The lung enriched transcription factor TTF-1 and the ubiquitously expressed proteins Sp1 and Sp3 interact with elements located in the minimal promoter of the rat Clara cell secretory protein gene

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The mechanisms that direct expression of the Clara cell secretory protein (CCSP) gene to the bronchiolar epithelial cells of the lung remain to be elucidated. Previous studies have identified a number of proteins which bind to a functionally important region (Region 1) located −132 to −76 bp from the transcription start site in the rat CCSP gene. Subsequently we have shown that while Region 1 is an important positive regulator of CCSP gene expression, sequences 3′ of this region (−75 to +38) are sufficient to confer tissue-specific expression of a reporter gene. In the present study we have used transient transfections with a deletion series of CCSP–CAT reporter plasmids (where CAT is chloramphenicol acetyltransferase) and gel mobility shift assays with a series of overlapping oligonucleotides covering the whole minimal promoter region to study protein–DNA interactions within this region. These studies have identified a conserved functional binding site for the lung and thyroid enriched homeodomain transcription factor TTF-1, located between positions −51 and −42 from the transcription start site. CCSP–CAT chimaeric reporters containing this region are specifically activated by TTF-1 in co-transfection assays, and nuclear extracts from cells which express TTF-1 bind to this region, as does *in vitro* translated rat TTF-1. Three additional conserved regions were identified, and in further gel mobility shift studies with an oligonucleotide spanning the conserved region immediately 5′ to the TTF-1 site we identified a binding site for the ubiquitously expressed zinc-finger-containing proteins Sp1 and Sp3. These studies suggest that cell-type-restricted and ubiquitous nuclear proteins may play a combined role in the regulation of the CCSP gene within the bronchiolar epithelium by interacting with the minimal promoter region.

INTRODUCTION

It is clear that cellular differentiation and development are the result of regulated gene expression due to co-ordinated activation and/or repression. This regulation occurs primarily at the transcriptional level through the action of *trans*-acting factors which exert their effects on *cis*-acting DNA motifs in the regulatory regions of target genes. Within the pulmonary epithelium such regulation gives rise to distinct subsets of genes being activated within certain specialized cell types. One such cell type is the Clara cell of the bronchiolar epithelium. Within this cell are expressed a number of cell-restricted gene products, one of the most significant of which is the Clara cell secretory protein (CCSP). This abundant protein of lung secretions is localized by immunohistochemistry to the secretory granules of Clara cells [1]. Although the function of CCSP is unknown, the abundance and cell-specific nature of this protein makes it an ideal model to elucidate the mechanisms of pulmonary epithelial cell-specific gene expression [2,3]. Studies have been performed on the rat, human and mouse CCSP genes, as well as on the rabbit homologue, uteroglobin, which in addition to being expressed in the lung is also expressed within the reproductive tract [4]. Expression of the CCSP gene in the bronchiolar epithelium is confined to non-ciliated cells and recapitulates the process of cellular differentiation in the proximal respiratory epithelium during development [5–7].

Transgenic studies indicated that *cis*-acting elements within the first 2.25 kb of the 5′ flanking region of the rat CCSP gene are sufficient to orchestrate this tissue- and cell-specific expression [8,9]. Initial characterization of the *cis*-acting elements that regulate CCSP gene expression identified a region 5′ to the transcription start site (positions −132 to −76; CCSP I) which is bound by a number of transcription factors, the most prominent of these being members of the *forkhead/winged helix*/hepatic nuclear factor-3 (HNF-3) transcription factor family [10,11]. Transfection studies suggest that the CCSP I region is not required for cell-type-specific transcription of the CCSP gene, as a CCSP–CAT construct (where CAT is chloramphenicol acetyltransferase) with sequence 3′ to the CCSP I region is active only in pulmonary cell lines [12]. More recent transgenic studies have shown that cell-type-specific expression of a reporter gene can be directed appropriately with as little as 87 bp of the mouse CCSP gene promoter [13]. It appears, therefore, that the proximal promoter region plays an important role in mediating the tissue-specific expression of the CCSP gene. Identification of the DNA elements within this region and the *trans*-acting factors which bind to them may help in elucidating the pathways regulating the tissue specificity of the CCSP gene.

In addition to members of the *forkhead/winged helix*/HNF-3 transcription factor family, other tissue-restricted and ubiquitous regulatory proteins are found within the lung. One such tissue-restricted regulator is the lung and thyroid enriched homeodomain-containing transcription factor TTF-1 (also known as NKX2.1) [14]. TTF-1 has been shown by *in situ*

Abbreviations used: CAT, chloramphenicol acetyltransferase; CCSP, Clara cell secretory protein; CMV, cytomegalovirus; HNF, hepatic nuclear factor; NF-κB, nuclear factor-κB; SP-A and SP-B, surfactant apoproteins A and B; TTF-1, thyroid transcription factor-1.

