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CAATT/Enhancer-binding Proteins α and δ Interact with NKX2-1 to Synergistically Activate Mouse Secretoglobin 3A2 Gene Expression*[S]

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Secretoglobin (SCGB) 3A2 is a small molecular weight secreted protein predominantly expressed in lung airways. We previously demonstrated that the expression of SCGB3A2 is regulated by homeodomain transcription factor NKX2-1. Here we show that CCAAT/enhancer-binding proteins, C/EBPα and C/EBPδ, regulate mouse *Scgb3a2* gene transcription *in vivo* and *in vitro* by binding to specific sites located in the *Scgb3a2* promoter and the activity is synergistically enhanced through cooperative interaction with NKX2-1. Six C/EBP binding sites lie within 500 bp of the *Scgb3a2* gene promoter, of which two sites, located at −44 to −54 bp and −192 to −201 bp, appear to be critical for the synergistic activation of *Scgb3a2* gene transcription with NKX2-1. All three transcription factors, C/EBPα, C/EBPδ, and NKX2-1, are expressed in the epithelial cells of airways, particularly the bronchus, where high expression of SCGB3A2 is found. The expression of these transcription factors markedly increases toward the end of gestation, which coincides with the marked increase of SCGB3A2, suggesting the importance of C/EBPα and C/EBPδ, and their synergistic interaction with NKX2-1 in mouse *Scgb3a2* gene transcription and lung development.

Secretoglobin 3A2 (SCGB3A2),[3] previously named as uteroglobin-related protein 1 (UGRP1), is a member of the SCGB gene superfamily that consists of secretory proteins of small molecular weight (1). SCGB3A2 is predominantly expressed in the conducting airway epithelium of the lung (2). The expression in mouse embryonic lung becomes detectable in embryonic day (E) 12.5, markedly increases by E16.5, and remains high throughout adulthood (2). SCGB3A2 suppresses the allergen-induced lung inflammation in a mouse model for allergic airway inflammation when intranasally administered with recombinant adenovirus expressing SCGB3A2 (3).

SCGB3A2 was originally identified as a downstream target for the homeodomain transcription factor NKX2-1 (previously called TITF1, TTF1, NXK2.1, or T/EBP) in *Nkx2-1*-null *versus* wild-type mouse embryo lungs using suppressive subtractive hybridization screening (2). The direct involvement of NKX2-1 in mouse *Scgb3a2* gene expression was further demonstrated by transfection analysis (2). NKX2-1 is one of the major transcription factors responsible for expression of many genes preferentially expressed in lung, including SCGB3A2, surfactant protein (SP)-A (4), SP-B (5), SP-C (6), and Clara cell secretory protein (CCSP), also named SCGB1A1 (7, 8). FOXA1 (HNF3α), FOXA2 (HNF3β), and C/EBPs are among other transcription factors that are critical for the expression of lung-specific genes and lung morphogenesis (9, 10). Whether these transcription factors are involved in SCGB3A2 expression is not known.

C/EBPs (CCAAT/enhancer-binding proteins) are a family of transcription factors containing the basic leucine zipper (bZIP) domain at the C terminus that is involved in dimerization and DNA binding (11). All six members have been cloned to date, and they play pivotal roles in controlling cellular proliferation and differentiation, metabolism, inflammation, and numerous other responses, particularly in hepatocytes, adipocytes, and hematopoietic cells (11). In lung, C/EBPα, C/EBPβ, and C/EBPδ are highly expressed in alveolar type II cells and bronchiolar epithelial cells with various degrees of expression depending on C/EBP subtype and developmental stages (10, 12–14). A role for C/EBPα in lung morphogenesis was demonstrated using a mouse model overexpressing or lacking C/EBPα expression in lung (14, 15). *In vitro* studies have shown a role for C/EBPs in the transcriptional regulation of lung-specific genes including SP-A, SP-D, and SCGB1A1 (10, 13, 16–19). In particular, the regulation of SCGB1A1 expression by C/EBPα and

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data, references, and Table S1.

