Identification of hepatocyte nuclear factor-3 binding sites in the Clara cell secretory protein gene

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To determine the mechanisms of cell-specific gene expression in the developing pulmonary epithelium the Clara cell secretory protein (CCSP) gene promoter was analysed by DNAase I footprinting. A prominent site of protein–DNA interaction was detected from nucleotides −132 to −76 using nuclear extract from mouse lung and human H441 cells. Mobility shift analysis revealed that an oligonucleotide corresponding to this region interacted with multiple proteins from lung and H441 cell nuclear extracts. Analysis of the nucleotide sequence of this region identified two potential binding sites for hepatocyte nuclear factor 3 (HNF-3), and consistent with this finding binding to this CCSP oligonucleotide was specifically competed for by an oligonucleotide corresponding to the HNF-3-binding site from the mouse transthyretin gene. Mobility shift of the CCSP oligonucleotide was supershifted using antisera specific to HNF-3α and HNF-3β, and HNF-3α and HNF-3β translated *in vitro* were found to bind specifically to this same oligonucleotide. Co-transfection of HNF-3α- and HNF-3β-expression plasmids increased cell-specific reporter gene activity in H441 cells transfected with a CCSP–CAT gene chimeric construct containing this −132 to −76 region. Taken together, these results suggest a role for HNF-3 in mediating cell-specific CCSP gene expression within the bronchiolar epithelium. These findings support the hypothesis that members of the HNF-3 'forkhead' family of transcription factors determine gene expression and cell fate in multiple cell lineages derived from the primitive gut endoderm.

INTRODUCTION

The pulmonary epithelium originates from an outpouching of embryonic gut endoderm into the surrounding fetal mesenchyme. Inductive signals mediated by cell–cell contact and secretion from this mesenchyme result in the formation of a contiguous epithelium from the trachea to the alveolus [1,2]. Geographic differentiation along this epithelium results in the development of specialized epithelial cells subserving the unique functions of the respiratory system. In the bronchiolar epithelium, such differentiation results in the formation of non-ciliated epithelial (Clara) cells which synthesize proteins essential for surfactant function and pulmonary host defence [3,4]. This epithelium is the site of expression of the cystic fibrosis transmembrane conductance regulator protein during human fetal development and is a common site of anaplastic transformation [5,6]. Despite the obvious importance of the bronchiolar epithelium in lung development and function little is known about the mechanisms of growth and differentiation in this tissue.

Clara cell secretory protein (CCSP) is an abundant 10 kDa protein present in lung secretions and shown by immunohistochemistry to localize to secretory vesicles within the bronchiolar epithelium [7]. Although the biological function of CCSP is unknown, the amino acid sequence of this protein is highly similar to rabbit uteroglobin and identical to a polychlorinated-biphenyl-binding protein isolated from rat lung [8]. The gene encoding CCSP has been isolated in the rat and studies using RNA-blot analysis indicate that CCSP gene expression is confined to the lung [8,9]. *In situ* hybridization studies reveal that within the lung parenchyma CCSP gene expression is cell-specific, being confined to Clara cells of the terminal and respiratory bronchioles [10,11].

CCSP gene expression is an informative developmental marker within the bronchiolar epithelium recapitulating the cellular differentiation in the distal respiratory epithelium during fetal and post-natal development [11]. Studies in transgenic mice have revealed that *cis*-acting elements within 2.25 kb of the 5′ flanking region of the rat CCSP gene are sufficient to direct tissue- and cell-specific expression of a chimeric CCSP-reporter gene [12,13]. Characterization of the *cis*-acting elements determining cell-specific gene expression can be a valuable approach to defining the mechanisms of cellular differentiation in tissues where lineage analysis is not available [14]. In this present study we have characterized the 5′ flanking region of the rat CCSP gene to elucidate the *cis*- and *trans*-acting factors responsible for cell-specific gene expression within the bronchiolar epithelium.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclei were isolated from organs of adult mice as described [15]. All procedures were performed at 4 °C. Isolated nuclei were lysed in a solution containing 10 mM Hepes, pH 7.9/100 mM KCl/3 mM $MgCl_2$/0.1 mM EGTA/1 mM dithiothreitol (DTT)/10% (v/v) glycerol and the proteins were precipitated with $(NH_4)_2SO_4$. The protein pellet was resuspended in 25 mM Hepes, pH 7.6/40 mM KCl/0.1 mM EDTA/1 mM DTT/10% glycerol and dialysed overnight against two changes of the same buffer. Precipitated proteins were cleared from the solution by spinning in a microfuge for 10 min. Protein concentration was determined from the supernatants using the Bradford assay [16].

