Synergistic Transactivation of the Differentiation-dependent Lung Gene Clara Cell Secretory Protein (Secretoglobin 1a1) by the Basic Region Leucine Zipper Factor CCAAT/Enhancer-binding Protein α and the Homeodomain Factor Nkx2.1/Thyroid Transcription Factor-1*

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The basic region-leucine zipper transcription factor CCAAT/enhancer-binding protein α (C/EBPα) and the homeodomain transcription factor Nkx2.1/thyroid transcription factor-1 are essential for normal lung morphogenesis. Nkx2.1 is expressed from the onset of lung development, whereas C/EBPα expression is turned on at later stages. The expression of C/EBPα correlates to the appearance of lung-specific proteins with differentiation-dependent expression patterns, such as the Clara cell secretory protein (secretoglobin 1a1 (Scgb1a1), CCSP). In this study, we demonstrate synergistic transactivation by C/EBPα and Nkx2.1 in the regulation of the CCSP gene. We show that the synergistic activity of C/EBPα and Nkx2.1 originates from cis-acting elements in the proximal promoter of CCSP and that the synergism is dependent on NH2-terminal transactivation domains of C/EBPα and Nkx2.1. Our results suggest that the cooperation of C/EBPα and Nkx2.1 is a major determinant for the high level, lung epithelial-specific expression of CCSP during the later stages of lung development and in the adult lung.

Lung development is initiated when the lung bud evaginates from the ventral wall of the anterior foregut endoderm (1). Mesenchymal tissue interaction with the lung bud induces a series of repeated branchings to establish the epithelially lined airways. As development proceeds, regional differentiation along the airway epithelium results in multiple cell types such as alveolar type I and type II cells and bronchiolar Clara cells. Differentiation is accompanied by expression of differentiation markers such as the pulmonary surfactant proteins-A, -B, and -D and the Clara cell secretory protein/secretoglobin 1a1 (CCSP/Scgb1a1).1

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‡ The abbreviations used are: CCSP, Clara cell secretory protein; C/EBP, CCAAT/enhancer-binding protein; SP, surfactant protein; HNF, hepatocyte nuclear factor.

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epithelium at high levels. Its expression is controlled by multiple transcription factors including HNF-3, Nkx2.1, and C/EBP factors (22). CCSP exhibits a differentiation-dependent expression pattern and is turned on late in lung development, in mouse around embryonic day E17 (23). Thus, CCSP provides a good model to study lung-specific gene expression and the interplay between lung-enriched transcription factors as well as the action of these transcription factors during lung morphogenesis. In this study, we demonstrate that Nkx2.1 and C/EBPα, but not the related family member C/EBPβ, synergistically transactivate the CCSP gene. We demonstrate that C/EBPα and Nkx2.1 act on cis-acting elements in the proximal CCSP promoter. The ability of C/EBPα and Nkx2.1 to act synergistically is specified by transactivation domains in the NH2-terminal part of both factors. Our results suggest that the cooperative action of C/EBPα and Nkx2.1 is a major determinant for the differentiation-dependent lung-specific expression of the CCSP gene.