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[3] The abbreviations used are: SCGB, secretoglobin; C/EBP, CAATT/enhancer-binding protein; CCSP, Clara cell secretory protein; E, embryonic day; SP, surfactant protein; RT, reverse transcriptase; EMSA, electrophoretic mobility shift analysis; PBS, phosphate-buffered saline; rtTA, reverse tetracycline responsive transactivator; ChIP, chromatin immunoprecipitation; IP, immunoprecipitation.

C/EBPδ, and their synergistic interaction with NKX2-1 have been extensively studied (13, 19).

NKX2-1 is expressed in the lung, thyroid, and brain, and plays a central role in structural development and regulation of tissue-specific (or enriched) genes in these organs (20). Activation of the tissue-specific genes by NKX2-1 often involves its interaction with other proteins (see Table S1 in supplemental materials). These include transcription factors such as the aforementioned FOXA1 (21, 22), FOXA2 (21), C/EBPs (19), and GATA6 (23) and NF I (24), and co-factors such as p300 (25), RAR (26), TAZ (transcriptional co-activator with PDZ-binding domain) (27), DREAM (28), calreticulin (29), and BR22 (30). It is of interest to examine whether NKX2-1 interacts with any other proteins, resulting in increased expression of SCGB3A2.

In this study, we demonstrate that C/EBPα and C/EBPδ regulate mouse *Scgb3a2* gene transcription *in vivo* and *in vitro* by binding to specific binding sites located in the *Scgb3a2* promoter and the activity is synergistically enhanced through cooperative interaction with NKX2-1. There are six C/EBP binding sites within 500 bp of the *Scgb3a2* gene promoter, of which two C/EBP sites located at −44 to −54 bp and −192 to −201 bp appear to be critical for the synergistic activation of *Scgb3a2* gene transcription with NKX2-1. The implication of this synergistic activation of mouse *Scgb3a2* gene transcription to the pattern and site of SCGB3A2 expression in mouse airways is discussed.

EXPERIMENTAL PROCEDURES

Construction of C/EBP Expression Plasmids—Total RNA was isolated from mouse embryonic lungs (embryonic day (E) 17.5) by using TRIzol (Invitrogen) and then treated with DNase I (Ambion, Austin, TX). DNase I-treated samples were utilized as a template for cDNA synthesis with reverse transcriptase (SuperScript II, Invitrogen). PCR were carried out using the following primers: 5′-TTAGGTACCATGGAGTCGGCCGACTTCTACGAG-3′ and 5′-TTATCTAGACGCGCCTCACGCGCAGTTG-3′ for C/EBPα, 5′-TAAGGTACCATGTCCGCGGGGGCGCAC-3′ and 5′-TTATCTAGACGCGCCTCACGCGCAGTTG-3′ for the dominant negative form of C/EBPα (C/EBPαDN), and 5′-TTAGGTACCATGAGCGCCGCGCTTTTCAG-3′ and 5′-TTATCTAGAGGTCGTTCAGAGTCTCAAAGGCCCAC-3′ for C/EBPδ. The PCR products were purified by a spin column (QIAquick PCR Purification Kit, Qiagen, Valencia, CA), digested with restriction enzymes, and subcloned into the KpnI-XbaI site of the cytomegalovirus driven mammalian expression vector, pcDNA3.1/myc-HisA (Invitrogen). DNA sequences were confirmed by sequencing analyses using CEQ-200XL (Beckman Coulter, Fullerton, CA).

Construction of NKX2-1 and NKX2-1-FLAG Expression Plasmids—To construct NKX2-1 expression plasmids, RT-PCR were carried out using 5′-TAAGAATTCGCCATGTCGATGAGTCCAGAAGCAC-3′ and 5′-AACTCTAGATCACCAGGTCCGACCATAAAG-3′ for NKX2-1, and 5′-TAAGAATTCGCCATGTCGATGAGTCCAGAAGCAC-3′ and 5′-AACTCTAGATTATCACTTGTCATCGTCGTCCTTGTAGTCCCAGGTCCGACCATAAAG-3′ for NKX2-1-FLAG. The amplified products were subcloned into the EcoRI-XbaI site of the pcDNA3.1/myc-HisA vector. The NKX2-1 construct was used for luciferase assay and electrophoretic mobility shift analysis (EMSA), whereas NKX2-1 with the C-terminal FLAG construct was used in co-immunoprecipitation assays.

