Transcriptional regulation of CCSP by interferon-γ in vitro and in vivo


Submitted 12 June 2002; accepted in final form 8 August 2002

Pulmonary inflammation is a physiological response to a variety of infectious and immune-mediated lung diseases. However, the ability of the lung to limit the inflammatory response to these processes is of critical importance in confining or preventing resultant lung injury. Therefore, the identification of intrinsic pulmonary mechanisms that can restrict or terminate pulmonary inflammation is of extreme importance in maintaining a homeostatic balance within the lung. One protein that may contribute to the lung’s modulation of inflammation is the Clara cell secretory protein (CCSP) produced by the nonciliated respiratory bronchiolar epithelial cells (Clara cells).

CCSP is a major component of the surface lining fluid in the lung (25), and the expression of CCSP is induced by a variety of proinflammatory mediators and cytokines, including interferon-γ (IFN-γ) (5, 13, 31, 32). The physiological function of CCSP in the alveolar lining fluid remains unclear. However, there is evidence for an anti-inflammatory role of CCSP. This evidence includes in vitro data that CCSP inhibits the activity of phospholipase A2 (6) and in vivo confirmation that CCSP deficiency in mouse models, generated by homologous recombination, increases the susceptibility to pulmonary inflammation secondary to viral, bacterial, or hyperoxic stimuli (9, 11, 14). These observations imply that CCSP may have an important role in modulating intrapulmonary inflammatory events. Therefore, elucidation of the regulatory mechanisms controlling CCSP gene expression in response to IFN-γ stimulation may help define the intrinsic mechanisms for modulating pulmonary inflammation in the lung.

The molecular events that regulate IFN-γ-induced expression of CCSP have not been fully defined. Previous analyses demonstrate that IFN-γ-induced expressions of CCSP are mediated in part by posttranscriptional mechanisms (31). However, the potential for transcriptional regulation of CCSP by IFN-γ remains to be determined. Many analyses performed to ascertain the regulatory mechanisms responsible for CCSP gene expression have been carried out in a variety of cell culture models. These analyses have been hampered by the lack of a cell line in which the endogenous expression of CCSP and a full complement of the respiratory cell-specific trans-acting factors are present. The known cis elements identified in the 5′-flanking region of the CCSP gene are demonstrated in Fig. 1. The CCSP promoter is divided into proximal and distal promoter regions, with each region containing multiple cis elements that may function in the regulation of...
CCSP gene expression (10, 17, 18, 26). The proximal 166-bp region is sufficient to maintain cell-specific expression of reporter genes in vitro and in vivo (16, 18, 22), whereas the distal promoter region is capable of driving cell-specific expression of a reporter gene to levels comparable to endogenous CCSP gene expression in vivo (15). The known cis elements contained in the proximal 166-bp fragment of the CCSP promoter that may be important in lung-specific gene expression include two thyroid transcription factor-1 (TTF-1) sites (17), two CCAAT/enhancer-binding protein (C/EBP) sites (3), an activator protein-1 (AP-1) binding site (27), and two hepatocyte nuclear factor-3 (HNF-3) consensus sites (22). Previous analyses of these cis elements suggest that there may be complex combinatorial regulation mediated by interactions of the complementary trans-acting elements (1, 22, 27, 28). The cis elements in the distal CCSP promoter that regulate the enhanced CCSP gene expression remain to be elucidated. However, it is known that three TTF-1 sites and a γ-interferon activation site (GAS) are localized to this promoter region (13, 17).

Although the molecular events that regulate IFN-γ-induced expression of CCSP have not been fully defined, it is known that IFN-γ effects are mediated by receptor-ligand interactions. These interactions precipitate the activation of a rapid signal transduction cascade of events, resulting in the activation and nuclear translocation of the signal transducer and activator of transcription (STAT1) protein. Nuclear STAT1 homodimers may affect the transcription of specific target genes directly by binding to specific DNA regulatory elements or indirectly through the increased transcription and activity of interferon regulatory factor-1 (IRF-1) transcription factor. Previous analysis of the CCSP gene promoter demonstrates at least two regions through which IFN-γ could mediate a change in CCSP gene expression. DNase I footprinting demonstrates an interferon activation site (GAS) spanning nucleotides –314 to –284, through which IFN-γ could directly affect a change in CCSP gene transcription (13). Alternatively, IFN-γ could indirectly affect CCSP gene expression through IRF-1-mediated interactions with the HNF-3 sites in the proximal promoter region, as observed for hepatic expression of the transthyretin gene (20).