EXPERIMENTAL PROCEDURES

Plasmids and DNA Manipulations—Plasmids were maintained in bacterial strains MC1061/pS for plasmid pCMV-ATAAA and yeast strains YEp13-2 and YEp13-1 for all other plasmids. The insect expression vectors pPAC-Nkx2.1 and pPAC-HNF-3α have been described previously (24). The insect expression plasmid for wild-type C/EBPα, pGAC-C/EBPα, has been described previously (25). The insect expression vector pPAC-C/EBPα was constructed by cleaving pCMV-C/EBPα (2) with BamHI and EcoRI. BamHI linkers were ligated to the C/EBPα cDNA and the resulting fragment was subcloned into the BamHI site of the pPAC expression vector. The pDNAI expression vectors for C/EBPα deletion mutants have been previously described (26). Construction of insect expression vectors for C/EBPα deletion mutants was performed by cleavage of the respective pDNAI vector with HindIII/XhoI restriction enzymes. Subsequently, each fragment corresponding to cDNA for C/EBPα deletion mutants was incubated with Klenow fragment (Amersham Biociences) and dNTPs (40 μM of each). BglII linkers were ligated to the blunt-ended cDNAs and fragments were subcloned into the BamHI site of the pGAC insect expression plasmid. No significant difference in expression levels between the insect expression vectors pPAC and pGAC was observed. The Nkx2.1 deletion mutant D1-93 was constructed by subcloning appropriate PCR-derived fragments into the BamHI site of pPAC. The 5′ deletion mutant was preceded by a methionine codon and a Kozak translation initiation sequence. The upper primer used was 5′-CGGGATCCGACCTGGGAGCGCAATCACTGCCCTCTACCTCTTGTGGGCTGCAAA-3′ (ATG start codon underlined) and the lower primer used was 5′-CGGGATCCCTATCACD46-152CGACCATAAACGAGG-3′. The internal deletion mutant (AmpR-102) was constructed by PCR using primers excluding amino acids 46–152 of Nkx2.1. The internally deleted fragment was replaced by a BamHI linker encoding proline and glycine. The primers used were: 5′-TCCCGGGGGGCGGCTGGCGCTGGTCTTGGATGAC-3′ and 5′-TCCCGGGGGCGGCGAGCCCGCGCGGGGACG-3′. Substitution of the C/EBPα NH2-terminal transactivation domain with that of C/EBPβ was done by cleaving the plasmid pPAC-C/EBPβ with restriction enzymes BspEI and BamHI to excise the 5′ basic-leucine zipper domain of C/EBPβ and by cleaving pGAC-C/EBPα with the restriction enzymes BamHI and MluI to excise the 5′ NH2-terminal transactivation domain of C/EBPα. Subsequently, these fragments were ligated and subcloned into the empty pGAC expression vector. The resulting construct, encoding a C/EBPα-C/EBPβ swap mutant, replaces amino acids 1–186 of C/EBPβ with amino acids 1–192 of C/EBPα. The wild-type CCSP promoter-reporter gene constructs and the construct with mutated C/EBP-binding sites have been described previously (2). The Nkx2.1-binding site mutations were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene), according to the protocol provided by the manufacturer. The oligonucleotides used for site-directed mutagenesis were as follows (complementary strands are not shown, mutated bases are underlined): distal Nkx2.1 site mutation, 5′-CCCGCTATTATAGCCAATCTCGTGGAGGCATCTGACCCATGCAG-3′; proximal Nkx2.1 site mutation, 5′-CAGGTCCGCTATCTCAGCGGTGGGCTGAAGAGACATTATAAGACCCG-3′. For the mutation of both Nkx2.1 sites, the plasmid with the proximal site mutation was subsequently mutated with the distal site mutation oligonucleotides. Following site-directed mutagenesis, the mutations were verified by DNA sequence analysis.

RESULTS

C/EBPα and Nkx2.1 Activate the CCSP Promoter Synergistically—Previous studies have identified cis-elements in the first 172 bp of the proximal CCSP promoter that confer lung cell specificity. The transcription factors Nkx2.1, HNF-3α, C/EBPα, and C/EBPβ, can act through this region (22) (Fig. 1A). To further investigate the action of these transcription factors on the CCSP promoter, we performed transfections in

Cell Culture—SL2 cells were cultured in Schneider medium (Invitrogen) supplemented with 10% fetal calf serum (insect cell qualified; Invitrogen) at 27 °C. COS-1 cells were cultured at 37 °C in a humidified atmosphere with 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. All medium were supplemented with l-glutamine.
Drosophila SL2 cells. These cells lack many mammalian transcription factor homologues and are thus a useful tool to study the activity of transcription factors on mammalian promoters (27). Nkx2.1 activated the CCSP promoter 3.5-fold, whereas HNF-3α, C/EBPα, or C/EBPδ were almost inactive (Fig. 1B). In pairwise co-transfections, a strong synergistic activity was seen between C/EBPα and Nkx2.1 that reached a 54-fold induction of the reporter gene (Fig. 1B). All other combinations of transcription factors resulted in levels of transactivation that were approximately additive except for C/EBPα and C/EBPδ that exhibited a slightly more than additive transactivation (Fig. 1B). The finding that C/EBPα and Nkx2.1 synergistically transactivate the CCSP promoter led us to perform further transfection studies with different amounts of expression plasmid for these transcription factors. Increasing amounts of transfected C/EBPα expression plasmid, along with 2000 ng of Nkx2.1 expression plasmid, activated the CCSP promoter in a dose-dependent manner (Fig. 2A), whereas C/EBPα on its own was inactive even at highest DNA levels (Fig. 2A). In the presence of C/EBPα, Nkx2.1 stimulated the CCSP promoter already at 50 ng of Nkx2.1 expression plasmid (Fig. 2B). The highest stimulation was obtained with 1000 ng of Nkx2.1 expression plasmid (up to 12.5-fold over the preservative value of Nkx2.1 and C/EBPα alone) (Fig. 2B). In summary, these data show that high level activation of the CCSP promoter is dependent on the simultaneous presence of Nkx2.1 and C/EBPα.