DNAase I footprinting

Individual fragments of the 5′ flanking region of the CCSP gene were prepared by restriction endonuclease digestion of genomic clones encoding defined regions of the rat CCSP gene [11]. The genomic clones corresponding to this region were isolated and

Abbreviations used: CCSP, Clara cell secretory protein; HNF-3, hepatocyte nuclear factor 3; DTT, dithiothreitol.

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characterized from a rat genomic library as described [11]. DNA fragments were isolated on DEAE paper, treated with calf-intestine alkaline phosphatase, end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase, digested with restriction endonucleases, and recovered by salt elution after electrophoresis through 20% (w/v) polyacrylamide gels [17]. DNAase I reactions were performed using 1–2 ng of labelled DNA (specific radioactivity approx. 10^8 c.p.m./μg) incubated in 50 μl of 25 mM Tris/HCl, pH 7.9/6.25 mM $MgCl_2$/0.5 mM EDTA/50mM KCl/0.5 mM DTT/10% (v/v) glycerol/1 μg poly (dI-dC) at 4 °C for 15 min in the presence or absence of nuclear extracts as indicated [18]. A predetermined amount of DNAase I was added to the reaction for 1 min and the reaction was then stopped by the addition of 100 μl of 20 mM NaCl/20 mM EDTA/1% SDS/250 μg/ml tRNA. DNA was phenol/chloroform-extracted, precipitated in ethanol and electrophoresed through 8% (w/v) acrylamide–urea gels. Nucleotide sequence reactions of the region being footprinted were run in parallel in these same gels for accurate identification of the size and nucleotide sequence of footprinted regions. After electrophoresis gels were fixed, dried and exposed to film at −80 °C.

Gel-mobility-shift assays

Overlapping complementary oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer for use as probes in the mobility-shift assays. The complementary oligonucleotides were annealed and labelled by filling in the overlapping regions with [^{32}P]dNTPs and Klenow polymerase. Each binding reaction used 1 ng of probe (specific radioactivity 10^7 c.p.m./μg). Binding reactions were performed at 20 °C in a 20 μl reaction mix containing 20 mM Hepes, pH 7.6, 40 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5 mM EGTA, 4% (w/v) Ficoll, 1 μg of poly(dI-dC) and nuclear protein (1–10 μg) [19]. The probe was added last and the reaction was allowed to proceed for 30 min. At the end of the binding reaction samples were electrophoresed through 6% (w/v) polyacrylamide gels [30:1, acrylamide/bis, 0.25 × Tris–borate–EDTA buffer (TBE)] at 20 °C. Gels were dried without fixing and exposed to film at −80 °C. In some reactions unlabelled competitor oligonucleotides were included at the concentration outlined in the text. Oligonucleotides used in these studies corresponded to sequences for the mouse transthyretin gene hepatocyte nuclear factor-3 (HNF-3) site [19] (5′-TGACTAAGTCAATAATCACAATCAGCAGGT-3′) and the human immunoglobulin-light-chain gene NF-κB site [20] (5′-ACAGAGGGGACTTTCCGTCTCC-3′). For antibody supershift experiments 1 μl of preimmune serum or 1 μl of antiserum specific to HNF-3 [21] or C/EBP [22] family members was added to the reaction and incubated for 15 min at 20 °C before the addition of the labelled probe. In all cases titration studies were performed using dilutions of these specific antibodies to be certain that the specificity of interaction was maintained.

Translation *in vitro* and gel-mobility-shift assays

Linearized plasmids containing full-length HNF-3α or HNF-3β cDNA sequences were used as templates for transcription using T7 RNA polymerase [23]. Translation *in vitro* was performed using nuclease-treated rabbit reticulocyte lysate with [^{35}S]methionine and reaction products were analysed by SDS/PAGE to confirm the size of the translated product. Reticulocyte lysate (2.5 μl), containing the translated protein, was then used directly in the mobility-shift assay as outlined above, except that the amount of poly(dI-dC) was increased to 2.5 μg. Two sheets of X-ray film were used to produce the autoradiographs with the film in proximity to the gel blocking the ^{35}S signal from the translated protein.