In the present study, we demonstrate that IFN-γ-induced expression of the CCSP gene is regulated, at least partially, at the level of transcription. Furthermore, we demonstrate that the IFN-γ responsiveness of the CCSP gene is localized to the proximal 166-bp region of the 5′-flanking region of the CCSP gene in vivo and in vitro. Moreover, we demonstrate that the IFN-γ responsiveness of the CCSP gene is mediated through a complex regulatory region in the proximal promoter region and that HNF-3β binding is a major component of this induction.

**MATERIALS AND METHODS**

Cell culture. Mouse transformed Clara cells (mtCC) were cultured at 37°C in a humidified atmosphere with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (0.1 mg/ml).

Footprinting. The murine CCSP (mCCSP) promoter sequence previously reported (26), spanning base pairs –291 to –11, was amplified by PCR. The PCR product was subcloned into the pCR2.1-TOPO vector (Invitrogen), and the fragment was isolated by digestion with HindIII and XbaI. The ends were then labeled with Klenow and [32P]dATP and [32P]dCTP. Unincorporated nucleotides were removed by passing the reaction mixture through a Sephadex G-25 spin-column (Boehringer Mannheim). The DNA was cut with BamHI, extracted with phenol-chloroform-isooamyl alcohol, and precipitated with 100% ethanol. The DNase I reaction was performed according to the manufacturer’s recommendation using the Promega Core Footprinting system.

Briefly, nuclear extracts obtained from mtCC were incubated in binding buffer (12 mM HEPES, 12% glycerol, 50 mM KCl, 0.12 mM EDTA, 3 mM MgCl₂, and 1.2 mM dithiothreitol) with 40 ng of poly(dI-dC) for 10 min on ice and then for 1 min at room temperature. Pancreatic DNase I (0.375 U) was incubated with increasing amounts of nuclear proteins (10 or 30 ng). After 1.5 min, the reaction was stopped by addition of 100 µl of stop buffer (10 mM EDTA, 0.1% SDS, and 50 ng/ml proteinase K). The samples were incubated at 37°C for 15 min and then subjected to phenol-chloroform-isooamyl alcohol extraction and ethanol precipitation. The reaction products were analyzed on a polyacrylamide-8 M urea sequencing gel. A nucleotide reaction for the region being footprinted was run in parallel in the same gel for accurate identification of the sizes and nucleotide sequences of the footprinted regions. After electrophoresis, the gels were fixed, dried, and exposed to X-ray film at –80°C.

Western blot analyses. For Western blot analyses, we utilized samples obtained from whole lung homogenates or mtCC whole cell extracts. Samples were supplemented with SDS loading buffer and boiled. The proteins were separated by 12% SDS-polyacrylamide gels and electrobotted onto a polyvinylidene difluoride membrane (Millipore) at 4°C. Western blot analysis was done using a number of primary antibodies. TNT-1 at a 1:6,000 dilution (Santa Cruz Biotechnology and the laboratory of F. J. DeMayo), HNF-3β (Santa Cruz Biotechnology), C/EBPα (Affinity Bioreagents and Santa Cruz Biotechnology), C/EBPβ (Affinity Bioreagents and Santa Cruz Biotechnology), or C/EBPδ (Santa Cruz Biotechnology) at a 1:3,000 dilution. This was followed by luminos-
cent detection according to the protocol of the manufacturer (Amersham Life Technologies).

**Transient transfection analyses.** At 1 day before transfection, mitomycin was plated at a density of 3 x 10^6/100-mm plate. Cells at 50–70% confluence were transfected on 100-mm dishes by using 30 µl of the Superfect transfection reagent (Qiagen) with 10 µg of reporter plasmid and 0.5 µg of cytomegalovirus (CMV)-β-galactosidase plasmid as an internal control. Transfected cells were incubated for 3 h and then washed with DMEM to remove the transfecting agent. Cells were then fed DMEM without fetal calf serum and treated with vehicle or recombinant mouse IFN-γ (Invitrogen) at 1,000 IU/ml DME. The cells were harvested, centrifuged for 5 min, and resuspended in 100 µl of 250 mM Tris (pH 7.5).

The cells were lysed by three cycles of freezing in liquid N2 and thawing at 37°C. The cell debris was cleared by centrifugation, and protein concentration was measured using Bradford reagent (Bio-Rad). Alternatively, for RNA analyses, the cells were harvested with TRIzol reagent (Invitrogen), and total RNA was quantified by measuring the absorbency at 260 nm using a spectrophotometer.