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hybridization to be localized within the bronchiolar epithelium of the lung [15], and more recently it has been shown that TTF-1, along with HNF-3 family members, plays an important role in the regulation of expression of the surfactant apoprotein A and B (SP-A and SP-B) genes [16–18]. In the present study we have used transient transfection and gel mobility shift studies to identify DNA binding proteins which interact with the minimal promoter region of the rat CCSP gene. In this manner we were able to identify a TTF-1 binding site located within 50 bp of the transcription start site of the gene. Additionally we have also identified a conserved DNA motif just 5′ to the TTF-1 binding site which we have shown to be bound by the ubiquitously expressed zinc-finger-containing transcription factors Sp1 and Sp3.

MATERIALS AND METHODS

Plasmids and construction of chimaeric genes

Specific regions of the rat CCSP gene promoter corresponding to positions −315, −166 and −75 from the transcription start site were amplified by PCR using oligonucleotide primers containing *Bam*HI sites, and subcloned into the *Bam*HI site of plasmid p0CAT-BS as described previously [11]. Additional constructs corresponding to positions −65 and −55 from the transcription start site were generated in the same manner. Full-length rat TTF-1 was amplified by PCR and inserted in sense and antisense orientation into a cytomegalovirus (CMV) expression vector, pCR III (Invitrogen) to yield pCMVrTTF-1 and pCMVrTTF-1 (AS). All chimaeric constructs were sequenced in both directions by the dideoxy chain-termination method to determine orientation and to confirm the fidelity of the amplification.

Tissue culture, transient transfections and reporter gene assays

Human lung-derived NCI-H441 and A549 cells and human liver-derived HepG2 cells were obtained from the American Tissue Culture Collection, and were maintained as monolayers in RPMI medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin and 150 μg/ml streptomycin. The human endometrial cell line Ishikawa was obtained from Dr. J. D. Croxtal (Biochemical Pharmacology, St. Bartholomew's Hospital), and was maintained as for the other lines. Growing cultures of the rat thyroid cell line FRTL-5 were obtained from Rose Davis (University of Sheffield, U.K.). For transfection studies, cells were transfected at 70% confluency in 35 mm-diam. dishes using 2.5 μg of CCSP–CAT chimaeric constructs, carrier DNA (pBS) up to 7.5 μg and 20 μl of Lipofectin (Gibco), except for the Ishikawa cells which were transfected with 10 μl of Lipofectamine (Gibco). Transfection mixtures were placed on cells for 5 h, after which time the cells were washed three times with PBS and re-fed with complete growth medium. In the appropriate experiments some carrier DNA was replaced with 2.5 μg of transcription factor expression plasmids. pSV2CAT-BS and −103Cp-CAT, containing the rat caeruloplasmin gene minimal promoter [19], were used as appropriate controls. CAT assays were performed as described previously [11] using a constant amount of protein. To control for efficiency of transfection, all transfections were performed at least four times.

Nuclear extract production, *in vitro* translation reactions and gel mobility shift assays

Nuclear extracts were prepared from confluent monolayers of tissue culture cell lines (1×10^7 cells), in the presence of proteinase inhibitors, as described previously [20]. Extracts were divided into aliquots and stored at −70 °C. *In vitro* transcription and translation of rat TTF-1 was performed with rabbit reticulocyte lysate using the TnT system (Promega) in the presence of ^{35}S-labelled amino acids. Reactions were primed with either SP6 (sense) or T7 (antisense) polymerase. SDS/PAGE was performed to ensure that the appropriate reaction products were generated.

For gel mobility shift experiments, overlapping complementary oligonucleotides were annealed and labelled by filling in regions with [^{32}P]dNTPs and Klenow polymerase. The following oligonucleotides were used in the present study. TTF-1 binding site from the thyroglobulin gene: wild type, 5′ CACTGCCCAGTCAAGTGTTCTTGA 3′; mutated, 5′ CACTGCCCAG**ATATC**TGTTCTTGA 3′ [21] (altered bases in **bold**); T/EBP binding site from the human thyroid peroxidase gene, 5′ GGGATTCTTCACACTTCATAGAGCTCGAGATC 3′ [22]; TTF-1 binding site from the human SP-B gene (SPBf1-SPBf2), 5′ CACCTGGAGGGCTCTTCAGAGCAAAGACAAACACTGAGGT 3′ [23]; Sp1 consensus binding site, 5′ ATTCGATCGGGGCGGGGCGAG 3′. The non-specific competitor oligonucleotide was an NF-κB site, 5′ ACAGAGGGGACTTTCCGAGAGG 3′ [11]. The specific CCSP gene oligonucleotides used in this study are shown in Figure 4 (upper panel). Approx. 1 ng of probe (specific radioactivity 10^7 c.p.m./μg) was used per binding reaction. Binding reactions were performed at room temperature in a 20 μl reaction mixture as described [11]. When *in vitro* translated proteins were used in gel mobility shift experiments, 1–2 μl of the translation reaction was used in place of nuclear extracts. Antisense primed *in vitro* translation reactions were used as controls. Following electrophoresis, gels were fixed, dried and exposed to film at −80 °C. In some reactions a 100-fold molar excess of unlabelled competitor oligonucleotides was included in the reactions. For Sp1/Sp3 antibody supershift studies, the gel shift reactions were prepared as above except that 1 μl of antiserum monospecific for human Sp1 or Sp3 [24], or 1 μl of preimmune serum, was added to the reaction 10 min prior to the addition of the labelled probe.