Construction of Mouse Scgb3a2 Gene Promoter-luciferase Plasmids—To make serial deletion mutant constructs, PCR were carried out using mouse genomic DNA and the following primers: forward primers, 5′-ATGACTAGTTAGGAAGATTGCCCTGCATGCTC-3′ for −113, 5′-ATTACTAGTGCCTTCACATCTGCTTTAGTGTTCTTC-3′ for −292, 5′-TTAACTAGTGGCGGTTCCAGTCAGCTAAATC-3′ for −387, 5′-ATAACTAGTAAACACATGCTCATTTTCCCCTGG-3′ for −506, and 5′-AGAACTAGTGGATCTTTAAAGCAATACC-3′ for −907 constructs, and common reverse primer 5′-ATTGGATCCTGTGATGTTTTCCGGGAC-3′. PCR products were double-digested by SpeI-BamHI and cloned into the NheI-BglII site of the firefly luciferase pGL4.11 vector (Promega, Madison, WI). Each construct has a *Scgb3a2* promoter region represented by a construct name in addition to 55 nucleotides in the 5′-untranslated region. The C/EBP binding element mutations were generated by the site-directed mutagenesis kit (Stratagene, La Jolla, CA). All plasmids were confirmed by nucleotide sequencing.

Reporter Assay—COS-1 cells were cultured in Dulbecco's modified Eagle's medium high glucose with L-glutamine (Invitrogen) supplemented with 10% fetal bovine serum, at 37 °C in a 5% CO_2 incubator. Cells were seeded in 24-well tissue culture plates. A transfection mixture contained 20 μl of serum-free Dulbecco's modified Eagle's medium, 1 μl of FuGENE 6 (Roche Applied Science), 250 ng of pGL4.11-based reporter construct (Promega), 5 ng of pGL4.74 having the *Renilla* luciferase gene connected to the herpes simplex virus-thymidine kinase promoter as an internal control (Promega), and a total 50 ng of pcDNA3.1-C/EBPα or C/EBPδ mammalian expression vector (Invitrogen). When the C/EBP construct was not incorporated, pcDNA3.1 vector (50 ng) was added to adjust the total DNA amount. This transfection mixture was added to a well, mixed briefly, and cells were incubated for 48 h. Cells were washed once with phosphate-buffered saline (PBS) and lysed with passive lysis buffer (Promega). Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Pharmingen, model monolight 3010).

Nuclear Extracts—COS-1 cells were grown in 150-mm dishes to 50–80% confluence. One ml of serum-free Dulbecco's modified Eagle's medium containing expression constructs (C/EBPα or C/EBPδ, 20 μg) was mixed with 50 μl of FuGENE HD (Roche Applied Science), the mixture was left to stand for 15 min at room temperature, and added dropwise to cells. Media were changed 8 h after transfection and cells were cultured for an additional 48 h before harvest. The cells were washed twice with 6 ml of cold PBS and harvested in 6 ml of cold PBS. The cell suspension was centrifuged at 1,000 × *g* for 5 min and the cell pellet was re-suspended in 2 ml of Buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). Cell-Buffer A was placed in 1.5-ml plastic tubes and spun by a table-top centrifuge at 2,500 × *g* for 3 min. The cell pellet was then suspended in Buffer B (Buffer A + 0.2% IGEPAL) followed by centrifugation at 2,500 × *g* for 3 min to obtain nuclei pellet. The

nuclei pellet was washed with Sucrose buffer (250 mM sucrose, 10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.5 M EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and mixed with Extraction buffer (50 mM HEPES, pH 7.9, 400 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) by rotating in a cold room for 30 min. Finally, nuclear extract was obtained by centrifugation and the protein concentration was determined using the Bradford protein assay (Bio-Rad) with bovine serum albumin as a standard. The final concentration was adjusted to 2 mg/ml.