Studies in transgenic mice have revealed that additional Nkx2.1-binding sites reside further upstream in the CCSP promoter (21). To investigate the relevance of these upstream Nkx2.1-binding sites for the synergistic activity with C/EBPα, we performed transient transfections in SL2 cells with a reporter plasmid containing 2.1 kb of the 5'-flanking region of the endogenous CCSP promoter. Activation of the −2.1-kb CCSP promoter fragment by Nkx2.1 or C/EBPα on their own, was 7.1- and 1.5-fold, respectively (S.D. 0.78 and 0.33, respectively). When Nkx2.1 and C/EBPα expression plasmids were transfected together, the induction of the reporter gene reached 47.4-fold (S.D. 4.0). Thus, the synergy conferred by Nkx2.1 and C/EBPα is essentially the same as with the shorter CCSP promoter construct. The somewhat higher transactivation by Nkx2.1 on its own on the longer −2.1-kb CCSP promoter as compared with the −172-bp CCSP promoter, likely reflects the action of Nkx2.1 through the more promoter-distal sites previously described (21). From these data we conclude that sequences within the first −172 bp of the CCSP promoter are sufficient for full synergistic activation by C/EBPα and Nkx2.1.

The Integrity of Both C/EBP- and Nkx2.1-binding Sites Is Important for Synergistic Transactivation by C/EBPα and Nkx2.1—We next investigated whether the C/EBP- and Nkx2.1-binding sites present in the proximal CCSP promoter are essential for the synergistic activation by C/EBPα and Nkx2.1. We performed transient transfections using the −172 bp CCSP promoter with mutated C/EBP-binding sites or mutated Nkx2.1-binding sites (Fig. 3). Inactivation of the proximal or distal C/EBP-binding site resulted in significantly reduced transactivation by C/EBPα and Nkx2.1 as compared with the wild-type promoter (Fig. 3). When both C/EBP-binding sites were mutated, activation by C/EBPα and Nkx2.1 together was 9-fold, whereas the induction by Nkx2.1 and C/EBPα on their own was 3- and 1-fold, respectively, representing a more than additive induction. More than additive induction was also observed when submaximal levels of expression vectors were used (data not shown). Possibly, Nkx2.1 recruits C/EBPα to the CCSP promoter, perhaps via a direct protein-protein interaction. However, we did not detect any strong direct protein-protein interaction of C/EBPα and Nkx2.1 in pull-down experiments (data not shown). Alternatively, interaction between Nkx2.1 and C/EBPα might be mediated by additional factors present in the cells. Inactivation of the proximal or distal Nkx2.1-binding site resulted in significantly reduced transactivation by C/EBPα and Nkx2.1 as compared with the wild-type promoter (Fig. 3). Inactivation of the distal Nkx2.1 site had a stronger effect than inactivation of the proximal site, however, synergistic activity was still observed. When both Nkx2.1-binding sites were inactivated, synergy was abolished. In summary, this demonstrates the requirement of both the C/EBP- and Nkx2.1-binding sites in mediating the synergistic transactivation by C/EBPα and Nkx2.1.