RESULTS

TTF-1 specifically transactivates the rat CCSP gene promoter

To begin to isolate potential TTF-1-responsive regions of the rat CCSP gene we performed transient transfections into HepG2 cells with the rat −315 CCSP–CAT plasmid, a construct equivalent to the human region which we have previously shown to be TTF-1 responsive (S. Gowan and C. D. Bingle, unpublished work), in the absence and presence of pCMVrTTF-1. When the −315 CCSP–CAT reporter was transfected alone, no CAT activity was detected (Figure 1, lane 3), but when pCMVrTTF-1 was co-transfected a marked induction of expression of the reporter was seen (Figure 1, lane 4). To ensure that this transactivation was the specific result of TTF-1 expression in these cells, further transfections were performed with the rat −315 CCSP–CAT construct and differing amounts of sense and antisense rat TTF-1 constructs, where the only difference in the effector construct was the orientation of the TTF-1 insert. As the amount of sense TTF-1 plasmid was decreased (Figure 1, lanes 4–7) the transactivation of the CCSP promoter was also decreased, and when antisense TTF-1 alone was used (Figure 1, lane 8) no CAT activity was seen, similar to when the CCSP construct was transfected alone (Figure 1, lane 3). These studies suggest that transactivation of the CCSP gene reporter is the result of expression of sense TTF-1 within the cells. To further demonstrate the specficity of TTF-1 transactivation of the CCSP promoter, similar transfections performed with rat caeruloplasmin gene reporter constructs [19] failed to show activation by TTF-1, and additional experiments which replaced the TTF-1

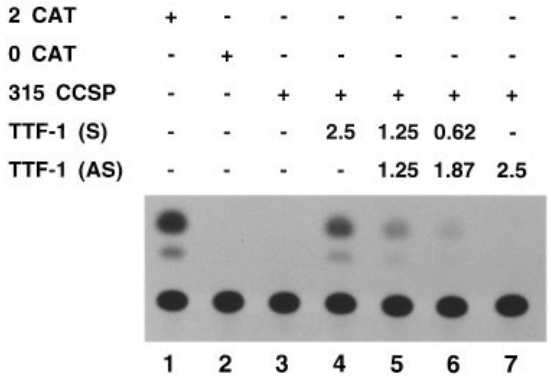

Figure 1 TTF-1 specifically transactivates the rat CCSP gene promoter

Transient transfections were performed by lipofection into HepG2 cells using 2.5 μg of SV2CAT-BS (2 CAT; lane 1), 0CAT-BS (0 CAT; lane 2) and rat −315 CCSP–CAT (315 CCSP; lanes 3–7) in the absence (lanes 1–3) or presence (lanes 4–7) of decreasing amounts of pCMVrTTF-1 [TTf-1 (S)]. As the amount of pCMVrTTF-1 was decreased it was replaced with the indicated amount of antisense pCMVrTTF-1 [TTF-1 (AS)] so that the total amount of pCMV vector was constant at 2.5 μg. CAT assays were performed using a constant amount of protein as outlined in the Materials and methods section. This experiment was performed twice with identical results.

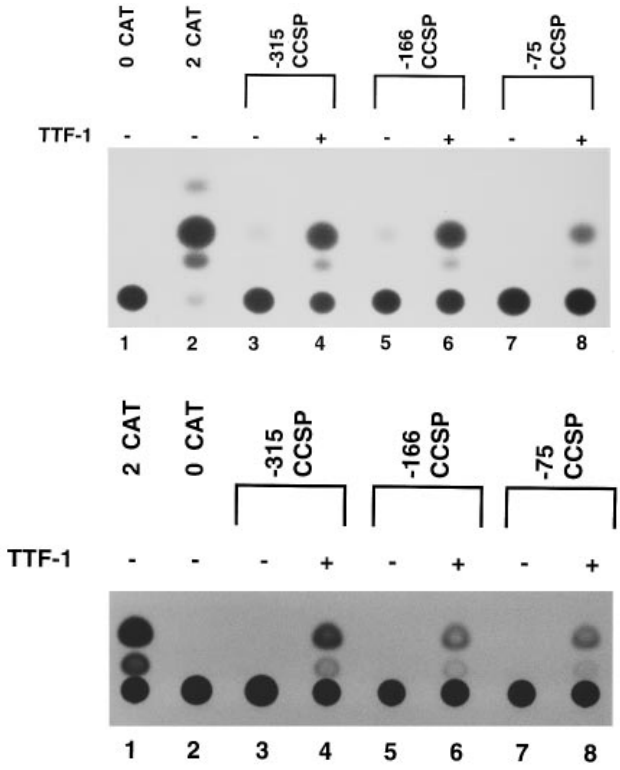

Figure 2 Characterization of CCSP 5′ flanking sequences in the rat gene mediating TTF-1 responsiveness in H441 and HepG2 cells