**Transfection analysis of CCSP-chloramphenicol acetyltransferase constructions.** The generation of the CCSP-chloramphenicol acetyltransferase (CAT) plasmids containing various lengths of the CCSP promoter ligated to the bacterial CAT gene has been reported previously (18). The linker-scanning mutation of the GAS site and the two HNF-3 binding sites were introduced into the 803-bp promoter fragment of the wild-type CCSP gene by modifications of the procedure previously described (12). Mutagenic oligonucleotides were generated to replace the GAS site or the proximal HNF-3 site with the sequences that compose a reporter gene. The amplification was performed using the 803-bp CCSP-CAT plasmid as a template. For each PCR, one of two anchor primers was used along with the specific mutagenic primer. A 5’ anchor, 1M, contains a HindIII overhang and anneals at position −803 of the CCSP promoter. A 3’ anchor primer, pBLCAT-3, annales a BglII restriction site within the CAT reporter gene. The amplified fragments were cloned into the pCR2.1 vector (Invitrogen) and sequenced to verify the appropriate pairs of fragments were then cloned into the HindIII-BamHI site of the pBluescript KS vector. Plasmid DNA for transfection was isolated using Maxi-Prep plasmid preparations (Qiagen).

**Liquid CAT assay.** The CAT activity of the lysate was assayed as described by Seed and Sheen (23). The activity of 50 µg of lysate was assayed at 37°C for 5 h with a mixture of 10 µl of butyryl CoA (2.5 mg/ml) and 20 µl of [³H]chloramphenicol (0.01 µCi/ml). The reaction was then extracted with 200 µl of tetrathylpentadecane-xylene (2:1), and 150 µl of the top aqueous phase were counted in a liquid scintillation counter with 4 ml of scintillation fluid.

**β-Galactosidase assay.** As an internal control, all CAT reporters were cotransfected with 1 µg of CMV-β-galactosidase plasmid, consisting of the CMV promoter driving the expression of the β-galactosidase gene. The β-galactosidase activity in each transfection was used to control for variability between transfections. Quantitation of β-galactosidase activity was achieved essentially as described by Sambrook et al. (21). In summary, equal amounts of protein in 30 µl from each transfection plate were mixed with the following reagents: 3 µl of 100× MgCl₂ (0.1 M MgCl₂ and 4.5 M β-mercaptoethanol), 66 µl of 1× ONPG (4 mg/ml of o-nitrophenyl-β-D-galactopyranoside dissolved in 0.1 M sodium phosphate, pH 7.5), and 201 µl of 0.1 M sodium phosphate, pH 7.5, and incubated for 30 min at 37°C. The reactions were stopped by the addition of 500 µl of Na₂CO₃, and the optical density of the reactions was read at a wavelength of 420 nm.

**Luciferase activity.** The CCSP-luciferase plasmid containing the 800-bp of the CCSP promoter ligated to the firefly luciferase reporter gene was generated by PCR using the 2.1-kb CCSP-CAT plasmid as a template and oligonucleotides with synthesized KpnI1 and HindIII sites 5’ and 3’, respectively, and TOPO TA cloned into the pCR2.1 vector (Invitrogen). The 800-bp promoter was then cut out with KpnI and HindIII and directionally ligated into the pGL3-basic luciferase reporter gene (Promega). The 166-bp CCSP-luciferase plasmid was generated by cutting the 800-bp CCSP-luciferase plasmid with SacI, blunt ended with T4 polymerase, and ligated with T4 DNA ligase (Boehringer Mannheim). Transient transfection analyses were then done utilizing the luciferase reporter genes in mitCC and harvested in 1× reporter lysis buffer (Promega). The cell lysate was subjected to a freeze-thaw cycle and centrifuged at 12,000 g in a microcentrifuge for 15 s at room temperature to pellet the cell debris. The luminescent signals generated by the firefly and Renilla luciferase reporter genes were measured using the dual-luciferase reporter assay (Promega) and a Monolight Luminometer (Pharmingen).

**Transgenic mouse analysis.** Previously generated transgenic mice that express a human growth hormone (hGH) reporter gene driven by a 166-bp segment of the CCSP promoter were utilized for the in vivo assessment of the effects of IFN-γ on CCSP gene expression (18). Adult transgenic mice were treated with 0.1 ml of saline or 0.1 ml of IFN-γ (1,000 IU/ml DME) via tracheotomy and allowed to recover on a warming pad for 30 min. The mice were then killed by lethal injection of tribromethanol (Avertin) at 24 or 48 h after treatment. Lung samples were harvested for RNA and protein analyses.

**IRF-1 mouse analysis.** Previously generated IRF-1-deficient mice (20) were utilized for in vivo assessment of the effects that the IRF-1 pathway was involved in the IFN-γ-induced expression of mCCSP, in addition to two wild-type controls: 129, which was the strain background of the IRF-1-deficient mice, and FVB, which is known to respond to IFN-γ with an increase in CCSP expression (13). Adult null mice and wild-type controls were treated with 0.1 ml of saline or 0.1 ml of IFN-γ (1,000 IU/ml DME) via tracheotomy and allowed to recover on a warming pad for 30 min. Mice were killed by lethal injection of tribromethanol 48 h after IFN-γ treatment. Lung samples were harvested for RNA and protein analyses.