EMSA —Synthetic oligonucleotides were radiolabeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase. Nuclear extract (1 μl) was diluted with Binding buffer (0.1 μg/μl poly(dI-dC), 10 mM Tris, pH 8.0, 1 mM dithiothreitol, 80 mM KCl, 20% glycerol, 0.04 μg/μl bovine serum albumin) and incubated 15 min at room temperature with radiolabeled probe in the presence or absence of cold competitor. For supershift assays, 1 μl of antibody was added to the sample solution prior to the probe. Samples were electrophoresed on a 4% polyacrylamide gel using 0.5× TBE buffer. Gels were dried and exposed to a phosphorimager screen and signals detected with Storm 840 (Amersham Biosciences). Sequences for probes and competitors are listed in Table 1. For antibody supershift analysis, anti-C/EBPα, anti-C/EBPδ, anti-TTF1 (sc-13040), and normal rabbit IgG (sc-2027) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Effect of A-C/EBP—Expression plasmid pcDNA3.1 containing the A-C/EBP cDNA (35) was transfected with FuGENE 6 into mtCC or embryonic lung primary cells prepared from lungs of C57BL/6 embryos at E16.5, which were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

A tetracycline-regulated A-C/EBP transgenic mouse (*TetO-A-C/EBP*) (36) and a transgenic mouse expressing the reverse tetracycline responsive transactivator (rtTA) under the CCSP (SCGB1A1) promoter (37) were interbred to produce *CCSP-rtTA*;*TetO-A-C/EBP* and *CCSP-rtTA* mice. The mice were fed food containing 200 mg/kg doxycycline for 1 month as soon as they were weaned. *C/EBP*δ-null mutant mice (38) were of C57BL/6 and 129S1 F1 genetic backgrounds and subjects were from intercrosses of heterozygous mice. All experiments with mice were conducted according to the NIH-approved protocols and NIH guidelines. The following primers were used for genotyping of *CCSP-rtTA*;*TetO-A-C/EBP* mice: 5′-CCA CGC TGT TTT GAC CTC CAT AG-3′ and 5′-ATT CCA CCA CTG CTC CCA TTC-3′ for A-C/EBP, and 5′-ACT GCC CAT TGC CCA AAC AC-3′ and 5′-AAA ATC TTG CCA GCT TTC CCC-3′ for CCSP-rtTA. The PCR condition used was 94 °C, 5 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s. The SCGB3A2 mRNA level was determined by quantitative RT-PCR using the following primers: 5′-ACA GGG AGA CGG TTG ATG AG-3′ and 5′-GTT GGG CTT TCT GAC TGC AT-3′ for SCGB3A2, and 5′-ATT GGA GCT GGA ATT ACC GC-3′ and 5′-CGG CTA CCA CAT CCA AGG AA-3′ for 18S. The condition used was 50 °C for 2 min, 54 °C for 30 s, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Analysis was carried out using SYBR Green master mixture and the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA).

Chromatin Immunoprecipitation (ChIP)—DNA was isolated from adult mouse lungs that had been fixed with 1% formaldehyde, followed by sonication. Endogenous NKX2-1, C/EBPα, or C/EBPδ together with fragmented DNAs was pulled down by anti-NKX2-1, anti-C/EBPα, or anti-C/EBPδ. After reverse cross-link, the *Scgb3a2* promoter region containing C/EBP binding sites 7 and 10, and two NKX2-1 binding sites, and exon 2 as a reference were PCR amplified. PCR primers used were as follows: 5′-GGGGAAATCCTTTGCCGTCTG-3′ and 5′-TCTGCTCGCTGTCACCTGATGG-3′ for amplification of the promoter region, and 5′-TCTTCAGTCCTGTCACCAGATGTTCTAC-3′ and 5′-CGAGAGGGATGGGATGGAGTCTTAG-3′ for the reference. Sample solution was mixed with each primer set and *Taq* polymerase, followed by PCR of 32 cycles with 95 °C denaturation, 15 s, 57 °C annealing, 15 s, and 72 °C extension, 30 s.