A deletion series of chimaeric rat CCSP–CAT constructs was prepared as described [10]. Transient transfections were performed by lipofection into H441 cells (upper panel) and HepG2 cells (lower panel) as outlined in the Materials and methods section using 2.5 μg of the CCSP–CAT constructs −315 CCSP–CAT (lanes 3 and 4), −166 CCSP–CAT (lanes 5 and 6) and −75 CCSP–CAT (lanes 7 and 8), or the positive and negative control plasmids 0CAT-BS (0 CAT) and SV2CAT-BS (2 CAT) (lanes 1 and 2), in the absence (−; lanes 1–3, 5 and 7) and presence (+; lanes 4, 6 and 8) of pCMVrTTF-1 (TTF-1). CAT assays were performed using a constant amount of protein as outlined in the Materials and methods section. Both series of transfections were performed on four occasions using different preparations of plasmid DNA, with identical results.

expression plasmids with an empty pCMV plasmid failed to activate trancription (results not shown).

Transactivation of the rat CCSP gene by TTF-1 requires sequence 3′ of position −75

To localize the region of the rat CCSP gene responsible for transactivation by TTF-1 we used a deletion series of CCSP–CAT chimaeric plasmids [12], corresponding to positions −315, −166 and −75 from the transcription start site, in further transient transfection studies in H441 cells. When these three constructs were transfected alone a low level of CAT activity was detected (Figure 2, upper panel, lanes 3, 5 and 7). Consistent with our previous findings, the activity of the −75 CCSP–CAT plasmid, the smallest construct that we have previously shown to display cell-type-specific expression [12], was very low. However, when pCMVrTTF-1 was co-transfected, the activity of all three constructs was markedly increased (Figure 2, upper panel, lanes 4, 6 and 8). Similar results were found when this experiment was repeated in HepG2 cells, a cell line in which we have previously shown that rat CCSP–CAT reporter plasmids are inactive [11,12] and which does not express TTF-1 (results not shown). In the absence of co-transfected pCMVrTTF-1 there was no detectable CAT activity from any of the reporter constructs (Figure 2, lower panel, lanes 3, 5 and 7). In contrast, when pCMVrTTF-1 was co-transfected all three of the reporter plasmids were strongly activated (Figure 2, lower panel, lanes 4, 6 and 8), as was the case with the H441 cells. These results suggest that a TTF-1-responsive region is located 3′ of position −75 in the rat gene, a region more proximal than the CCSP I region. Further studies using Ishikawa and A549 cells gave identical results (results not shown) and confirmed that expression of TTF-1 allows the normally tissue-restricted CCSP promoter to escape this cell-type-specific restriction.

A genomic element between −54 and −37 in the rat CCSP gene promoter binds to TTF-1 and contains a TTF-1 binding site

As an initial step to see if a TTF-1 binding site was localized to the −75 region of the rat CCSP promoter, we turned to gel mobility shift studies. We reasoned that if this region contained a TTF-1 binding site then this would compete for binding of TTF-1 to a well characterized TTF-1 binding site. When nuclear extracts from H441 cells, which contain abundant TTF-1 [25], interacted with a ^{32}P-labelled TTF-1 binding site from the thyroglobulin gene, a single retarded complex was formed (Figure 3, lane 2) which was specifically competed with an excess of unlabelled probe (Figure 3, lane 3) but not with a probe in which the TTF-1 binding site had been mutated (Figure 3, lane 4). An oligonucleotide corresponding to positions −75 to −30 of the rat CCSP gene was also an effective competitor (Figure 3, lane 5), as was an oligonucleotide containing a further TTF-1 binding site from the SP-B gene (Figure 3, lane 6), whereas an excess of an NF-κB oligonucleotide failed to compete for binding (Figure 3, lane 8). *In vitro* translated rat TTF-1 and nuclear extracts from the rat thyroid cell line FRTL-5, which also expresses TTF-1 [22], gave identical results (results not shown).