Plasmids and construction of chimeric genes

Full-length cDNAs corresponding to rat HNF-3α and HNF-3β inserted in either pBluescript or a CMV expression plasmid were used for translation *in vitro* and transfection respectively [21]. Cells were transfected as 70% confluent monolayers using 2.5 μg of CCSP–CAT constructs and an equal amount of carrier DNA (pBluescript) and 20 μl of Lipofectin. Lipofectin/DNA mixtures were left on cells for 5 h, after which time the cells were washed three times with PBS and re-fed with complete growth medium [24]. In some experiments carrier DNA was replaced with expression plasmids for HNF-3α or HNF-3β. To control for efficiency of transfection 1 μg of pSV-βgal was co-transfected into cells during all transfections. Reporter gene assays were performed as previously described [17].

RESULTS

DNAase I footprinting of the CCSP promoter

To identify potential *cis*-acting elements determining cell-specific CCSP gene expression the 5′ flanking region of the CCSP gene was analysed by DNAase I footprinting. Footprinting studies using nuclear extracts from mouse lung identified four prominent footprint regions using DNA fragments containing nucleotides −274 to +37 and −1106 to −658 from the rat CCSP gene (Figure 1, lanes 1–8). Additional footprints were not observed in the remaining 5′ flanking sequence up to and including −2072.

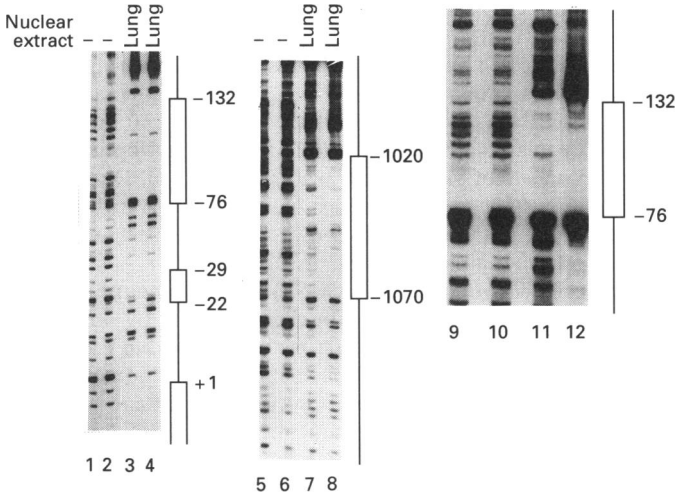

Figure 1 DNAase I footprinting of the rat CCSP promoter

DNAase I footprinting was performed as described in the text in the absence (lanes 1, 2, 5, 6, 9 and 10) or presence of 50 μg (lanes 3 and 7) or 100 μg (lanes 4, 8 and 11) of mouse lung nuclear extract, and 100 μg of H441-cell nuclear extract (lane 12) using end-labelled DNA probes corresponding to nucleotides −313 to +37 (lanes 1–4 and 9–12) or −1143 to −886 of the 5′ flanking region of the rat CCSP gene, labelled on the non-coding strand. After DNAase I digestion the reaction products were resolved on an 8% (w/v) acrylamide sequencing gel, fixed, dried and analysed by autoradiography. The locations of the regions protected from DNAase I digestion are indicated at the side of the figure. The −132 to −76 region is referred to as CCSP region I.

