Role of hepatocyte nuclear factor-3 α and hepatocyte nuclear factor-3 β in Clara cell secretory protein gene expression in the bronchiolar epithelium

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The 5' flanking region of the Clara cell secretory protein (CCSP) gene contains two *cis*-acting elements which bind hepatocyte nuclear factor (HNF)-3 α and HNF-3 β *in vitro*. To determine the role of these proteins in mediating CCSP gene expression in the bronchiolar epithelium, chimeric CCSP-reporter gene constructs containing various regions of the CCSP 5' flanking region were co-transfected into H-441 cells with HNF-3 α or HNF-3 β expression plasmids. These studies indicate that each of these transcription factors positively regulates CCSP gene expression and revealed that CCSP region I (-132 to -76) is sufficient to mediate this effect. Gel-mobility-shift assays with oligonucleotides corresponding to CCSP region I, nuclear extract from bronchiolar epithelial cells and HNF-3-specific antibodies indi-

cate that HNF-3 α and HNF-3 β are the only proteins in bronchiolar epithelial cells which directly interact with this region. Consistent with these observations, HNF-3 α and HNF-3 β transcripts were found to be enriched in this cell population and *in situ* hybridization of adult lung revealed HNF-3 gene expression in non-ciliated bronchiolar epithelial cells expressing the CCSP gene. Finally, experiments with CCSP region I and a heterologous promoter indicate that this region acts in a promoter-specific context, suggesting that additional factors interacting via the minimal CCSP promoter region are essential in determining the effects of HNF-3 on cell-specific CCSP gene expression in the bronchiolar epithelium.

INTRODUCTION

During development the primitive respiratory epithelium forms a contiguous layer of cells derived as an outpouching from the foregut endoderm. Subsequently regional differentiation along this epithelium results in multiple cell types which subserve the unique secretory and metabolic functions of the lung [1]. Understanding the process of pulmonary epithelial cell growth and differentiation is paramount to devising appropriate therapeutic strategies following lung injury as well as rational approaches for the somatic gene therapy of lung disease [2]. In addition, the pulmonary epithelium is the most common site of environmentally related carcinogenesis and understanding the normal controls of pulmonary epithelial cell growth and differentiation will be essential to the prevention and treatment of this anaplastic process [3]. Despite the importance of epithelial cell differentiation in lung development and function, little is currently known about the molecular events which determine this process.

Clara cell secretory protein (CCSP) is an abundant protein of lung secretions which is localized by immunohistochemistry to the secretory granules of non-ciliated (Clara) cells in the bronchiolar epithelium [4]. 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 [5,6]. 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 [7–9]. Studies using transgenic mice indicate that *cis*-acting elements within 2.25 kb of the 5' flanking region of the rat CCSP gene are sufficient to orchestrate this tissue- and cell-specific expression [10,11].

Differentiation is characterized by the expression of unique cell-specific genes and defining the mechanisms of cell-specific gene expression can provide insight into the molecular events determining cell differentiation [12]. For example, defining the factors determining growth hormone gene expression in the anterior pituitary led to the elucidation of a novel transcription factor involved in cell-fate determination during the differentiation of this tissue [13]. Initial characterization of the *cis*-acting elements determining cell-specific CCSP gene expression identified a region (termed CCSP I) 5' of the transcription start site which appears to be important in the regulation of this gene [14,15]. Using gel-mobility-shift assays, with nuclear extracts prepared from the human cell line NCI-H441, two binding sites for the forkhead/winged helix transcription factor family members hepatocyte nuclear factor (HNF)-3 α and HNF-3 β were identified in the CCSP I region [15]. Recent cloning of the mouse CCSP gene reveals evolutionary conservation of these HNF-3 elements supporting a potential role in CCSP gene expression [16,17]. In the present study we have characterized the CCSP 5' flanking region responsible for HNF-3-induced transactivation and identified the proteins within the pulmonary epithelium responsible for this activation.