These results suggested that a TTF-1 binding site is present between positions −75 and −30 in the rat gene. To localize this site more precisely we generated a series of overlapping rat CCSP oligonucleotides for use as competitors in gel mobility shift assays (Figure 4, upper panel). When these oligonucleotides were

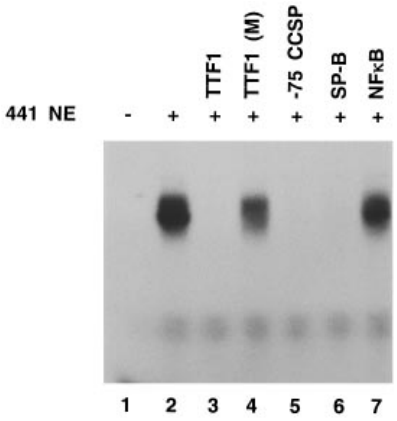

Figure 3 A genomic element between positions −75 and −30 of the rat CCSP gene acts as a competitor for binding of H441 cell nuclear proteins to a TTF-1 binding site

Gel mobility shifts were performed as outlined in the Materials and methods section using 1 μl of H441 cell nuclear extract. A ^{32}P-labelled TTF-1 binding site oligonucleotide from the thyroglobulin gene was used as a probe in the absence (lane 1) or presence (lanes 2–7) of nuclear extract (441 NE) and in the absence (lanes 1 and 2) or presence of a 100-fold molar excess of the following unlabelled double-stranded oligonucleotides: TTF-1, wild type (lane 3), TTF-1 mutant (lane 4), −75−30 rat CCSP (lane 5), SP-B TTF-1 site (lane 6) and the NF-κB site (lane 7).

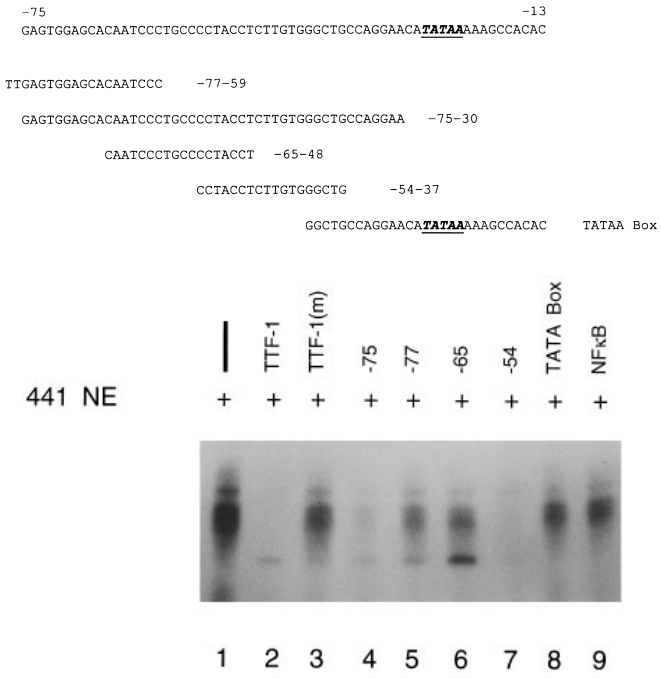

Figure 4 Sequences between positions −54 and −37 of the rat CCSP gene compete for TTF-1 binding

Upper panel: the sequence of the rat proximal promoter from positions −75 to −13 is given, with the TATAA box shown (underlined bold italics). The sequences of the overlapping oligonucleotides used in the gel mobility shift experiments are shown below. Lower panel: gel mobility shifts were performed as outlined in the Materials and methods section using 1 μl of H441 cell nuclear extract (441 NE) and the ^{32}P-labelled TTF-1 binding site oligonucleotide from the thyroglobulin gene as a probe in the absence (lane 1) or presence (lanes 2–9) of a 100-fold molar excess of the following unlabelled double-stranded oligonucleotides: wild-type TTF-1 (lane 2), mutant TTF-1 (lane 3), −75−30 rat CCSP (lane 4), −77−59 rat CCSP (lane 5), −65−48 rat CCSP (lane 6), −54−37 rat CCSP (lane 7), rat TATAA box (lane 8) and the NF-κB site (lane 9).

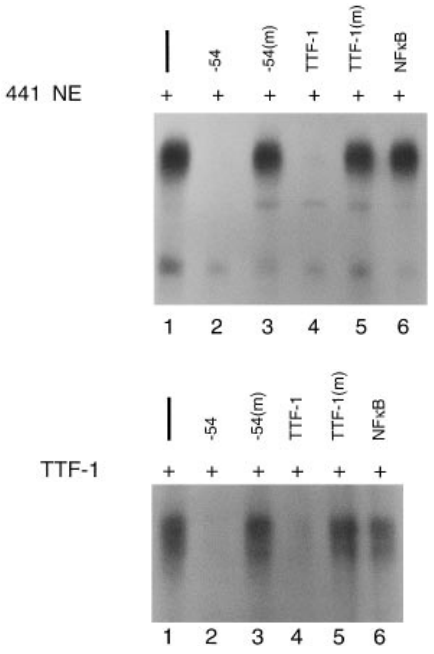

Figure 5 Sequences between positions −54 and −37 of the rat CCSP gene directly bind TTF-1 and contain a divergent TTF-1 binding site