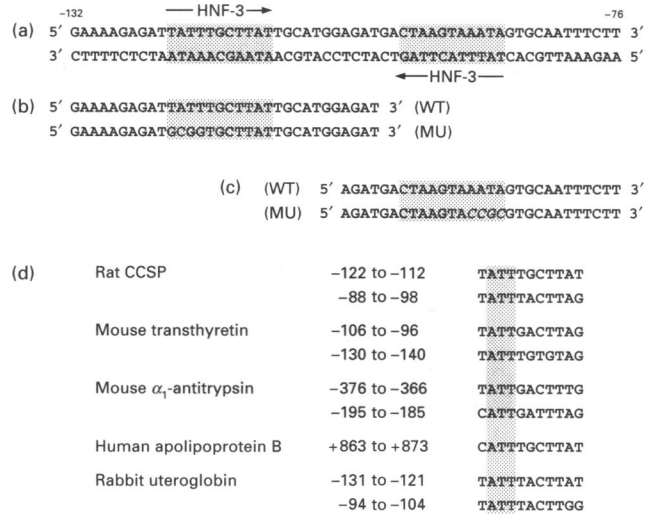

Figure 2 Nucleotide sequence of the CCSP region I

The sequence of the CCSP region (**a**) showing potential binding sites for HNF-3 (highlighted) as well as the sequences of the shorter 5′CCSP I (**b**) and 3′ CCSP I (**c**) oligonucleotides used in the gel-mobility-shift studies are shown. Both wild-type (WT) and mutated (MU) oligonucleotide sequences are shown. (**d**) The sequences represent a comparison of the putative HNF-3-binding sites from the rat CCSP gene with known HNF-3-binding sites as well as recently identified sequences in the rabbit uteroglobulin promoter. Highlighted nucleotides represent the most conserved bases.

A prominent site of protein–DNA interaction was detected from −132 to −76 (CCSP region I), which also footprinted using nuclear extract from H441 cells (Figure 1, lanes 9–12). We focused initially on this region because nucleotide sequence analysis revealed two potential sites for the liver-specific transcription factor HNF-3 arranged in opposite directions along the DNA. Comparison of these regions with HNF-3 sites shown previously to bind HNF-3 *in vitro* revealed a high degree of similarity, consistent with a derived consensus sequence (Figure 2).

Interaction of CCSP I site oligonucleotides with nuclear proteins

A double-stranded oligonucleotide corresponding to CCSP I (Figure 2a) was end-labelled with [γ-^{32}P]ATP and used as a probe in gel-mobility-shift reactions with nuclear extracts from H441 cells. This reaction gave rise to a number of specific retarded complexes which were competed with a 100-fold molar excess of unlabelled oligonucleotide (Figure 3a, lanes 2 and 3). As with the footprint studies similar results were obtained using mouse lung nuclear extract (results not shown); thus for the remainder of these studies H441-cell extracts were used. Competition was also performed using oligonucleotides corresponding to known binding sites for HNF-3 and NF-κB. Each retarded complex was specifically inhibited by 100-fold molar excesses of unlabelled CCSP I and HNF-3 oligonucleotides (Figure 3a, lanes 3 and 4). Competition for all bands was also achieved using the 3′ and 5′ CCSP I wild-type oligonucleotides shown in Figure 2 (Figure 3a, lanes 5 and 6) which contain a single HNF-3-binding site. These effects were specific because no competition was observed with an oligonucleotide corresponding to the NF-κB binding site (Figure 3a, lane 7). Gel-mobility-shift assays performed using a

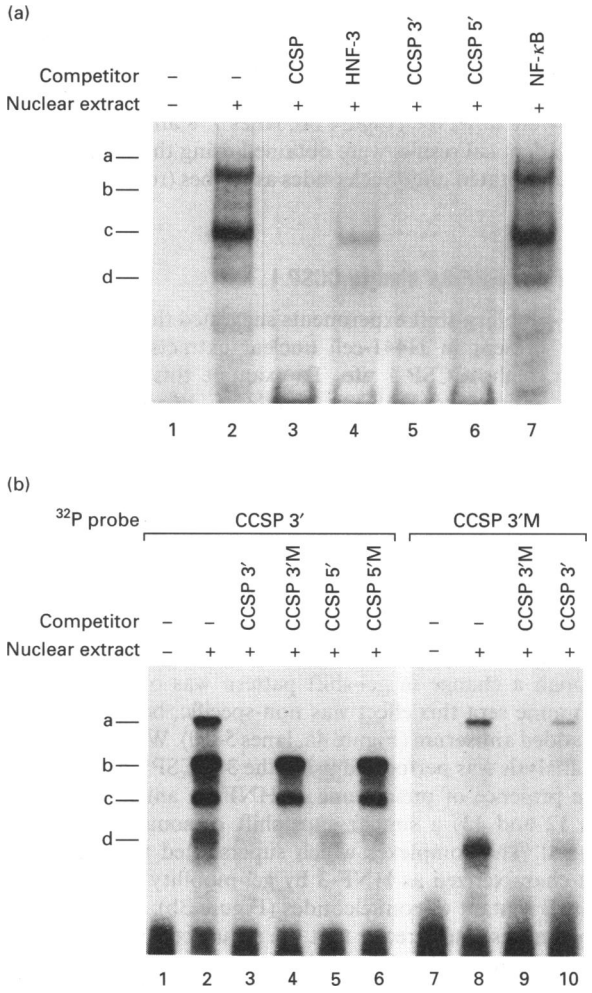

Figure 3 Gel mobility shift of H441-cell nuclear extracts with ^{32}P-labelled CCSP I oligonucleotides