Nkx2.1 and C/EBPα Bind Simultaneously to the Proximal CCSP Promoter—The C/EBPα and Nkx2.1 sites in the CCSP promoter are located in close proximity to each other (see Fig. 1A). To investigate whether C/EBPα and Nkx2.1 are capable of simultaneous binding to their respective adjacent sites, a 58-bp oligonucleotide, spanning −94 bp to −36 bp of the CCSP pro-
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Fig. 2. Titrations of Nkx2.1 and C/EBP\(\text{a}\) for synergistic activity on the CCSP promoter. A, increasing amounts of expression plasmids for C/EBP\(\text{a}\) were cotransfected in Drosophila SL2 cells with or without 2 \(\mu\)g of expression plasmid for Nkx2.1 along with the CCSP promoter-reporter plasmid (−172 bp to +7 bp). B, increasing amounts of expression plasmids for Nkx2.1 were cotransfected in the absence or presence of 500 ng of expression plasmid for C/EBP\(\text{a}\) along with the CCSP promoter-reporter plasmid. Luciferase activities were normalized against \(\beta\)-galactosidase activity. The total amount of plasmid DNA was kept constant. The values represent the averages of at least three independent experiments. Error bars indicate ± S.D.

Fig. 3. Effect of mutation of the C/EBP- and Nkx2.1-binding sites for the synergistic activity of Nkx2.1 and C/EBP\(\text{a}\) in the transactivation of the CCSP promoter. The −172 to +7 CCSP promoter-reporter gene construct was subjected to site-directed mutagenesis to inactivate the C/EBP- and Nkx2.1-binding sites. 500 ng of each expression plasmid was transfected in SL2 cells alone or in pairwise combination together with a \(\beta\)-galactosidase expression plasmid, and with a constant amount of plasmid DNA. Luciferase activities were normalized against \(\beta\)-galactosidase activity. The values represent the averages of at least three independent experiments. Error bars indicate ± S.D.

moter and including both C/EBP-binding sites and the Nkx2.1-binding site (Fig. 4A) was incubated in the presence of nuclear extracts from COS-1 cells, transiently transfected with expression plasmids for Nkx2.1 or C/EBP\(\text{a}\) or with mock DNA. Prominent complexes were detected in the presence of both C/EBP\(\text{a}\) and Nkx2.1 (Fig. 4A, lanes 3 and 5, respectively). Upon the inclusion of the respective antibody, these complexes were efficiently abolished and supershifts appeared (Fig. 4A, lanes 4 and 6, respectively). In the presence of both C/EBP\(\text{a}\) and Nkx2.1 containing nuclear extracts, a strong slowly migrating shift appeared (Fig. 4A, lane 7, marked with an arrowhead) as compared with C/EBP\(\text{a}\) alone (Fig. 4A, lane 3). The appearance of the strong shift was accompanied by the loss of the Nkx2.1 shift (Fig. 4A, lane 7), suggesting that a majority of the Nkx2.1 molecules bind together with C/EBP\(\text{a}\). Inclusion of Nkx2.1 or C/EBP\(\text{a}\) antibodies in the presence of both Nkx2.1 and C/EBP\(\text{a}\) containing nuclear extracts, resulted in a supershift of the slow migrating complex (Fig. 4A, lane 8 and 9, respectively). These results suggest that the slow migrating complex represents simultaneous binding of Nkx2.1 and C/EBP\(\text{a}\). Inclusion of increasing amounts of unlabeled oligonucleotide abolished all complexes formed in both C/EBP\(\text{a}\) and Nkx2.1 containing nuclear extracts (Fig. 4B, lanes 2–4). Competition with an oligonucleotide with the C/EBP-binding sites intact and the Nkx2.1 sites mutated, abolished the C/EBP\(\text{a}\) containing complex as well as the slowly migrating complex representing simultaneous binding of Nkx2.1 and C/EBP\(\text{a}\), but left the Nkx2.1-containing complex intact (Fig. 4B, lane 7). When higher amounts of competing oligonucleotide were included, the Nkx2.1 containing complex was reduced as well (Fig. 4B, lane 8). Competition with an oligonucleotide with intact Nkx2.1-binding sites and mutations in the C/EBP sites did not affect the C/EBP\(\text{a}\) containing complex and reduced, but did not abolish, the slowly migrating Nkx2.1/C/EBP\(\text{a}\) complex (Fig. 4B, lanes 5 and 6). Formation of the slowly migrating Nkx2.1/C/EBP\(\text{a}\) complex even though excess of competing unlabeled oligonucleotide was included (Fig. 4B, lanes 5 and 6), indicates that Nkx2.1 binds stronger to the CCSP promoter together with C/EBP\(\text{a}\) than alone. A possible explanation for this could be a protein-protein interaction between the two factors. In conclusion, these results show that C/EBP\(\text{a}\) and Nkx2.1 can bind simultaneously to the proximal CCSP promoter and suggest that the two transcription factors interact.