MATERIALS AND METHODS

Plasmids and construction of chimeric genes

Specific regions of the rat CCSP gene 5' flanking region were amplified by PCR [11] using oligonucleotide primers with synthetically introduced *Bam*HI sites, and subcloned into the *Bam*HI site of p0CAT-BS as described previously [18]. Single-strand oligonucleotides corresponding to CCSP region I were synthesized with *Bgl*II and *Bam*HI sites at the 5' and 3' ends

Abbreviations used: CCSP, Clara cell secretory protein; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; DTT, dithiothreitol; β Gal, β -galactosidase; HNF, hepatocyte nuclear factor.

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respectively, phosphorylated with T4 polynucleotide kinase, annealed and ligated for 3 h at room temperature, and digested with *Bgl*II and *Bam*HI. Single and triple copies of the CCSP I region were recovered and ligated into pTKCAT which had been digested with *Bgl*II and *Bam*HI, and plasmids were selected by their resistance to *Bgl*II and *Bam*HI digestion. Triple copies of the CCSP I region were also blunt-end-ligated into the *Spe*I site of CCSP-75 CAT to yield plasmid 3X CCSP-75 CAT. Full-length cDNAs corresponding to HNF-3 α and HNF-3 β were inserted into a cytomegalovirus (CMV) expression plasmid [19]. All chimeric constructs were sequenced by the dideoxy-chain-termination method to determine orientation and to confirm the fidelity of the amplification.

Transfections and reporter gene assays

NCI-H441 and HepG2 cells were obtained from the American Tissue Culture Collection and maintained as monolayers in RPMI medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin and 150 μ g/ml streptomycin. Cells were transfected at 70% confluency, in 35-mm-diam. dishes using 2.5 μ g of CCSP-CAT chimeric constructs, carrier DNA (pBS) up to 7.5 μ g and 20 μ l of lipofectin. Lipofectin/DNA mixtures were placed on cells for 5 h after which time the cells were washed three times with PBS and refed with complete growth medium. In some experiments carrier DNA was replaced with 2.5 μ g of expression plasmid for HNF-3 α or HNF-3 β . To control for efficiency of transfection 1 μ g of pSV- β Gal was co-transfected into cells during all transfections. Reporter gene assays were performed as previously described [18]. Results were expressed as pmol of chloramphenicol acetyltransferase (CAT) acetylated/h per unit of β -galactosidase (β Gal).

Gel-mobility-shift assays

Populations of lung epithelial cells enriched to contain Clara cells were isolated from Sprague-Dawley rat lungs by centrifugal elutriation as described elsewhere [20]. Modifications of the original procedure resulted in cells where > 50% contained characteristics of Clara cells (C. D. Bingle, M. Moxley, W. Longmore and J. D. Gitlin, unpublished work). Nuclear extracts were prepared from 1×10^7 primary cells as described in [22]. Overlapping complementary oligonucleotides were annealed and labelled by filling in regions with 32 P-dNTPs and Klenow polymerase [15]. CCSP I-region oligonucleotides used are described in detail elsewhere [15]. 1 ng of probe (specific radioactivity 10^7 c.p.m./ μ g) was used per binding reaction. Binding reactions were performed at 20 °C in a 20 μ l reaction mixture containing 20 mM Hepes, pH 7.6, 40 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EGTA, 4% Ficoll, 1 μ g of poly dI-dC and nuclear protein (1–10 μ g). 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% polyacrylamide gels (30:1, acrylamide:bisacrylamide, 0.25 $\times$ Tris/borate/EDTA) at 4 °C. Gels were fixed, dried and exposed to film at –80 °C. In some reactions unlabelled competitor oligonucleotides were included at the concentration outlined in the text. For antibody supershift experiments 1 μ l of preimmune serum or 1 μ l of specific antiserum to HNF-3 family members was added to the reaction and incubated for 15 min at 20 °C prior to the addition of the labelled probe. The HNF-3 antisera used were polyclonal rabbit antibodies raised against specific HNF-3 α or - β peptide sequences and shown to be specific for each family member [19].