(**a**) Gel-mobility-shift reactions were performed with 5 μg of H441-cell nuclear extract and ^{32}P-labelled CCSP I oligonucleotide as a probe alone (lane 1), in the absence of competitor oligonucleotide (lane 2), or in the presence of 100-fold molar excess of unlabelled specific (lanes 3–6) and non-specific competitors (lane 7) including CCSP oligonucleotides containing a single putative HNF-3-binding site. Reactions were resolved in 6% 0.25 × TBE gels as outlined in the Materials and methods section. (**b**) Gel-mobility-shift reactions were performed as outlined using 5 μg of H441-cell nuclear extract and ^{32}P-labelled CCSP I 3′ oligonucleotide alone (lane 1), in the absence (lane 2) or presence of 100-fold molar excess unlabelled 3′ wild-type (lane 3), 3′ mutated (lane 4), 5′ wild-type (lane 5), or 5′ mutated (lane 6) oligonucleotide. Also shown is the ^{32}P-labelled CCSP I 3′ mutated oligonucleotide alone (lane 7), in the absence (lane 8) or presence of 100-fold molar excess unlabelled 3′ mutated (lane 9), or wild-type (lane 10) oligonucleotide.

^{32}P-labelled HNF-3 oligonucleotide resulted in the formation of multiple complexes using H441-cell nuclear extract, each of which was specifically competed with by the CCSP I oligonucleotides (results not shown).

To examine further the nature of the individual HNF-3 sites in the CCSP promoter 3′ oligonucleotide was ^{32}P-labelled and used as a probe in mobility-shift assays (Figure 3b). Four distinct bands were observed using the 3′ oligonucleotide (Figure 3b, lane 2) and each was competed for by a 100-fold molar excess of 3′ and 5′ CCSP I oligonucleotides (Figure 3b, lanes 3 and 5). In contrast only some of the complexes were competed for using 5′ and 3′ oligonucleotides in which the putative HNF-3-binding site

had been mutated as shown in Figure 2 (Figure 3b, lanes 4 and 6). Consistent with this observation, when mobility shift was performed using the ^{32}P-labelled 3′ mutated oligonucleotide, only those protein–DNA complexes not competed for in the above studies were observed (Figure 3b, lanes 7, 8 and 9 versus lanes 4 and 6). Identical results were obtained using the CCSP I 5′ wild-type and mutated oligonucleotides as probes (results not shown).

HNF-3α and HNF-3β bind to CCSP I

The gel-mobility-shift experiments suggested that an HNF-3-like protein present in H441-cell nuclear extracts was capable of binding to the CCSP I site. To examine this in greater detail specific antisera to HNF-3α and HNF-3β were used in gel-mobility-shift experiments (Figure 4a). As seen previously the CCSP I oligonucleotide retarded four specific complexes from H441-cell nuclear extracts, each of which were competed for by a 100-fold molar excess of the specific CCSP I oligonucleotide but not an equivalent excess of an oligonucleotide corresponding to NF-κB (Figure 4a, lanes 1–4). When antisera were added to these reactions, a supershift of the CCSP I complexes was observed with antiserum to HNF-3α but not antiserum to HNF-3β or C/EBPα (Figure 4a, lanes 6 versus lanes 8 and 10). Although a change in gel-shift pattern was observed with the preimmune sera this effect was non-specific, being present with each added antiserum (Figure 4a, lanes 5–10). When gel-mobility-shift analysis was performed using the 3′ CCSP I oligonucleotide in the presence of preimmune of HNF-3α antisera (Figure 4a, lanes 12 and 13) a similar supershift of some complexes was observed. The complexes which supershifted were identical to those characterized as HNF-3 by gel mobility shift using wild-type and mutant oligonucleotides (Figure 3b).