The \(NH_2\)-terminal Transactivation Domain of C/EBP\(\text{a}\) Is Essential for the Synergistic Activity with Nkx2.1—Earlier studies have demonstrated that C/EBP\(\text{a}\) contains three separate transactivation domains termed TEI, TEII, and TEIII (26). To elucidate the role of these domains for the synergy with Nkx2.1, we used a series of C/EBP\(\text{a}\) deletion mutants in transient transfections together with Nkx2.1. As shown in Fig. 5A, deletion of the first 11 \(NH_2\)-terminal amino acids of C/EBP\(\text{a}\) did not significantly affect the synergy with Nkx2.1. When amino acids 1–70 of C/EBP\(\text{a}\) were deleted, removing TEI, a significant decrease of synergistic activity with Nkx2.1 was
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Fig. 4. Binding of Nkx2.1 and C/EBPα to the CCSP promoter. A, end-labeled oligonucleotide representing the -94 to -36 DNA sequence of the CCSP promoter (left panel) was incubated in the presence of nuclear extract containing C/EBPα (lanes 3 and 4), Nkx2.1 (lanes 5 and 6), Nkx2.1 and C/EBPα in combination (lanes 7–9), or with nuclear extract from cells transfected with mock DNA (lane 2). In lanes 4 and 6, antibodies against C/EBPα and Nkx2.1 were added, respectively. In lanes 8 and 9, antibodies against Nkx2.1 and C/EBPα, respectively, were added in the presence of both C/EBPα and Nkx2.1 containing nuclear extracts. Lane 1 contains free probe only. B, end-labeled oligonucleotide representing the -94 to -36 DNA sequence of the CCSP promoter was incubated in the presence of nuclear extract containing Nkx2.1 and C/EBPα in combination. In lanes 3 and 4, excess unlabeled wild-type oligonucleotide was included (100- and 200-fold excess, respectively). In lanes 5 and 6, excess unlabeled oligonucleotide with mutations in the C/EBP-binding sites was included (100- and 200-fold excess, respectively), and in lanes 7 and 8, excess unlabeled oligonucleotide with mutations in the Nkx2.1-binding sites was included (100- and 200-fold excess, respectively). Lane 1 contains free probe only. Dot indicates the C/EBPα shift, asterisk indicates the Nkx2.1 shift, and the arrowhead indicates the Nkx2.1 + C/EBPα shift.

Fig. 5. A, cotransfection studies in Drosophila SL2 cells to determine the synergistic activity between Nkx2.1 and deletion mutants of C/EBPα. 500 ng of each C/EBPα deletion mutant expression plasmid was cotransfected with 500 ng of Nkx2.1 expression plasmid together with the reporter plasmid CCSP promoter (-172 to +7 bp). Luciferase activities were normalized against β-galactosidase activity. The values represent the averages of at least three independent experiments using the constructs indicated at the left. B, Western blot of whole cell extracts from Drosophila SL2 cells, transfected with the C/EBPα expression vectors in A.

The NH2-terminal Part of CEBPα Is Sufficient for Synergistic Activity with Nkx2.1—C/EBPα and C/EBPδ are highly similar in the DNA-binding basic region and dimerization of the leucine zipper domain. Consequently, C/EBP proteins bind to similar cis-acting elements (2, 31, 32). C/EBPα and C/EBPδ interact with similar efficiency to each of the two C/EBP-binding sites of the proximal CCSP promoter and both are expressed in lung (2). In contrast, C/EBPα and C/EBPδ differ significantly in the NH2-terminal transactivation domain. This conveys differences in target gene activation as exemplified by our finding that C/EBPδ does not act synergistically with Nkx2.1 (Fig. 1B). To test if the C/EBPα NH2-terminal transactivation domain is sufficient to give synergy with Nkx2.1, we constructed a C/EBPα-C/EBPδ swap mutant. Nkx2.1, together with a mutant protein containing the C/EBPα transactivation domain fused to the C/EBPδ basic region-leucine zipper domain synergistically activated the CCSP promoter with equal efficiency as wild-type C/EBPα (Fig. 6). This result, together with the data from the C/EBPα deletion studies, shows that the synergistic activation of the CCSP promoter by C/EBPα and Nkx2.1 is dependent on
transactivation domains present in the NH2-terminal part of C/EBPα.

