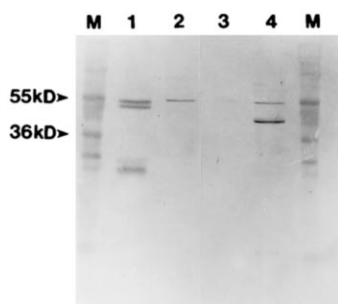


FIG. 10. **Characterization of TTF-1 protein expression in SV40TII, IMR-90, and MLE-15 cell lines.** Western analysis was performed with nuclear protein (20 μ g) from cell lines or total adult lung protein and blotted with anti-TTF-1 detected with an alkaline phosphatase-labeled secondary antibody. Lane 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 masses 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).

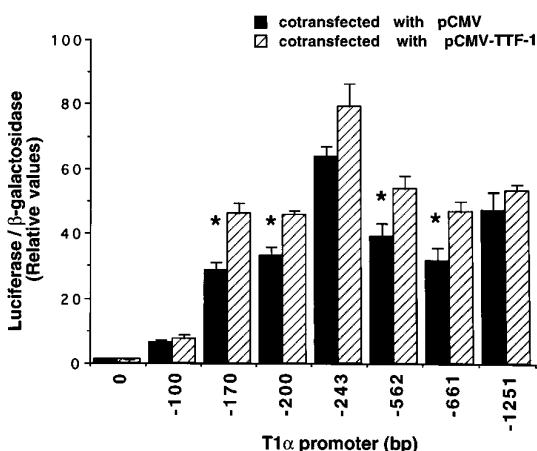


FIG. 11. **TTF-1 protein transactivates *T1α* promoter deletion constructs when co-transfected in SV40TII cells.** Normalized luciferase activity in SV40TII cells transiently transfected with the indicated 5' deletion constructs in the presence of coexpressed pCMV-TTF-1 (hatched bars) or pCMV alone (black bars). Data, relative to the promoterless plasmid 0bp-Luc (pGL3) in each condition, are expressed as the mean $\pm$ S.E. of two different transfections analyzed in duplicate. Asterisks indicate statistically significant differences determined by the *t* test; $p \leq 0.05$.

(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-3 β 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-3 β protein (Fig. 12A). HNF-3 β 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-3 α or anti-HNF-3 β fails to disrupt the SV40TII protein-DNA complexes, although anti-HNF-3 β supershifts the complex formed by the recombinant protein (data not shown). Anti-HNF-3 α and anti-HNF-3 β also impaired complex formation with MLE-15 nuclear extracts (data not shown) that have HNF-3 α and HNF-3 β 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-3 α or HNF-3 β 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-3 β 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-3 β 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-3 α or anti-HNF-3 β 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