To demonstrate directly that the sequence within the CCSP I site was capable of binding to HNF-3 proteins, gel-mobility-shift assays were performed using HNF-3α and HNF-3β proteins translated *in vitro*. Each protein was translated from cRNA transcribed from specific HNF-3 plasmids and the translated product was shown to be of the correct size (α, 50 kDa; β, 47 kDa) by SDS/PAGE (results not shown). A retarded complex of identical size was detected when reticulocyte lysates containing either HNF-3α or HNF-3β were incubated with ^{32}P-labelled oligonucleotides corresponding to the HNF-3 site (Figure 4b, lanes 1–4) or the CCSP I site (Figure 4b, lanes 5–10). In each case the complex was inhibited by an excess of the specific competitor but not by an oligonucleotide corresponding to the NF-κB site. An identical specific complex was detected in these studies using the CCSP I 3′ oligonucleotide and reticulocyte lysate containing HNF-3α (Figure 4b, lane 12) and this was competed by an excess of wild-type but not mutated 3′ oligonucleotide (Figure 4b, lanes 13 and 14). The differences in intensity of bound HNF-3 with each oligonucleotide reflected differences in specific activity of the oligonucleotides used as probes. The additional bands found with the CCSP I 3′ oligonucleotide represent degradation products of HNF-3α following storage of lysate at −20 °C as each band was eliminated in competition experiments.

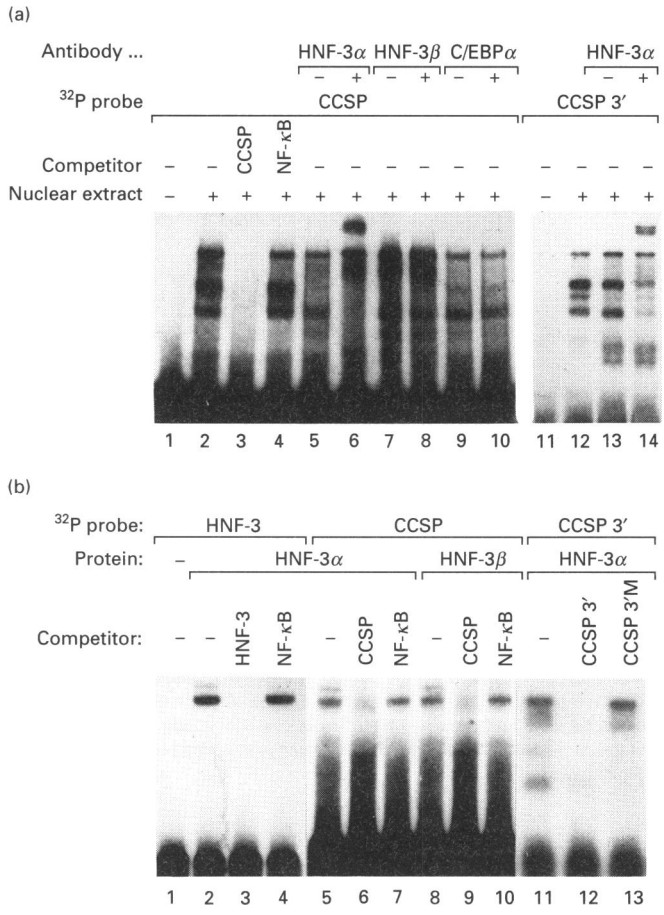

Figure 4 Gel-mobility-shift assay of H441-cell nuclear extracts and translated proteins

(**a**) Gel-mobility-shift reactions were performed as outlined in the Materials and methods section with 5 μg of H441-cell nuclear extract and the ^{32}P-labelled CCSP I oligonucleotide alone (lane 1), in the absence (lane 2) or presence of 100-fold molar excess unlabelled CCSP I (lane 3) or NF-κB (lane 4) competitor oligonucleotides and in the presence of 1 μl of either preimmune serum (lanes 5, 7 and 9) or 1 μl of antiserum to HNF-3α (lane 6), HNF-3β (lane 8) or C/EBPα (lane 10). Gel-mobility-shift assay was also performed using the ^{32}P-labelled CCSP I 3′ oligonucleotide alone (lane 11) or with H441-cell nuclear extract alone (lane 12) and in the presence of either preimmune serum (lane 13) or antiserum to HNF-3α (lane 14). Reactions were resolved in 6% 0.25 × TBE gels as outlined in the Materials and methods section. (**b**) Translation reactions *in vitro* were performed using cRNAs for HNF-3α and HNF-3β as outlined in the Materials and methods sections. A sample (2.5 μl) of reaction products was used to perform gel-mobility-shift assays using either ^{32}P-labelled HNF-3 (lanes 1–4) or CCSP I (lanes 5–10) oligonucleotides as probes. In lanes 2–7 the translation reaction was primed with HNF-3α RNA and in lanes 8–10 the translation reaction was primed with HNF-3β RNA. Reactions were performed in the absence (lanes 2, 5 and 8) or presence of unlabelled specific (lanes 3, 6 and 9) or non-specific (lanes 4, 7 and 10) competitor oligonucleotides. Mobility shift was also performed using ^{32}P-labelled CCSP I 3′ oligonucleotide with *in vitro* translated HNF-3α in the absence (lane 11) or presence of wild-type (lane 12) or mutated (lane 13) CCSP I 3′ oligonucleotide. The reactions were resolved in 6% 0.25 × TBE gels as outlined in the Materials and methods section.