NH2-terminal Transactivation Domains of Nkx2.1 Confer Synergistic Activity with C/EBPα—A transactivation domain of Nkx2.1 is located at the NH2-terminal region between amino acid residues 51 and 123 (33, 34). To investigate the role of this transactivation domain in the synergistic activity with C/EBPα, we constructed two Nkx2.1 deletion mutants lacking amino acid residues 1–92 and 46–152, respectively. As shown in Fig. 7A, the deletion of amino acid residues 1–92 of Nkx2.1 affected synergy with C/EBPα to a minor extent. Maximal induction reached 32-fold, representing a 40% decrease in synergistic activity. When performing transient transfections with the Nkx2.1 deletion mutant lacking amino acid residues 46–152, however, the synergistic activity with C/EBPα was completely abolished (Fig. 7A). The expression levels of the wild-type and mutant Nkx2.1 protein were examined with electrophoretic mobility shift assay using a Nkx2.1 consensus binding site and were found to be similar (Fig. 7B). These results show that the transactivation domain(s) in the NH2-terminal part of Nkx2.1 is vital to give full synergistic activity with C/EBPα.

DISCUSSION

Regulation of eukaryotic transcription is intricate and involves a variety of transcription factors to promote tissue- and cell-specific expression of a given gene. During development, the exact temporal and spatial expression of transcription factors are imperative steps to control the differentiation of multipotent progenitors into specialized cell types. A variety of transcription factors, including HNF-3α and -β, C/EBPα, C/EBPβ, and Nkx2.1, have been shown to be important for lung-specific gene expression (35, 36). Currently, less is known of how lung-enriched transcription factors act together to control cell differentiation and differentiation-dependent gene expression in the lung epithelium (37, 38).

The importance of Nkx2.1 for normal lung development was demonstrated in the Nkx2.1 (−/−) mice (16, 17). These mice exhibit severe abnormalities in lung morphogenesis. The lungs completely fail to undergo branching morphogenesis. In addition, epithelial cell differentiation is severely impaired as expression of the differentiation markers SP-A and CCSP is absent in the mutant lungs (17). An impaired cellular differentiation in Nkx2.1 (−/−) mice is in line with previous studies suggesting an important role for Nkx2.1 in the gene regulation of SP-A and CCSP (19, 21). Both genes start being expressed late in lung development, correlating to the extensive cellular differentiation that takes place during this period (35, 36). However, as the expression of Nkx2.1 is confined to epithelial cells from an early time point of lung morphogenesis and is sustained through development (15), additional transcription factors are likely to be important to promote the extensive differentiation program of late lung development.

The importance of C/EBPα in lung development was demonstrated in C/EBPα (−/−) mice, which have morphological abnormalities with hyperproliferation of alveolar type II cells...
(11). That C/EBPβ and Nkx2.1 synergistically transactivate the lung-specific CCSP gene suggests that combinatorial action of these two transcription factors is vital for high level expression of CCSP. Following this, the developmental expression patterns of C/EBPβ and Nkx2.1 could explain the differentiation-dependent expression pattern of CCSP during lung development. In rat developing lung, C/EBPβ expression is detected from embryonic day 18 in temporal connection to the initiation of epithelial differentiation (14) and high level expression of CCSP (39) (Fig. 8). A similar expression pattern of these proteins is seen in mice (23).2 The strong synergy between C/EBPβ and Nkx2.1 in the regulation of CCSP suggests that the onset of C/EBPβ expression in the developing lung is the key to high level expression of CCSP (Fig. 8). Based on our data, together with the results of targeted inactivation of C/EBPβ and Nkx2.1, we hypothesize that Nkx2.1 serves to specify lung epithelial cell lineage early in lung development and regulates lung-specific gene expression. C/EBPβ has its main role in late development to serve as a regulator of cellular differentiation and proliferation, and thus governs differentiation-dependent gene expression. A gene such as CCSP, which is specifically expressed in the lung epithelium and exhibits a differentiation-dependent expression pattern, would then be predicted to require the simultaneous presence of Nkx2.1 and C/EBPβ to be highly expressed (Fig. 8).