RNA preparation and analysis

Total RNA was isolated from rat tissues and cell lines by cesium chloride gradient centrifugation after dissolution of tissues in guanidinium isothiocyanate [23]. RNA samples were electrophoresed, transferred to membranes and hybridized as described previously [11]. HNF-3 α - and HNF-3 β -specific probes were produced by PCR using full-length rat HNF-3 α and HNF-3 β plasmids as templates. The forkhead region corresponds to amino acids 168–266 of the HNF-3 α protein, the HNF-3 α -specific probe corresponds to amino acids 287–371 of the HNF-3 α protein and the HNF-3 β -specific probe corresponds to amino acids 275–357 of the HNF-3 β protein (see Figure 3a) [19]. PCR products were subcloned into pCR II (Invitrogen) and fully sequenced using the dideoxy-chain-termination method. Specific cRNA probes were prepared by *in vitro* transcription with [32 P]CTP as described [24].

In situ hybridization

Lungs from adult Sprague-Dawley rats were removed en bloc, washed extensively in PBS and placed in ice-cold fixative solution containing 4% (w/v) paraformaldehyde, 0.1 M Na₂B₄O₇ and 0.4% NaOH for 30 min at 4 °C. Tissue was then transferred to the same solution with 10% (w/v) sucrose and 5 μ m frozen sections were cut on to superfrost (Fisher) slides [8]. Sense and anti-sense cRNA probes were transcribed from linearized plasmid templates using digoxigenin-UTP (Boehringer) and the sections were hybridized overnight as previously described [25]. To detect hybridized transcripts sections were hybridized at 4 °C for 16 h with a 1:10000 dilution of alkaline phosphatase-conjugated anti-digoxigenin antibody and following incubation the slides were washed multiple times in 100 mM NaCl, 100 mM Tris/HCl (pH 9.5), 50 mM MgCl₂ and 0.1% Tween-20 for 10 min each at 20 °C. The histochemical reaction to detect alkaline phosphatase was carried out by incubation with NitroBlue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for 20 min in the dark at 20 °C. Colour developing reactions were allowed to proceed for 12 h and photographs were taken with light-field microscopy.

RESULTS

CCSP I is required for HNF-3 responsiveness of the CCSP promoter

To precisely localize the regions required for HNF-3 responsiveness we constructed a series of chimeric CCSP CAT plasmids containing progressively smaller regions of the 5' flanking sequence. These constructs were used in transient transfections of the adenocarcinoma cell line NCI-H441 in the absence and presence of co-transfected HNF-3 α or HNF-3 β . Transfections in the absence of HNF-3 revealed the presence of a number of positive- and negative-acting regions in the CCSP 5' flanking sequence (Figure 1a). Thus, removal of sequences from -2333 to -1144 resulted in an increase in reporter gene activity, while removal of a further region down to -735 caused a reduction of reporter activity (Figure 1a). The sequence from -1070 to -1020 (boxed) represents a footprinted region using lung nuclear extract [15]. The sequence between -315 and -75 (boxed), which contained the footprinted region CCSP I contains positive-acting regions. Removal of the 5' most half of the CCSP I region resulted in a loss of 50% of reporter gene activity while removal of the complete region led to a loss of most of the reporter gene activity. These data suggest that the majority of elements required to support expression of CCSP are located 5' of -75. Co-transfection

Figure 1 Characterization of CCSP 5' flanking sequences mediating HNF-3 responsiveness

A deletion series of chimeric CCSP-CAT constructs were prepared as outlined in The Materials and methods section. (a) 2.5 µg of each construct was transiently transfected into NCI-H441 cells. For co-transfection experiments the cells were also transfected with 2.5 µg of pCMV-HNF-3α. All transfections contained 1 µg of pSV-βGal as an internal control and pBluescript up to a total of 7.5 µg of DNA per reaction. After 42 h cellular extracts were assayed for CAT and βGal activity as described in the Materials and methods section. Activity expressed as pmol of CAT converted/h per unit of βGal. Induction by HNF-3α is expressed as fold-induction over control values which are the mean ± S.D. of six independent transfections. (b) Transient transfections were performed into either H441 cells (lanes 1–6) or Hep G2 cells (lanes 7–12). Constructs used are shown in (a). The Figure represents an autoradiograph of the developed CAT assay as described in the Materials and methods section and is representative of six separate experiments.