**RNase protection assay.** Total RNA was extracted from mouse lungs by using TRIzol reagent (Invitrogen). Expression of the mRNAs for the CCSP and hGH was accomplished by RNase protection with a [³²P]UTP (ICN)-labeled probe using an RNase protection assay (RPA) kit (Ambion). The template for the CCSP mRNA was generated by insertion of a 327-bp BamHI and NotI fragment of the mouse CCSP cDNA into pCR21 (Invitrogen). An antisense riboprobe for RPA was generated by digesting the plasmid with BamHI, and T7 RNA polymerase was used for in vitro RNA synthesis. A template for hGH mRNA was generated by insertion of a 2.0-kb EcoRI fragment of the hGH cDNA into pBluescript (Stratagene). An antisense riboprobe for RPA was generated...
by digesting this plasmid with BglII and by using T7 RNA polymerase for in vitro RNA synthesis. A cyclophilin probe was used as an internal control for all RPA analyses.

EMSAs. A synthetic oligonucleotide was generated that contained 30 bp of the CCSP promoter from -110 to -81, which includes the overlapping binding sites for AP-1, HNF-3, and C/EBP. Subsequent oligonucleotides were generated with mutations in the AP-1, HNF-3, or C/EBP binding sites and followed by oligonucleotides, which contained double mutations in the AP-1/HNF-3, AP-1/C/EBP, or HNF-3/C/EBP binding sites. Also, a triple-mutant synthetic oligonucleotide that contained mutations in all three binding sites was generated to be utilized as a nonspecific competitor (Fig. 2C). In addition, the oligonucleotides contained a 5’ overhang and were end-labeled with [32P]dATP and [32P]dCTP using a Sequenase reaction kit (US Biochemicals). EMSA was performed by incubating 5 × 10^4 cpm of labeled oligonucleotide with 5–10 μg of nuclear extract from the mtCC in gel shift binding buffer (Promega) treated with vehicle or IFN-γ for 4–24 h. The formation of complexes was performed at room temperature for 15 min. The complexes were separated by electrophoresis through a 6% nondenaturing polyacrylamide gel, dried on filter paper, and exposed to autoradiographic film.

RESULTS

mtCC express a full complement of respiratory epithelial trans-acting factors. Previous analysis of the mCCSP promoter was conducted in H441 cells, which do not express CCSP or recognize the distal promoter elements required for in vivo expression of CCSP (18). With the use of tumor cells derived from mice expressing the simian virus 40 large T antigen gene under the control of the CCSP promoter, an mtCC line was generated. This cell line expresses IFN-γ-regulatable CCSP, albeit at low levels (13). Before these cells were used in transfection analysis, a survey was conducted to determine the nuclear trans-acting proteins expressed in these cells; these proteins would bind to the endogenous mCCSP promoter. DNase I footprinting analyses demonstrate three distinct regions, RI, RII, and RIII, within the proximal CCSP promoter that contain the DNA binding sequences for the known respiratory epithelial transcription factors, HNF-3, C/EBP, and TTF-1 (Fig. 2A, Table 1). Western blot analyses were done to establish whether the mtCC...
lines contained these endogenous trans-acting factors expressed in native respiratory epithelium. mtCC do express the transcription factors HNF-3β, C/EBP-α, C/EBP-β, C/EBP-δ, and TTF-1 (Fig. 2B). mtCC maintain the expression of these trans-acting factors through >175 passages, although HNF-3β protein content diminishes with increasing passage number (Fig. 2B). The passage number does not appear to influence expression of TTF-1 or the three C/EBP isoforms. The high level of expression of C/EBP-δ and the low level of expression of C/EBP-α are consistent with previous observations in bronchiolar epithelium (3). Our observation of C/EBP-β expression in mtCC is the first demonstration of this transcription factor in bronchiolar epithelium.