Co-immunoprecipitation—The TnT T7-coupled reticulocyte lysate systems (Promega) were used to generate NKX2-1, C/EBPα, and C/EBPδ proteins using pcDNA3.1/myc-HisA-C/EBPα, C/EBPδ, and NKX2-1 expression plasmids, respectively, which have T7 promoter that allows T7 polymerase to transcribe genes of interest, followed by translation. The [^{35}S]methionine (GE Healthcare) was incorporated into the reaction mixture. Translated proteins were combined and immunoprecipitated by anti-FLAG-agarose resin (Sigma, A2220). After rigorous washing, the eluted fraction was subjected to SDS-PAGE, and 10% PAGE gel was fixed, treated with amplifier (NAMP100 Amplify Fluorographic Reagent, GE Healthcare), dried, and exposed to phosphorimager screen.

Northern Blot—Total RNA (3 μg) isolated from adult lung was electrophoresed on 1% agarose gel containing 0.22 M formaldehyde and transferred onto nitrocellulose membrane (Immobilon-Ny+, Millipore, Billerica, MA). Filters were hybridized with C/EBPα, C/EBPδ, and ribosomal protein B36 (loading control) as a probe. Hybridization was performed in Perfect Hybridization solution (Amersham Biosciences) at 68 °C overnight. The membrane was washed twice with 2× SSC containing 0.1% SDS at 68 °C for 30 min, followed by exposure to a phosphorimager screen. Data processing was carried out using ImageQuant TL 2005 software (GE Healthcare).

Western Blot—COS-1 cells after transfection of C/EBPα, C/EBPδ, or NKX2-1 expression plasmids (50 ng each/well of 24 well plate) were washed with PBS and lysed in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate) with Protease Inhibitor Mixture tablets (Complete Mini; Roche Applied Science). The protein lysates were mixed with SDS sample loading buffer containing β-mercaptoethanol, electrophoresed on 10% SDS-PAGE, which were then electrotransferred to a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare). Membranes were blocked with PBS, 5% bovine serum albumin, and incubated with first antibody in PBS + 5% bovine serum albumin overnight at 4 °C. Membranes were then washed with PBS containing 0.1% Tween 20 (PBST) three times before proceeding to the second antibody (NA9340V, GE Healthcare) incubation.

Protein bands were detected using chemiluminescence reagent (NEL 104001EA, PerkinElmer Life Sciences) and the CCD camera system (Alpha Innotech Fluor Chem HD2, San Leandro, CA).

Immunohistochemical Analysis—Mouse embryos at E16.5 or adult lungs were fixed in 4% paraformaldehyde and embedded in paraffin. Serial 4-μm sections were mounted on glass slides and deparaffinized with xylene and graded ethanol. Antibodies used for immunohistochemistry were all rabbit polyclonal antibodies against C/EBPα (Santa Cruz, 1:800), C/EBPδ (Santa Cruz, 1;200), NKX2-1 (1:1000), and mouse SCGB3A2 (1:1600) as previously described (2). NKX2-1 antibody was produced using 3 peptides (MSPKHTTPFSVSD, NMSELPPYQDTMR, PGWYGANPDPRFP) as an antigen by Global Peptide Services (Fort Collins, CO). In brief, rehydrated sections were first pretreated by incubation in PBS for 5 min for permeabilization and immersed in 0.3% hydrogen peroxide in 100% methanol for 30 min. The sections were blocked by 5% skim milk for 30 min and incubated with first antibody at 4 °C overnight. Sections were then incubated with biotinylated anti-rabbit IgG for 30 min, and avidin-biotinylated peroxidase complexes (Vector Laboratory, Burlingame, CA) for 30 min. The bound peroxidase activity was visualized by incubation with the DakoCytomation Liquid DAB Substrate Chromogen System (DAKO). Sections were rinsed in water after each step of the immunostaining procedure. Finally, the sections were counterstained with Hematoxylin QS (Vector Laboratories), dehydrated, and mounted in permanent mounting medium.