Gel mobility shifts were performed as outlined in the Materials and methods section using the ^{32}P-labelled rat −54−37 CCSP oligonucleotide as a probe and 1 μl of H441 cell nuclear extract (441 NE; upper panel) or 1 μl of *in vitro* translated full-length rat TTF-1 (lower panel), in the absence (lane 1) or presence (lanes 2–7) of a 100-fold molar excess of the following unlabelled double-stranded oligonucleotides: −54−37 rat CCSP wild type (lane 2), −54−37 rat CCSP mutant (lane 3), TTF-1 wild type (lane 4), TTF-1 mutant (lane 5) and the NF-κB site (lane 6).

used as competitors for binding to the TTF-1 binding site, the −77−59, −65−48 and TATA box CCSP oligonucleotides (Figure 4, lower panel, lanes 5, 6 and 8) as well as the mutant TTF-1 (lane 3) and NF-κB (lane 9) oligonucleotides were unable to compete for binding of H441 cell TTF-1. In contrast the −75−30 oligonucleotide (lane 4) and the −54−37 oligonucleotide (lane 7) were as effective competitors for TTF-1 binding as the TTF-1 oligonucleotide itself (lane 2). These results suggest that a TTF-1 binding site is localized between positions −54 and −37 in the rat CCSP gene. Because of the overlap of sequences with the −65−48 and the TATA box oligonucleotides (which covers nucleotides −41 to −13), this suggests that the TTF-1 site is located around positions −48 to −41. Consistent with this finding, a CCSP–CAT chimaeric reporter containing sequence 3′ of −54 was specifically transactivated by TTF-1 (results not shown).

To test whether the −54−37 oligonucleotide was a direct binding site for TTF-1, we used it as a probe for a further series of gel mobility shift reactions. With H441 cell nuclear extracts a single retarded complex was observed (Figure 5, upper panel, lane 2) which was competed for by an excess of unlabelled probe as well as by the TTF-1 binding site (lanes 3 and 5), but not by an excess of the −54−37 oligonucleotide in which the six unshared nucleotides had been mutated (lane 4) or by the mutant TTF-1 and NF-κB oligonucleotides (lanes 6 and 7), suggesting that this site bound to a protein which has binding characteristics indistinguishable from those of TTF-1. Additional studies with the same labelled oligonucleotide and *in vitro* translated rat TTF-

1 showed that the −54−37 oligonucleotide formed a complex with TTF-1 (Figure 5, lower panel, lane 2) which was competed for by an excess of unlabelled probe (lane 3) but not by the NF-κB oligonucleotide (lane 4).

These results clearly show that a region between positions −54 and −37 of the rat CCSP gene is a specific binding site for TTF-1. However, initial analysis of the sequence within this region failed to identify a clear TTF-1 consensus binding site on the sense DNA strand using a consensus TTF-1 binding site generated from 14 known TTF-1 binding sites, including those identified in the SP-A and SP-B genes. When this sequence, CA/TCTC/TAAGT/GG/A, was aligned with the antisense strand of the −54−37 oligonucleotide a match of eight out of ten of the bases was found, C*C*CTCAAG*A*G (mismatches are italicized). The mutated oligonucleotide used in the gel shift studies completely disrupts this region.

The −65 region of the rat CCSP gene contains a conserved non-classical Sp1 binding site

Alignment of the minimal promoters of the rat, human and rabbit genes identifies a further conserved region. This motif, spanning positions −61 to −53 in the rat gene, contains a GC-rich sequence which has the hallmarks of a binding site for the zinc-finger-containing transcription factor Sp1 [26]. The major divergence is the C to T substitution at position 5 in all three promoters. To directly test if Sp1-like proteins are able to bind to this region we used the −65−48 oligonucleotide as a probe in gel mobility shift assays with specific Sp1 competitors and monospecific antibodies to human Sp1 and Sp3 proteins [24]. When this probe interacted with H441 cell nuclear extracts, three distinct retarded complexes were seen (Figure 6, upper panel, lane 2), which were competed for by a 100-fold molar excess of unlabelled −65−48 (lane 3) and Sp1 (lane 4) oligonucleotides, but not by a similar amount of the non-specific competitor (lane 5). Identical results were also found with HepG2, Ishikawa and A549 cell nuclear extracts (results not shown), which is consistent with the widespread distribution of Sp1-related proteins [27]. The inclusion of specific antibodies to Sp1 and Sp3 [24] confirmed that these proteins were responsible for the formation of the shifted complexes. Preimmune serum added to the reaction did not alter the gel shift pattern (Figure 6, upper panel, lane 6), whereas when antiserum to Sp1 was included in the reaction the uppermost complex was supershifted (lane 7), and when antiserum to Sp3 was included the lower two complexes were supershifted (lane 8).