mtCC nuclear proteins interact with the HNF-3 binding site contained within a region of overlapping cis elements in the CCSP proximal promoter. There are two cis elements for each of the trans-acting factors TTF-1, HNF-3, and C/EBP proteins in the proximal 144 bp of the CCSP promoter. To determine whether these proteins were functionally interactive with the cis elements in the CCSP proximal promoter in mtCC and whether the protein-DNA interactions were altered by IFN-γ, EMSAs were performed. The region of the CCSP promoter investigated was a complex sequence of nucleotides spanning base pairs −110 to −81, which contains overlapping cis elements for AP-1, HNF-3, and C/EBP. To determine whether the potential protein-DNA interactions at this region of the CCSP promoter were affected by IFN-γ, we utilized the double-stranded oligonucleotides shown in Fig. 2C. The representative EMSA shown in Fig. 2D demonstrates three specific protein-DNA complexes (B1, B2, and B3) after incubation of nuclear extracts from mtCC with the labeled intact AHC probe (lane 2). These three specific protein-DNA complexes are competed away by the addition of increasing amounts (10× and 50×) of intact cold AHC probe (lanes 3 and 4). To identify whether the trans-acting factors were binding specifically to the AP-1 (22), HNF-3 (22), or C/EBP (4) binding sites, we generated additional mutations to abolish the individual binding sites for AP-1, HNF-3, and C/EBP. These mutations were termed mAHC, AmHC, and AHmC, respectively. Increasing amounts of unlabeled mutant oligonucleotides were used in an EMSA competition assay to interact with the mtCC nuclear extract (lanes 5–10). Increasing amounts of unlabeled AHmC oligonucleotides, which contain a mutation in the C/EBP binding site but retain the capacity to bind AP-1 and HNF-3, showed competition with bands B2 and B3 (lanes 5 and 6). This indicates that B1 interacts with the C/EBP binding site. Competition with mAHC, a mutation in the AP-1 binding site (lanes 7 and 8), demonstrated that there is very little, if any, difference in competition between the wild-type AHC oligonucleotides (lane 4) and the mutant mAHC oligonucleotides (lane 8). Thus interaction of the AP-1 site in the AHC probe with mtCC nuclear extract could not be demonstrated. Competition with unlabeled AmHC, which contains a mutation in the HNF-3 binding site, showed that complexes B2 and B3 remained, indicating that these complexes interact with the HNF-3 binding sites (lanes 9 and 10). These results demonstrate that mtCC contain multiple nuclear proteins that bind specifically to the CCSP proximal promoter region spanning nucleotides −110 to −81 upstream from the start of transcription.

The minimal IFN-γ-responsive region of the CCSP promoter is localized to the proximal promoter in vitro. To localize the minimal IFN-γ-responsive region of the CCSP promoter, a variety of CCSP deletion reporter genes were transiently transfected into mtCC and then treated with vehicle (DMEM without fetal calf serum) or IFN-γ (1,000 IU/ml DMEM) without fetal calf serum. As shown in Fig. 3A, basal expression of the CAT reporter gene under the control of 803 bp of the CCSP 5′-flanking region is greater than that for the 166 bp of 5′-flanking DNA. This demonstrates that, unlike H441 cells, mtCC recognize the elements in the distal region of the mCCSP promoter. Figure 3A also shows IFN-γ responsiveness in the 803-, 166-, and 144-bp CCSP promoter reporter constructs. However, when the CCSP promoter was deleted to −87 bp, the baseline level of expression of the CCSP reporter gene was greatly reduced and failed to demonstrate a response to IFN-γ treatment. These results demonstrate that IFN-γ can regulate expression of a CAT reporter gene, mediated by 144 bp of 5′-flanking DNA of the CCSP gene.

IFN-γ activates the CCSP gene in vivo via the proximal 166-bp segment of the CCSP promoter. The ability of 166 bp of the 5′-flanking region of the CCSP promoter to mediate the effects of IFN-γ on CCSP gene expression in vivo was confirmed using previously generated adult transgenic mice with the 166-bp segment (166CCSP-hGH) of the CCSP promoter ligated to the hGH coding region (18). Quantification of representative RPA results are shown in Fig. 3, B and C, which demonstrates a time-dependent increase in endogenous CCSP and hGH RNA transcripts in the IFN-γ-treated 166CCSP-hGH transgenic mice. These results demonstrate that the minimal IFN-γ-responsive elements are contained in the proximal 166 bp of the CCSP promoter.

HNF-3β binding to the overlapping cis elements in the CCSP proximal promoter is increased by IFN-γ. Additional EMSAs and antibody inhibition analyses were performed to determine whether exposure to