RESULTS

NKX2-1 Interacting Proteins—To identify NKX2-1 interacting proteins that may be responsible for activation of mouse *Scgb3a2* gene expression, the yeast two-hybrid system and anti-FLAG immunoprecipitation (anti-FLAG IP) using liquid chromatography (31), followed by tandem mass spectrometry (LC/MS/MS) were employed. Seventeen genes were identified from the yeast two-hybrid system, whereas 12 genes were from anti-FLAG IP-LC/MS/MS as NKX2-1 interacting proteins. Interestingly, none of the genes identified by these two methods overlapped. Separately, several transcription factors/co-factors were chosen based on prior literature that interact with NKX2-1 and are involved in the expression of thyroid- or lung-specific (or enriched) genes (see supplemental Table S1). Expression plasmids for these NKX2-1 interacting proteins were prepared in plasmid pcDNA3.1, which were then subjected to transfection analysis with the mouse *Scgb3a2*-promoter (−907 bp) luciferase reporter construct using mtCC (32), MLE 15 (33), TC-1, and/or COS-1 cells. The former three cell lines are derived from mouse lung epithelial cells. Within the −907 bp of the promoter sequence, two NKX2-1 binding sites, located at −120 to −125 and −182 to −187 bp from the transcription start site of the gene, were previously found to be critical for expression of the mouse *Scgb3a2* gene (2). Among 44 interacting proteins examined (listed in Table S1), C/EBPα was the only protein that demonstrated significant activation of *Scgb3a2* gene expression in the presence as well as absence of the NKX2-1 expression plasmid in mtCC, MLE15, and COS-1 cells (see below). C/EBPδ was also found to activate transcription of the *Scgb3a2* gene. We therefore focused on C/EBPα and C/EBPδ in further studies.