studies performed with pCMV-HNF-3α revealed that the CCSP I region, which includes the two previously identified HNF-3-binding sites, is absolutely required for the inductive effect of HNF-3. Reporter gene activity of all constructs containing the CCSP I region was increased approximately 3-fold (Figure 1a). Removal of the 5' HNF-3-binding site (-105) caused a reduction in HNF-3 responsiveness and suggests that in the CCSP promoter the two HNF-3 sites can be activated independently. Co-transfections performed with pCMV-HNF-3β yielded identical results but these could not be accurately controlled for transfection efficiency since HNF-3β had a negative effect on βGal plasmid expression (data not shown). To identify the sequences that mediate cell-specificity of CCSP gene expression the deletion series was transfected into H441 cells and the hepatoblastoma cell line HepG2 (Figure 1b). These data reveal that all constructs down to and including CCSP-75 CAT were active only in the H441 cell line (compare Figure 1b, lanes 3–6 with lanes 9–12). Similar levels of transfection efficiency were

Figure 2 Identification of HNF-3α and HNF-3β in Clara cell nuclear extracts

Gel-mobility shifts were performed as outlined in the Materials and methods section using 5 µg of Clara cell nuclear extracts. (a) CCSP I 3' oligonucleotide was used as probe in the absence (lane 1) or presence of Clara cell (lanes 2 and 3) or H441 (lanes 4 and 5) nuclear extract and in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of a 100-fold excess of specific competitor. (b) A 32P-labelled CCSP 3' oligonucleotide was used as probe with Clara cell nuclear extract in the absence (lane 1) or presence of 100-fold molar excess of specific (lane 2) or non-specific (lane 3) competitors and in the presence of antiserum to HNF-3α (lane 4), HNF-3β (lane 5), HNF-3γ (lane 6) or HNF-3α and HNF-3β together (lane 7).

achieved in both cell lines as evidenced by activity of the pSV-2CAT transfections (Figure 1b, lanes 2 and 8).

HNF-3α and HNF-3β bind to the CCSP I sequence in lung epithelial cells

In view of the fact that H441 cells support the expression of CCSP-CAT chimeric constructs but do not express the endogenous CCSP gene, populations of rat lung epithelial cells enriched to > 50% Clara cells were isolated and used to produce nuclear extracts for gel-mobility-shift assays. When these extracts were used in gel-mobility-shift assays using the 32P-labelled CCSP 3' oligonucleotide as a probe a distinct pattern was observed (Figure 2a). Whereas H441 cell nuclear extract gives

Figure 3 RNA blot analysis of HNF-3 expression

A probe to the forkhead region was used to hybridize total RNA from lung (lane 1), liver (lane 2), Clara cells (lane 3) and H441 cells (lane 4). Rat lung poly A⁺ RNA was hybridized with cRNA probes specific to HNF-3 α (lane 5) and HNF-3 β (lane 6). RNA blots were hybridized and washed as described and exposed to X-OMAT film at -70°C for 9 h.

rise to four specific retarded complexes (letters a–d note bands as observed previously) [15] (Figure 2a, lanes 4 and 5), Clara cell

nuclear extract produced a broad band (Figure 2a, lane 2) which specifically competed with unlabelled oligonucleotide (Figure 2a, lane 3). Further analysis of this region originally observed as a single band using prolonged electrophoresis revealed that it contained three distinct components (Figure 2b, lane 1). Competition with the specific competitor sequence blocked the formation of all of this complex (Figure 2b, lane 2) whereas competition with a non-specific competitor led to the loss of the lower portion of the complex (Figure 2b, lane 3), indicating that the uppermost band was derived from non-specific interactions. To directly identify the proteins interacting with the CCSP 3' oligonucleotide, specific antisera to the different HNF-3 family members were utilized. The addition of antiserum to HNF-3 α or HNF-3 β caused a supershift of the top or middle part of the complex respectively (Figure 2b, lanes 4 and 5), suggesting that HNF-3 α and HNF-3 β were causing these retarded complexes. Consistent with this, when antisera to both HNF-3 α and HNF-3 β were added to the same reaction the top two complexes were supershifted leaving only the lower non-specific complexes bound to the probe (Figure 2b, lane 7). These supershifts were shown to be specific by the addition of antiserum to HNF-3 γ (Figure 2b,