Table 1. Known binding sequences protected in DNase I footprinting analysis

<table>
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<tr>
<th>Protected Sequence (5′–3′)</th>
<th>Known Transacting Factors</th>
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<tbody>
<tr>
<td>Region 1 ATTATTG</td>
<td>HNF-3</td>
</tr>
<tr>
<td>Region 2 CCAAGTAATAAA</td>
<td>HNF-3</td>
</tr>
<tr>
<td>Region 2 TAATGCAAT</td>
<td>C/EBP</td>
</tr>
<tr>
<td>Region 3 GGAGCAGCA</td>
<td>C/EBP</td>
</tr>
<tr>
<td>Region 3 TCTACCTCTTG</td>
<td>TTF-1</td>
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HNF-3, hepatocyte nuclear factor-3; C/EBP, CCAAT/enhancer-binding protein; TTF-1, thyroid transcription factor-1.
IFN-γ would increase the protein-DNA interactions within the proximal −110 to −81 bp of the CCSP promoter and, if so, to identify the proteins interacting with the specific binding site. For these analyses, we utilized nuclear extracts from mtCC treated for 4–24 h with vehicle or IFN-γ and a radiolabeled mAHmC probe spanning base pairs −110 to −81, which contains an intact HNF-3 binding site but mutant AP-1 and C/EBP binding sites. We observed a time-dependent increase in protein-DNA interactions at the HNF-3 binding site (Fig. 4A, lanes 2–4). Competition with the cold probe (lane 5) demonstrates the formation of three specific protein-DNA complexes, H1, H2, and H3, with H1 and H2 running as a doublet. Antibodies were added during the incubation of hot probe and nuclear proteins for the samples in lanes 7–9. The addition of an antibody specific for HNF-3α (lane 7) shows some minor inhibition of the upper bands (H1 and H2) in the nuclear protein-DNA complex. The addition of an antibody for HNF-3β (lane 8) shows a complete inhibition of complex H1 and partial inhibition of complexes H2 and H3. The addition of a nonspecific antibody (lane 9) does not show any inhibition or supershift. These results suggest that HNF-3β is a major component within the protein-DNA complex binding at the complex overlapping the HNF-3 site.

Previously, it was shown that HNF-3β mRNA in mtCC is increased by exposure to IFN-γ (13). Therefore, we performed Western blot analyses to determine whether the protein content of HNF-3β was increased as well. The representative Western blot shown in Fig. 4B demonstrates a significant increase in nuclear protein expression of HNF-3β. To determine whether HNF-3β was sufficient to mediate an increase in CCSP gene activity, we performed transient cotransfections with HNF-3β and the 166-bp CCSP reporter gene (Fig. 4C). HNF-3β expression resulted in a significant increase in CCSP reporter gene expression. Additional transient cotransfections with HNF-3β and the 166-bp CCSP reporter gene plus IFN-γ treatment did not show any additional activation above the level of expression induced by HNF-3β alone (data not shown). These findings clearly demonstrate that HNF-3β can mediate an induction in the expression of CCSP in mtCC, that HNF-3β protein content is increased by IFN-γ, and that HNF-3β interactions with the CCSP proximal promoter increase with IFN-γ exposure.

Proximal and distal HNF-3 cis-acting elements are required for CCSP gene expression. To determine the contribution of the GAS site in the distal promoter region and the two HNF-3 binding sites in the proximal promoter to IFN-γ induction of CCSP gene expression, transient transfections were conducted utilizing specific linker-scanning mutations to the GAS, distal HNF-3, and proximal HNF-3 sites. Figure 5 demonstrates that mutation of the distal GAS site resulted in an attenuation of the baseline expression of the CCSP reporter gene but that this construct remained responsive to IFN-γ. However, mutation of the distal or proximal HNF-3 cis-acting element reduced the expression of the CCSP reporter gene constructs to background.

Fig. 3. Localization of interferon-γ (IFN-γ) responsiveness to the proximal CCSP promoter in vitro and in vivo. A: transfection analysis of mtCC with deletion constructions with 803, 166, 144, 87, and 23 bp of the CCSP 5′-flanking DNA fused to the chloramphenicol acetyltransferase (CAT) reporter gene. Cells were treated with vehicle or IFN-γ (1,000 IU/ml DMEM). Transfection efficiency was normalized by cotransfection with cytomegalovirus (CMV)-β-galactosidase (β-gal). Values (means ± SE for each construct) are representative of several independent transfections and are normalized for β-galactosidase activity. *P < 0.05. B and C: quantification of RNase protection assay (RPA) of in vivo expression of endogenous CCSP and human growth hormone (hGH) reporter gene activity under direction of the CCSP promoter in transgenic mice after 0, 24, or 48 h of intratracheal instillation of 0.1 ml of IFN-γ (1,000 IU/ml DMEM). Values (means ± SD) are normalized to cyclophilin for each exposure group. *P < 0.05.
levels and further eliminated the IFN-γ responsiveness of the CCSP promoter. These results demonstrate that the distal and proximal HNF-3 cis elements in the CCSP promoter are required for CCSP gene expression, as well as IFN-γ responsiveness.