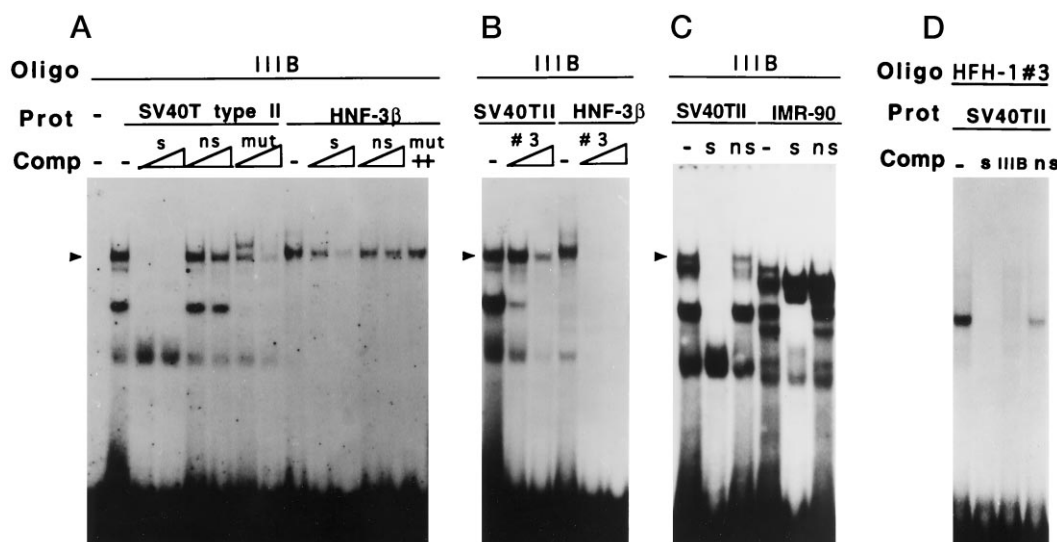


FIG. 12. A TGT3 site in the bp -96 to -112 fragment binds SV40TII cell nuclear extracts and HNF-3 β protein. A, oligonucleotide IIIB was incubated with 10 μ g of SV40TII nuclear extract or $\sim$ 3 ng of recombinant HNF-3 β protein. Competition assays were performed in the presence of a 100- and 500-fold 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 IIIB. 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 IIIB, or nonspecific oligonucleotide (ns). Gel was exposed 5 h.

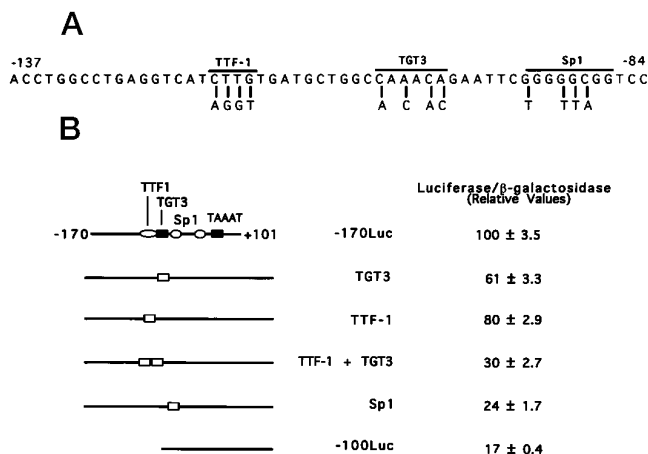


FIG. 13. Mutational analysis of the bp -84 to -137 region of the *T1α* promoter. A, TTF-1, TGT3, and Sp1 sites are highlighted by bars, and the bases mutated in each site are indicated below by vertical lines. B, scheme of the mutated promoters used to drive expression of the luciferase reporter gene. 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 β -galactosidase activity and expressed relative to the wild type promoter activity (100%). Data are the mean of three or four experiments done in triplicate $\pm$ S.E.

restricted sites in the adult (2, 3). This temporal and spatial restriction of *T1α* expression suggests that cell-specific mechanisms regulate transcription of this gene. Preliminary observations of transgenic animals (1.25-kb *T1α* promoter-chloramphenicol acetyltransferase) suggest that the 1.25-kb *T1α* promoter contains much of the information needed for this complex pattern of expression in both fetuses and adults.³ Others have reported that 13 kb of 5'-flanking sequence is required to limit expression of SP-C to alveolar type II epithelial cells (52).

Using the 1.25-kb *T1α* 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 *T1α* 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 TGAGGTCA 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 *T1α* 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 *T1α* promoter and that there is a moderate increase in expression of a -170 bp *T1α*-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 *T1α* expression. Mutation of

³ 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 *T1 α* 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 *T1 α* -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 MLE-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-3 α and HNF-3 β do not interfere with the complex formation. Furthermore, we found that HNF-3 α and HNF-3 β mRNA and protein were marginally detectable or not detectable in SV40TII nuclear extracts, although they were readily detected in MLE-15 cells. As a positive control for the conditions of DNA-protein binding and electrophoresis, we determined that HNF-3 β protein and MLE-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-3 α or HNF-3 β but having similar DNA binding specificities to the HNF-3/Fkh family are expressed in SV40TII cells and interact with the *T1 α* 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/HFH-8 and FREAC2 (forkhead-related 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-3 α and HNF-3 β 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 *T1 α* via this TGT3 site, a possibility that would explain some level of expression of *T1 α* 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 *T1 α* , by forkhead proteins, but this information is currently incomplete. HNF-3 α and HNF-3 β 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 *T1 α* 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 *T1 α* 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 *T1 α* 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 *T1 α* 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 *T1 α* 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 *T1 α* 5'-flanking sequence. Additional studies of the *cis*- and *trans*-acting elements that regulate the *T1 α* promoter will be directed toward identifying molecular mechanisms that differentiate between these two cells.

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