Figure 4 Clara cells express HNF-3 α and HNF-3 β

In situ hybridization of adult rat lung tissue with HNF-3 α - and HNF-3 β -specific probes using digoxigenin-labelled cRNA probes as outlined in the Materials and methods section. Shown are serial sections hybridized with CCSP (a and c), HNF-3 α (b) and HNF-3 β (d). (Abbreviations: a, alveolus; b, bronchiole; v, vessel) (Magnification in a–d = $\times 130$). Also shown is a higher power view of a serial section through a bronchiole hybridized with CCSP (e) or HNF-3 α (f) (Magnification in e and f = $\times 260$).

lane 6) or preimmune sera (data not shown) which caused no supershift of any of the bands. These data indicate that HNF-3 α and HNF-3 β are the only proteins in this nuclear extract that specifically interact with the CCSP 3' region under these conditions. Identical results were obtained when these experiments were repeated using oligonucleotides corresponding either to the 5' or the entire CCSP region I (data not shown).

HNF-3 α and HNF-3 β are expressed in Clara cells

The supershift experiments described above suggested that the Clara cell population contains appreciable levels of HNF-3 α and HNF-3 β . To ensure that these proteins were expressed in Clara cells rather than in other cell types within the isolated cell population specific cRNA probes for HNF-3 α and HNF-3 β were utilized for RNA blot analysis. An HNF-3 α forkhead probe identified two RNA transcripts from rat lung and liver (Figure 3, lanes 1 and 2) identified as HNF-3 α (top band) and HNF-3 β (lower band) based upon size analysis. RNA from the Clara-cell-enriched population contained both these transcripts (Figure 3, lane 3) and the abundance greatly increased compared with the levels seen in total lung. In contrast RNA from H441 cells was found to contain only the HNF-3 α transcript (Figure 3, lane 4). HNF-3 α - and HNF-3 β -specific probes confirmed the presence of these individual transcripts in the Clara-cell-enriched population (Figure 3, lanes 5 and 6).

The data presented above suggested that the enriched population of Clara cells contains both HNF-3 α and HNF-3 β . Nevertheless it remained formally possible that some other cell type that is also enriched in this population may be the source of the HNF-3 proteins identified in the gel-shift and RNA experiments. To directly address the source of HNF-3 α and HNF-3 β , *in situ* hybridization was performed using specific antisense cRNA probes. Figure 4 shows that both HNF-3 α (Figure 4b) and HNF-3 β (Figure 4d) transcripts are localized in the epithelial cells which line the respiratory bronchioles and not in other regions of the lung including the blood vessels and mesenchyme. When serial sections of lung were hybridized with a probe for CCSP a similar pattern of expression was observed (Figures 4a and 4c). On examination of serial sections at higher power, bronchiolar epithelial cells with the morphological and histological characteristics of Clara cells were positive for both CCSP and HNF-3 α (Figures 4e and 4f). In all cases, hybridization performed with sense strand probes was negative (data not shown).

CCSP I region acts in a promoter-specific context

The data presented above suggest that the CCSP I region is the site of binding for two members of the HNF-3 family which are enriched in Clara cells and act as positive regulators of CCSP gene transcription. However, since HNF-3 α and HNF-3 β are expressed in a variety of cell types and tissues the mechanisms for cell-specific expression of CCSP clearly must involve additional factors. To determine whether CCSP region I was capable of acting alone as a positive regulator, single or triple copies of the CCSP I region were placed upstream of the HSV TK minimal promoter and transfected into H441 cells. Although single or triple copies of CCSP I upstream of the CCSP -75 minimal promoter were active in H441 cells (Figure 5) no activity was detected using the TK promoter in these cells. Furthermore, as seen previously with larger constructs, none of these elements were active when transfected into Hep G2 cells (data not shown).

Figure 5 CCSP region I acts in a promoter specific context

Single and multiple copies of CCSP I were ligated into CCSP 75-CAT and pTKCAT as described in the Materials and methods section. The resulting constructs (see Figure 1) were transfected into H441 cells and assayed as described in the legend to Figure 1. Results are expressed as pmol of CAT converted/h per unit of β Gal and are the means of eight independent transfection experiments.