**IFN-γ-stimulated expression of mCCSP expression in vivo is mediated through IRF-1.** IFN-γ signaling is known to involve cytokine binding to the IFN-γ receptor on the cell surface, which leads to activation of the Janus kinases and phosphorylation and homodimerization of STAT1 proteins. Previously, it was shown that HNF-3β is induced by IFN-γ in hepatocytes through mechanisms involving STAT1 and IRF-1 (19). Therefore, to determine whether the underlying mechanism for IFN-γ responsiveness, localized to the proximal mCCSP promoter, was mediated through a similar mechanism involving IRF-1, we analyzed CCSP expression in IRF-1-deficient mice exposed to intratracheal administration of IFN-γ. Figure 6A is a representative RPA demonstrating the CCSP mRNA expression after intratracheal administration of saline or IFN-γ. We observed no change in CCSP expression in the IRF-1-deficient mice after administration of IFN-γ, in contrast to the significant increase in CCSP expression in the 129 and FVB wild-type mice. Quantification of CCSP mRNA expression after administration of saline or IFN-γ to IRF-1-deficient mice, i.e., 129 and FVB wild-type mice, is shown in Fig. 6B. To further clarify whether IFN-γ responsiveness was mediated through IRF-1-mediated induction of HNF-3β, Western blot analyses were done to determine the level of HNF-3β in whole lung homogenates obtained from the IRF-1−/− and 129 wild-type mice after administration of 0.1 ml of saline or 0.1 ml of IFN-γ (1,000 IU/ml DMEM). There was no detectable HNF-3β in the whole lung homogenates from the IRF-1−/− mice with saline or IFN-γ.
molecular mechanisms that mediate the increase in CCSP expression secondary to exposure to IFN-γ. The present investigations utilized a variety of in vitro and in vivo techniques to determine that IFN-γ-induced expression of CCSP is mediated, in part, at the level of transcription. Our findings extend the findings of Yao et al. (31), who showed that IFN-γ treatment of human BEAS-2B cells increased CCSP protein production in a dose- and time-dependent manner. They found the greatest stimulation in CCSP expression at 1,000 IU/ml IFN-γ, the same dose used in this study; at a lower dose (300 IU/ml), however, these same investigators found that the increase in CCSP protein expression was mediated by an increase in CCSP mRNA stability, with the half-life of CCSP mRNA increased from 15 to 40 h. The transcriptional regulation of the CCSP promoter in mtCC in the present study highlights the utility of this cell line to respond to stimuli in a manner similar to endogenous Clara cells. This is the first report of an in vitro tool that mirrors the responsiveness of the in vivo Clara cells.

In addition, there is the potential that IFN-γ treatment affects specific gene expression by multiple mechanisms, resulting in an alteration in the rate of tran-

Fig. 5. Intact HNF-3β cis elements are mandatory for IFN-γ-induced expression of the CCSP gene. Wild-type (WT) 803 bp of the CCSP promoter as well as linker-scanning mutations of the 803-bp promoter with mutations in the GAS (mGAS), distal HNF-3 (mdH) binding site, and proximal HNF-3 (mH) binding site fused to the CAT reporter gene were transiently cotransfected with an internal control, CMV-β-gal, into mtCC. At 24 h after transient transfection, mtCC were treated with vehicle or IFN-γ (1,000 IU/ml DMEM) in the absence of fetal calf serum. Data represent independent transfections, which are normalized for absence of fetal calf serum. Data represent independent transfections, which are normalized for absence of fetal calf serum. Values are means ± SD for each construct. *P < 0.05.

DISCUSSION

The cell-specific expression of respiratory epithelial cell genes is not regulated by lung-specific transcription factors. This lung-specific gene regulation is, in fact, conferred by complex interactions of general transcription factors with distinct cis elements in the 5′-flanking regions of the respiratory epithelial cell genes (2, 8, 30). The cell-specific gene expression of the CCSP gene represents an interesting model to decipher the molecular mechanisms involved in cell-specific transcriptional control of pulmonary genes. However, these analyses have been hampered by the lack of a faithful cell line to mirror the in vivo Clara cell environment and function. In this study, we utilized CCSP reporter genes in transgenic mice and mtCC to elucidate the transcriptional control of CCSP in response to IFN-γ stimulation. In addition, we have further clarified the utility of the mtCC line as a faithful model for analysis of CCSP expression mediated by the proximal and distal promoter regions with greater fidelity than present models for CCSP gene expression.

The importance of elucidating the molecular mechanisms that regulate IFN-γ responsiveness of the CCSP gene may exemplify a cell-specific scheme for the control of host defenses and modulation of pulmonary inflammation. The observation that CCSP inhibits the production and biological activity of IFN-γ by mononuclear cells further supports the speculation that IFN-γ induction of CCSP expression in vivo may function to restrict intrapulmonary inflammation (33). Extensive investigations have failed to clearly characterize the...
scription and/or an alteration in message stability (24). These results, in combination with those of Yao et al. (31), would suggest that IFN-γ treatment regulates CCSP gene expression through multiple mechanisms, including an increase in gene transcription, message stability, and protein production. The use of multiple mechanisms to ensure an increase in CCSP expression may signify the importance of limiting intrapulmonary inflammatory events.