To confirm that the −65−48 rat CCSP region bound an identical complement of proteins to those that bind to a classical CG-containing Sp1 binding site, we used the Sp1 oligonucleotide in a further series of gel mobility shifts. The Sp1 probe also generated three retarded complexes when it interacted with H441 cell nuclear extracts (Figure 6, lower panel, lane 2). These complexes had identical mobilities to those formed with the labelled −65−48 oligonucleotide and were competed for by the Sp1 and −65−48 CCSP oligonucleotides (lanes 3 and 4) but not by the non-specific competitor (lane 5). Inclusion of specific antisera to human Sp1 and Sp3 proteins produced identical results to those with the −65−48 CCSP probe (lanes 6–8). These results, along with the observations that the mutated −65−48 CCSP oligonucleotide failed to bind H441 cell nuclear proteins and also failed to compete for binding of proteins to both the wild-type CCSP and Sp1 oligonucleotides (results not shown), support the conclusion that Sp1 and Sp3 are the only proteins present in H441 cells that can bind to Sp1-like binding sites.

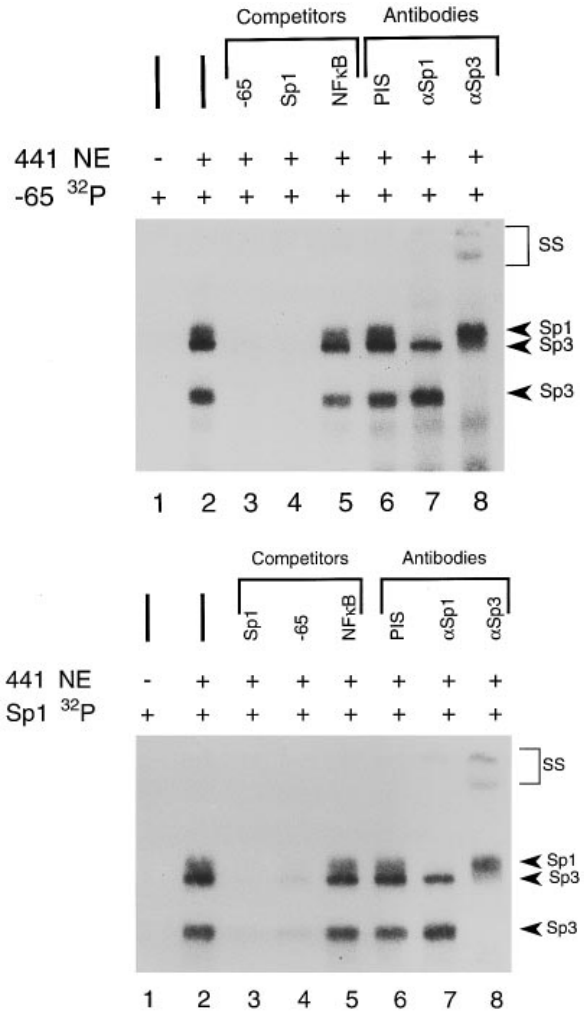

Figure 6 The −65 region of the rat CCSP gene contains a conserved motif which is a binding site for proteins of the Sp1 family of transcription factors

Gel mobility shifts were performed as outlined in the Materials and methods section. Upper panel: ^{32}P-labelled −65−48 rat CCSP oligonucleotide (-65 ^{32}P) was used as a probe, in the absence (lane 1) or presence (lanes 2–8) of 1 μl of H441 cell nuclear extract (441 NE) and in the absence (lanes 1 and 2) or presence of a 100-fold molar excess of the following unlabelled double-stranded oligonucleotides: −65−48 rat CCSP (lane 3), Sp1 site (lane 4) or the NF-κB site (lane 5), or 1 μl of the following antibodies: preimmune serum (PIS; lane 6), anti-Sp1 (lane 7) or anti-Sp3 (lane 8). Lower panel: the ^{32}P-labelled Sp1 oligonucleotide (Sp1 ^{32}P) was used as a probe, in the absence (lane 1) or presence (lanes 2–8) of 1 μl of H441 cell nuclear extract (441 NE) and in the absence (lanes 1 and 2) or presence of a 100-fold molar excess of the following unlabelled double stranded oligonucleotides: Sp1 site (lane 3), −65−48 rat CCSP (lane 4) or the NF-κB site (lane 5), or 1 μl of the following antibodies: preimmune serum (PIS; lane 6), anti-Sp1 (lane 7) or anti-Sp3 (lane 8). In both gels the positions of the Sp1 and Sp3 proteins are marked with arrowheads and the supershifted complexes are marked by the brace (SS).