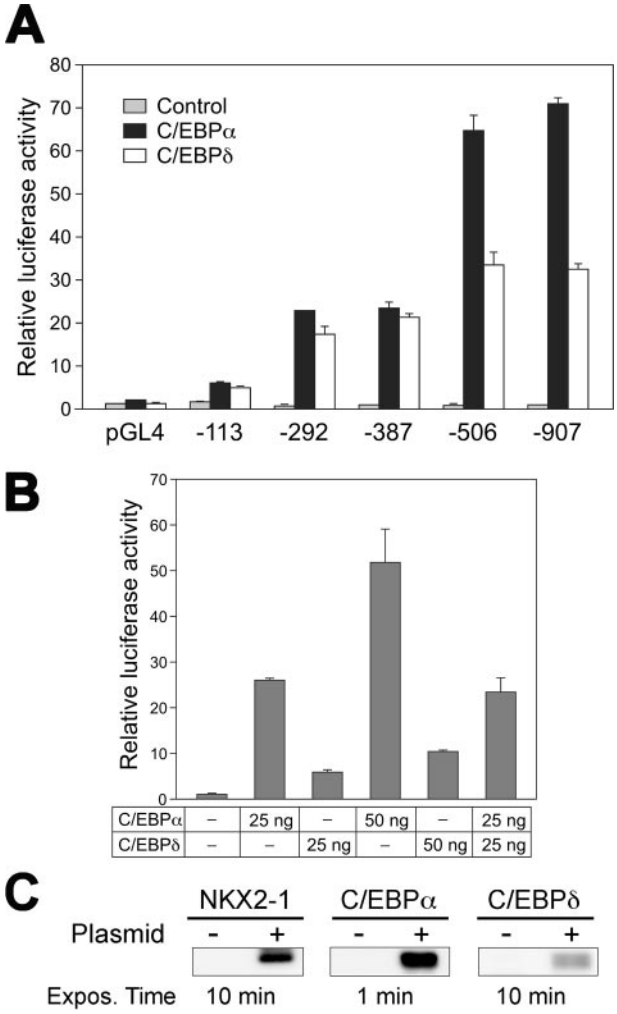

FIGURE 1. **Regulation of mouse *Scgb3a2* gene transcription by C/EBPα and C/EBPδ.** *A*, a series of *Scgb3a2* gene promoter deletion constructs were co-transfected with C/EBPα or C/EBPδ expression plasmid in COS-1 cells. Each deletion construct has the promoter sequence from −113, −292, −387, −506, or −907 to +55 bp. *pGL4*, vector only used as a control. *B*, relative luciferase activity was determined using construct −506 and increasing amounts of C/EBPα or C/EBPδ expression plasmid or both plasmids together. Relative luciferase activity is shown as the mean ± S.D. based on the activity of control vector pGL4 as 1 from three independent experiments, each carried out in duplicate. *C*, levels of overexpressed NKX2-1, C/EBPα, and C/EBPδ in COS-1 cells were determined by Western blotting using cell lysates with (+) and without (−) transfection of expression plasmid for each transcription factor. Exposure time was 1 min for C/EBPα and 10 min for NKX2-1 and C/EBPδ.

C/EBPα and C/EBPδ Are Involved in Mouse Scgb3a2 Gene Promoter Activity—When a series of *Scgb3a2* promoter deletion constructs (vector only, −113, −292, −387, −506, and −907) were subjected to co-transfection analysis into COS-1 cells that do not express NKX2-1 with C/EBPα or C/EBPδ in the absence of the NKX2-1 expression plasmid, three tiers of robust increase of promoter activity were obtained with the construct, −113, −292, and −506, respectively (Fig. 1*A*). These results suggest that C/EBP binding sites may be present between the transcription start site and −113 bp, −113, and −292 bp, and −387 and −506 bp of the mouse *Scgb3a2* gene promoter. C/EBPα appears to be more effective than C/EBPδ in activation of the mouse *Scgb3a2* promoter, which became evi-

dent with constructs −506 and −907. This was further demonstrated by a titration analysis in which the −506 construct was co-transfected with an increasing amount of C/EBPα or C/EBPδ expression plasmid individually or together (Fig. 1*B*). Three- to 4-fold higher reporter activity was obtained with C/EBPα than C/EBPδ at the same concentration, and co-transfection with 25 ng each of C/EBPα and C/EBPδ expression plasmids together produced a reporter activity slightly lower to that obtained with 25 ng of C/EBPα expression plasmid alone. When actual levels of expressed proteins were examined by Western blotting, C/EBPα appeared to be expressed at higher levels than C/EBPδ (Fig. 1*C*). It has to be pointed out, however, that the protein expression level determined by Western blotting does not necessarily reflect the actual expression level because of the specificity of antibody used. Nevertheless, these results may suggest that in this experimental system, the lower reporter activity obtained with the C/EBPδ expression plasmid as compared with the C/EBPα expression plasmid, regardless of the presence of NKX2-1, could be due to a lower expression of C/EBPδ protein (see also Figs. 3*B*, 4, and 5).

Analysis of C/EBP Binding Sites in the Mouse Scgb3a2 Gene Promoter—C/EBP protein has a wide spectrum of DNA binding preferences. Within −506 bp of the mouse *Scgb3a2* promoter, 11 C/EBP binding sites were predicted using the AliBaba2.1 (darwin.nmsu.edu/~molb470/fall2003/Projects/solorz/aliBaba_2_1.htm) (Fig. 2*A*). Thus, 11 oligonucleotides harboring putative C/EBP binding sites in the mouse *Scgb3a2* promoter were used as a competitor for EMSA using nuclear extracts prepared from COS-1 cells overexpressing C/EBPα or C/EBPδ and an oligonucleotide containing the C/EBP binding site present in the rat *Mrp* (multidrug resistance-associated protein) 3 gene proximal promoter region (−141 to −160 bp) as a probe (Fig. 2*B* and Table 1). The intensity of a specific protein-DNA shifted band was similar between C/EBPα and C/EBPδ under the same experimental conditions, considering that the gel for examining the C/EBPδ binding was exposed twice as long as the gel for C/EBPα binding. The addition of 100-fold excess of competitor oligonucleotides 1, 2, 6, 7, 9, and 10 abolished or greatly reduced the intensity of mobility shifted bands for both C/EBPα and C/EBPδ. Oligonucleotides 1 and 2 are present within the −506/−387-bp region, oligonucleotides 6 and 7 are within the −113/−292-bp region, and oligonucleotides 9 and 10 are between −113 bp and the transcription start site of the gene. These areas are the ones that showed increased reporter activity as described in the legend to Fig. 1.