Our promoter deletion and linker-scanning analyses demonstrate that the proximal 144 bp of the CCSP promoter were sufficient to maintain IFN-γ responsiveness of the CCSP gene. The mutational analyses were critical in verifying our conclusions from the promoter deletions, the results of which may be misleading because of the potential for disruption of specific protein-protein or protein-DNA interactions of enhancing elements in the 5′-flanking region of the gene. Further verification of our results, in addition to recognition of the physiological importance of the cis elements in vivo, was obtained from our analyses in the transgenic mouse model. The consistency in the results from these different models further strengthens our hypothesis that increased HNF-3β protein-DNA interactions mediate the IFN-γ-induced transcriptional increase in CCSP gene expression. Moreover, the observation that both HNF-3 sites are critical to the basal and IFN-γ-induced CCSP gene expression is consistent with emerging evidence that combinatorial action of transcription factors may provide a method of stimulus- and cell-specific gene regulation through complex protein-DNA and protein-protein interactions (7).

The complex regulatory region of the CCSP proximal promoter between nucleotides −110 and −81, represented by R2 on the DNase footprinting analysis, includes the overlapping consensus sites for HNF-3 and C/EBP proteins. We have shown that IFN-γ stimulation of mtCC results in an increase in HNF-3β levels and an increase in functional protein-DNA interactions at the HNF-3 binding site. We identified HNF-3β as the major protein contained within two of the protein-DNA complexes with a minor contribution by HNF-3α. Interestingly, HNF-3 proteins have been shown to mediate the IFN-γ regulation of the transthyretin gene in the liver (19). In the cytokine regulation of transcription, similarities can be drawn between the liver-specific gene and respiratory epithelial-specific genes. Analysis of cytokine regulation of transcription of transthyretin showed that HNF-3β and C/EBP binding sites are involved in the cytokine regulation of this gene. Thus the use of these transcription factors to mediate cytokine regulation of lung and liver genes may be conserved.

C/EBP-α and C/EBP-δ proteins have been shown to be involved in the regulation of the CCSP gene through complex protein-DNA interactions at the overlapping C/EBP binding site in conjunction with a second C/EBP binding site in close proximity (3). This temporal arrangement of cis elements in the CCSP proximal promoter for the C/EBP binding sites is strikingly similar to the dual binding sites for the HNF-3 proteins. Our findings that both HNF-3 binding sites are critical for CCSP gene regulation in mtCC, in conjunction with the previous reports of required interactions at both C/EBP binding sites, suggest that such compound regulatory units may serve as a more universal molecular mechanism for organ- or cell-specific gene expression.

Our results support the hypothesis that CCSP induction by IFN-γ is mediated by the interaction of multiple trans-acting factors interacting with a compound regulatory unit in the proximal promoter. One potential molecular mechanism that illustrates the observed interactions of HNF-3β in the IFN-γ-induced expression of CCSP is shown in Fig. 7. The proposed mechanism for IFN-γ induction of CCSP expression mediated by the proximal mCCSP promoter was suggested by previous studies, which demonstrated that IFN-γ induced the expression of IRF-1 (15), which then induced an increase in HNF-3β expression and functional DNA interactions. Our findings clearly demonstrate that IFN-γ responsiveness of mCCSP is lost in the absence of IRF-1 in association with a lack of induction of HNF-3β.

In summary, the present study demonstrates that CCSP gene regulation by IFN-γ is, in part, regulated at the level of transcription. Furthermore, it shows
that the IFN-γ responsiveness of the CCSP gene is mediated through a complex regulatory region in the proximal promoter and that HNF-3β binding to this region is a major component of the induction of CCSP transcription. This report proposes a mechanism by which IFN-γ mediates an increase in mCCSP expression. Moreover, this report reveals the utility of the mCC line as a model for further investigation of the mechanistic features of cell-specific transcriptional regulation of gene expression in respiratory epithelium. Although the present study was focused on the determination of the minimal cis elements responsive to IFN-γ regulation for the CCSP gene, we also noted that the distal CCSP promoter was important in CCSP gene expression to a level >10 times the expression mediated by the proximal promoter alone. These findings are important for future investigations of CCSP gene regulation and the identification of enhancing elements in this distal regulatory region.

Dr. R. Costa (Dept. of Molecular Genetics, University of Illinois, Chicago, IL) generously provided the antibodies for HNF-3 and HNF-3α as well as the HNF-3 expression vector. J. Wang, M. Gu, and J. DeMayo provided technical assistance. J. Ellsworth aided in preparation of the manuscript. This work was supported by National Heart, Lung, and Blood Institute Grant HL-61406.

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