Transactivation of a CCSP–CAT construct

The data so far suggested that HNF-3α and β interacted *in vitro* with the CCSP I region. To determine if the HNF-3 sites contained within the CCSP I region were functional, transient transfection studies were performed using a CCSP–CAT gene chimeric construct containing 1.17 kb of the rat CCSP gene 5′ flanking region. As can be seen in Figure 5, the CCSP 1.17 CAT plasmid but not a pOCAT-BS construct was transcriptionally active in H441 cells (Figure 5, lanes 1–3). When this construct was co-transfected with expression plasmids for either HNF-3α or HNF-3β there was a marked increase in CAT activity in H441-cell extracts (Figure 5, lanes 4 and 5). No increase in CAT activity was observed when these same transfections were repeated using an equivalent amount of an expression plasmid for the transcription factor GATA-4 (Figure 5, lane 6). The induction

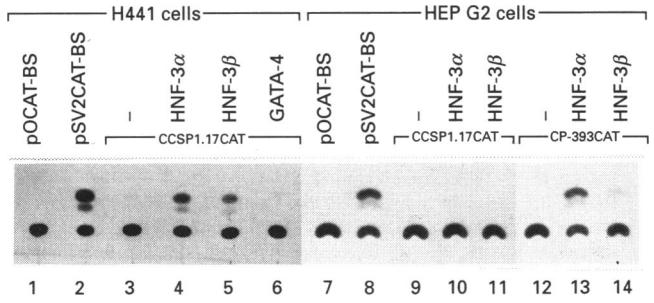

Figure 5 Co-transfection of CCSP–CAT chimeric construct with HNF-3 expression plasmids

Transient transfections were performed as outlined in the Materials and methods section in either H441 (lanes 1–6) or Hep G2 (lanes 7–14) cells using a CCSP–CAT chimeric construct containing 1.17 kb of 5′ flanking region of the rat CCSP gene. Cells were transfected with 2.5 μg of pOCAT-BS (lanes 1 and 7), pSV2CAT-BS (lanes 2, 8), pCCSP1.17CAT (lanes 3, 9), pCCSP1.17CAT and pCMV-HNF-3α (lanes 4, 10), pCCSP1.17CAT and pCMV-HNF-3β (lanes 5, 11), pCCSP1.17CAT and pGATA-4 (lane 6), pCP-393CAT (lane 12), pCP-393CAT and pCMV-HNF-3 α (lane 13), pCP-393CAT and pCMV-HNF-3β (lane 14). All reactions also contained 1 μg of pSVβ-Gal as a control, and pBS-SK such that each transfection reaction contained the same amount of DNA. After incubation for 42 h the cellular extracts were assayed for CAT activity and the reaction products were separated by t.l.c. as described.

of expression seen in H441 cells with the HNF-3 plasmids was cell specific as a similar effect was not seen when identical transfections were performed in Hep G2 cells. In this case the CCSP 1.17 CAT construct was inactive, either alone (Figure 5, lane 9) or when co-transfected with the HNF-3 expression plasmids (Figure 5, lanes 10 and 11). This lack of induction with HNF-3 was not due to inactivity of the HNF-3 constructs in Hep G2 cells because a rat ceruloplasmin gene–CAT construct containing HNF-3-binding sites was effectively transactivated in these cells (Figure 5, lanes 12–14). The effect of HNF-3 on the CCSP 1.17 region was highly reproducible, being consistently observed in six independent transfection assays utilizing different plasmid preparations. In all experiments variations in transfection efficiency were controlled as described in the Materials and methods section.