One concern in regard to the hypothesis that C/EBPβ is a major determinant of high level CCSP expression is the results from the Clebpa (−/−) mice. Although these mice exhibit a phenotype related to a defective control of proliferation, the expression pattern of CCSP and other differentiation markers was seemingly unaltered at the time of birth (11). However, there are several potential explanations for this discrepancy in relation to the results of this study. The levels of CCSP have not reached adult levels at the time of birth and adult levels are not reached until several days postnatally (23). Following this, it is possible that the major C/EBPβ action takes place after birth. In addition, the expression levels of CCSP were measured using in situ hybridization, making quantitation of the expression ambiguous.3 Another possible explanation is the addition of additional C/EBP factors in the lung epithelium. In addition to C/EBPβ, we have demonstrated that C/EBPβ can act together with Nkx2.1 to synergistically transactivate the CCSP promoter.4 C/EBPβ is also expressed in the lung epithelium (40), however, the C/EBPβ/Nkx2.1-mediated synergistic activation of CCSP expression was lower than that of C/EBPβ/Nkx2.1.4 These results suggest that C/EBPβ, in addition to C/EBPα, may play a role in the regulation of CCSP and also suggest that the CCSP expression seen in Clebpa (−/−) mice could stem from C/EBPβ transactivation. In other tissues, the interplay between different members of the C/EBP transcription factors and their expression during tissue development appears to be a complex process. An overlapping role for C/EBPα and C/EBPβ has been demonstrated in genetically modified mice in which C/EBPβ is expressed from the Clebpa locus (41). In these mice, C/EBPβ can functionally replace C/EBPα in liver, supporting an overlapping role for these transcription factors.

The question how different members of a family of transcription factors, which display similar or identical DNA-binding specificity, are able to regulate distinct sets of target genes is an important part in the study of promoter-specific transcription factors. In this study, we demonstrate that C/EBPβ, but not C/EBPα, is capable of synergistic transactivation with Nkx2.1. The inability of C/EBPα to act synergistically with Nkx2.1 results from differences of the NH2-terminal transactivation domain compared with C/EBPβ. This was demonstrated with the C/EBPα–C/EBPβ swap mutant, showing that a hybrid protein containing the C/EBPα transactivation domain and the C/EBPβ DNA-binding and dimerization domain, was capable of synergistic action with Nkx2.1. Thus, the ability of C/EBPβ to act in synergistic transactivation with Nkx2.1 is specified by the NH2-terminal transactivation domain. From the deletion studies of C/EBPβ, we show that the main synergistic activity with Nkx2.1 stems from TEI and TEII of C/EBPβ (Fig. 5). TEI and TEII of C/EBPβ have previously been shown to be important for interaction with TBP and TFIIB (42). These domains are not conserved in C/EBPβ (31) explaining the lack of synergy between Nkx2.1 and C/EBPβ. As the individual members within the C/EBP and Nkx2 transcription factor families exhibit similar binding site preferences, promoter-specific activation cannot be explained by the individual action of single factors. In this respect, the synergy of C/EBPβ and Nkx2.1 described here provide insight into how promoter specificity is achieved by specific combinatorial action of a subset of transcription factors.

In this study, we demonstrate a synergistic transactivation by C/EBPα and Nkx2.1 of the lung-specific CCSP gene. During development, this finding provides an explanation for the onset of high level expression of CCSP as the appearance of C/EBPβ in the developing lung epithelium is temporally correlated to extensive cellular differentiation and the expression of CCSP. On the transcriptional regulation level, the synergy between C/EBPα and Nkx2.1 provides an example of how promoter-specific transcription factors act in concert to enhance the rate of transcription. Together, these findings provide insight into the well organized program that coordinates gene transcription in individual cells and during organ formation.

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