DISCUSSION

Previous studies have indicated that *cis*-acting elements within the 2.25 kb of the 5′ flanking region of the rat CCSP gene are sufficient to direct appropriate tissue-specific expression of reporter genes to the bronchiolar epithelium [8,9]. Subsequent observations suggested that sequences 3′ to position −75 are important in mediating tisssue specificity. We were therefore interested in identifying other proteins that are capable of

interacting with the minimal promoter region and are possibly involved in regulating the tissue-restricted expression pattern of this gene.

The homeodomain-containing transcription factor TTF-1, originally identified as a thyroid enriched transcriptional regulator [14], is also expressed in the pulmonary epithelium [15,25]. Molecular studies have identified TTF-1 as a potent regulator of the SP-A and SP-B genes [16–18] and we have also shown that a human CCSP–CAT chimaeric reporter containing sequences between −322 and +42 of CCSP is specifically transactivated by human TTF-1 (S. Gowan and C. D. Bingle, unpublished work). The present transfection studies have clearly established that the −75 region of the rat CCSP gene is transactivated by TTF-1 in both pulmonary and non-pulmonary cell lines. This suggests that TTF-1 is a very potent transcriptional activator that is capable of driving expression of promoters in cell lines which do not normally support expression of the CCSP gene, a finding consistent with similar reports using TTF-1 and SP-A or SP-B gene reporter constructs [16–18]. The present results do not exclude the possibility that the region 5′ of −75 contains additional TTF-1 binding sites, which again would be consistent with the observation of multiple TTF-1 binding sites in the promoters of both the SP-A and SP-B genes [16–18]. Further studies are under way to investigate this possibility. In the present study we chose to concentrate on identifying the TTF-1-responsive region(s) 3′ of −75. Using gel mobility shift competition we have localized a TTF-1 binding site to between positions −54 and −37. These results clearly show that TTF-1 interacts with this region and, coupled with the observation that a −54 CCSP–CAT chimaeric reporter is specifically activated by TTF-1 in transient transfections (results not shown), suggest that this site may have a functional role in regulating expression of the CCSP gene. We are presently investigating the effects of mutations of the TTF-1 site on activation by TTF-1, and are also studying whether the −54 region can confer TTF-1 responsiveness to a heterologous promoter.

The TTF-1 binding site is located in one of four conserved regions within the minimal promoter. The region −77 to −59 generated a weak retarded complex with H441 nuclear extracts when it was used as a labelled probe in gel shift reactions (results not shown), and a further conserved region contains the TATAA box. The observation that the region −65 to −48, just 5′ to the TTF-1 binding site, is a non-typical binding site for proteins of the Sp1 family (Sp1 and Sp3) is consistent with the recent report that these proteins bind to the same region in the rabbit uteroglobin gene [28]. Functional studies of this region had identified this site as being important in the basal expression of the uteroglobin gene [29], with mutations in this site reducing basal activity to 5% of wild-type. Introduction of a similar mutation into the rat minimal promoter had no significant effect on basal activity (results not shown). This difference may be context-dependent, as our mutation was made in the −65 CCSP–CAT minimal promoter whereas the previous studies with the rabbit gene were performed with mutations made in the −395 uteroglobin–CAT reporter in a vector which included a simian virus 40 enhancer 3′ to the CAT gene. Transient transfection studies performed with 3.3 and 0.4 kb of the rabbit uteroglobin promoter without a simian virus 40 enhancer have failed to yield measurable activity [13]. Additional studies with the −65 CCSP–Sp1 mutant–CAT construct have shown that it is still TTF-1-responsive (results not shown) suggesting that, contrary to the findings with the uteroglobin gene, a functional Sp binding site is not absolutely required for CCSP gene minimal promoter activity.

In view of the fact that the minimal promoter of the rat CCSP gene retains tissue-specific activity and contains binding sites for TTF-1 as well as Sp1 and Sp3 proteins, the question arises as to whether these proteins are sufficient to direct expression of CCSP in a Clara-cell-specific fashion. Within the lung, expression of TTF-1 is restricted to the epithelial cells of the bronchiolar and alveolar regions [15,25], whereas CCSP expression is restricted to the Clara cells [1]. The exact cellular localization of Sp1-related proteins within the lung has not been determined. It remains, therefore, a formal possibility that expression of all three proteins only occurs within the Clara cell, giving rise to the cell type restriction of the CCSP gene either alone or in combination with additional cell-type-restricted co-binding (or co-activating) proteins. Indeed, recent studies of TTF-1-mediated transactivation of lung and thyroid target genes in a variety of different cell types suggests that TTF-1 may interact with lung- and thyroid-specific co-activators [30], in a manner similar to that recently reported for the B-cell-specific activation of immunoglobulin promoters by ubiquitiously expressed Oct-1 acting in concert with B-cell-specific co-factors [31,32]. We cannot, however, exclude the possibility that other regions of the minimal promoter bind to additional factors which are ultimately responsible for the tissue restriction of the CCSP gene. We are at present performing studies to directly address these possibilities.

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