DISCUSSION

Transgenic experiments reveal that *cis*-acting elements within 2.25 kb of the 5′ flanking region of the CCSP gene were sufficient to direct appropriate lung- and Clara-cell-specific expression of reporter genes [12,13]. This current study focused on this region with DNAase I footprinting identifying several specific footprints consistent with transient transfection studies in H441 cells [12]. Sequence analysis of the CCSP I region revealed the presence of putative *cis*-acting elements for HNF-3 and consistent with this observation the mobility gel shift, antibody supershifts and co-transfection studies presented here all support a role for HNF-3 in this region. It is important to note that the CCSP I oligonucleotides interacted with multiple proteins in H441-cell nuclear extracts. While it is possible that each complex represents a different form of HNF-3 it seems most likely that other transcription factors also interact with the CCSP I region. Experiments using HNF-3-specific antisera and the CCSP oligonucleotide, in which the HNF-3-binding sites were mutated, support the latter concept.

Interestingly, two HNF-3 sites are found within CCSP region I oriented in opposite directions. The orientation of these sites and their proximity to each other is similar to the arrangement observed in the HNF-3 sites in the mouse transthyretin gene which is also transactivated by HNF-3 [19,21]. Although there are currently no data available to suggest whether one or both of these sites is necessary for HNF-3 function, this arrangement is intriguing since an oligonucleotide to each of these regions successfully competed in the gel mobility studies for the HNF-3-retarded complexes. Since no model currently exists for the mechanism of HNF-3 binding to DNA it will be important in future studies to determine whether the bidirectional sites observed here for the CCSP gene are essential for HNF-3 activity *in vivo*. The absence of the HNF-3β-specific supershift in H441-cell nuclear extract is due to a lack of expression of HNF-3β in these cells, as determined by immunoprecipitation (results not shown) as opposed to a specific inhibition of CCSP I–HNF-3β interaction. The CCSP I oligonucleotide did interact with both HNF-3α and HNF-3β in mouse lung extracts and in HNF-3β-transfected H441 cells (results not shown), but it is not clear from the current studies which of these proteins specifically interacts with the CCSP gene promoter *in vivo*. Previous studies have demonstrated the presence of HNF-3α and HNF-3β transcripts in mouse lung [21] and thus further studies will be required to determine the role of each HNF-3 member in CCSP gene expression *in vivo*.

The results shown here clearly demonstrate an increase in CAT activity after co-transfection with either HNF-3α or -3β. The cell specificity of these results, as indicated by the Hep G2 transfections, suggests that H441 cells contain additional factors essential for Clara-cell-specific expression of the CCSP gene. Although the H441 cells have many Clara cell characteristics, these cells do not express the endogenous CCSP gene. Nevertheless, the H441-cell-specific expression of CCSP gene chimeric constructs observed here is consistent with recent observations that endogenous CCSP gene expression is actively repressed early in lung development and that the *cis*-acting elements responsible for this repression are not contained within 2.25 kb of the 5′ flanking region (B. P. Hackett and J. D. Gitlin, unpublished work). If such mechanisms are operative in H441 cells this may explain the transient expression observed using this region, despite the lack of endogenous CCSP gene expression. Consistent with this, transfection of the HNF-3α or HNF-3β expression plasmids did not activate endogenous CCSP gene expression in H441 cells.

HNF-3 transcription factors are members of the recently described forkhead family named after the original member identified as a *Drosophila* homeotic mutation [25]. Additional family members are the *Drosophila* sloppy paired loci [26], an activin-inducible *Xenopus* gene XFKH1 [27], two distinct HIV- and interleukin-binding factors from human T-cells [28,29], a rodent brain-specific transcription factor BF1 [30], a *Drosophila* forkhead homologue in *Saccharomyces cerevisiae* [31], as well as multiple new family members recently identified in *Drosophila* embryo [32]. Each of these factors, including HNF-3α, -3β and -3γ all have a highly conserved 110 amino acid 'forkhead' domain which has been shown by deletion studies to be a site of DNA binding as well as two other conserved domains mediating transcriptional activation. Many of the forkhead members play a role in determinative events of cell fate during embryogenesis. The studies presented here are the first to demonstrate a role for this transcription-factor family in the regulation of pulmonary epithelial cell-specific gene expression. Given the embryologic origins of the pulmonary epithelium our findings are consistent with the hypothesis that HNF-3 proteins may be important in

determining gene expression and cell-type determination in multiple epithelial cell-types derived from the primitive gut endoderm. Recent studies identifying unique forkhead family members specific to the lung also support the concept that these transcription factors may play a role in cell type determination in the developing pulmonary epithelium [33].

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