DISCUSSION

Previous studies using transgenic mice 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 [7-9]. Initial characterization of this promoter by DNase I footprinting identified a number of sites of protein-DNA interaction, the most prominent of which (CCSP I) is located between -132 and -76 from the transcription start site [10,11]. Gel-mobility-shift assays performed with nuclear extracts from a variety of lung and non-lung cell lines identified a complex pattern of nuclear proteins interacting with this region and suggested that members of the forkhead/winged helix family of transcription factors may play a role in the regulation of the CCSP gene through this sequence [10,11]. Functional data suggesting a direct role of these proteins in the regulation of the CCSP gene was obtained using a chimeric CCSP-CAT plasmid containing 1.17 kb of CCSP 5' flanking sequence, which was positively transactivated by HNF-3 α and HNF-3 β [11].

In this current study, we have extended these observations and provided evidence that the CCSP region I is necessary and sufficient for positive transactivation by HNF-3 α and HNF-3 β in H441 cells. H441 cells are a human adenocarcinoma cell line with characteristics of Clara cells and are the only cell line shown to support expression of CCSP-CAT chimeric reporter plasmids [10,11]. However, these cells do not express the CCSP gene and therefore we sought to directly identify proteins which play a role in regulating CCSP gene expression in the lung. For this approach rat lung epithelial cells enriched for Clara cells were used as the source of nuclear extracts for gel-mobility-shift studies. These data indicate that these cells contain a different complement of nuclear proteins capable of interacting with the CCSP I sequence compared with those found in H441 cells (Figure 2). Using such nuclear extracts and specific antibodies, HNF-3 α and HNF-3 β are identified as the only proteins which specifically interact with the CCSP region I. Although previous studies identified a potential Ap1 consensus sequence in the CCSP region I no binding to this site was obtained using the Clara cell nuclear extract (data not shown) and, consistent with this observation, functional studies suggest no role for Ap1 in regulation of CCSP gene expression [26]. RNA blotting and *in situ* hybridization

confirmed that the expression of HNF-3 α and HNF-3 β is within the Clara cell population expressing the CCSP gene (Figures 4 and 5) and this is consistent with *in situ* studies of HNF-3 expression during mouse development [27,28].

HNF-3 α and - β are members of the winged helix/forkhead family of transcription factors and are so named from the structure of the DNA-binding domain and the nature of the homeotic mutation in the original family member [29]. Many diverse family members have been identified in mammals, flies, worms, fish and yeast and shown to play roles in cell-specific gene expression and fate determination during development [29]. The data presented here are consistent with a potential role for HNF-3 α and - β in cell-fate decision in the pulmonary epithelium and this is supported by the early developmental expression of these factors in the lung [27,28]. Most recently a potential role for HNF-3 in regulation of expression of surfactant B has been reported, lending further support to the concept that these proteins may play an essential role in determining cell-specific gene expression in the pulmonary epithelium and cell-fate decisions in the developing foregut endoderm [30,31]. In contrast to the results reported here and by others [27,28] a recent *in-situ* analysis of HNF-3 β expression in the adult rat lung localized signal to smooth muscle [31]. The reasons for a discrepancy in findings are not apparent at this time.

Finally, although the data presented here indicate that the rat CCSP gene is regulated in a tissue-specific manner by HNF-3 α and HNF-3 β via a *cis*-acting element located between -132 and -76, regions of the promoter more proximal to the CCSP I site are also essential for cell-type specificity. The CCSP region is incapable of acting as positive activating region when placed upstream of a heterologous promoter, indicating that CCSP I acts in concert with sequences within the minimal promoter as a cell-type-specific activating region. These data are consistent with similar observations using luciferase-based reporter constructs and the CCSP minimal promoter region [26]. Further studies will be required to understand the nature of this interaction and to identify the factors responsible for cell-specific CCSP gene expression [20]. Recent studies underscore the complexity of this process, indicating that developmental timing of CCSP expression is dependent upon elements outside the 2.25 kb region and that novel winged helix/forkhead family members may participate in cell-specific gene expression in the bronchiolar epithelium [31,32].

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