To determine which C/EBP binding sites are most critical for activation of the mouse *Scgb3a2* promoter, a mutation was introduced into the C/EBP binding site located in oligonucleotides 1, 2, 6, 7, 9, and 10, which were then subjected to EMSA and reporter analysis (Fig. 3 and Table 1). Because competitor oligonucleotides 1 and 9 had two potential C/EBP binding sites in their sequences, two mutant oligonucleotides having each C/EBP binding site mutated (m1 and m2) were prepared (Table 1). The EMSA results demonstrated that in both C/EBPα and C/EBPδ, mutant oligonucleotides 1m2, 2m1, 6m1, 7m1, 9m1, and 10m1 did not have any effect on formation of a specific DNA-protein band, suggesting that C/EBPs cannot bind to these mutated sites (Fig. 3*A*). Reporter assays using COS-1 cells

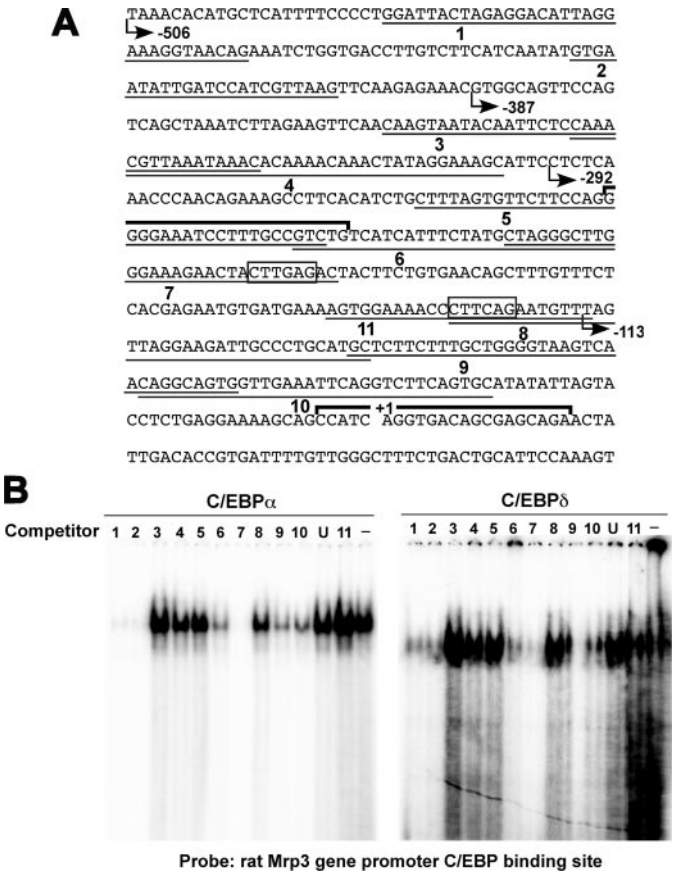

FIGURE 2. **Putative C/EBP binding sites in the mouse *Scgb3a2* gene promoter.** *A*, mouse *Scgb3a2* gene promoter sequence. The starting position of deletion mutants used in Fig. 1 is indicated by an *arrow*. Eleven oligonucleotides containing putative C/EBP binding site(s) are *underlined*. The actual C/EBP binding sites predicted by the computer analysis are listed in Table 1. NKX2-1 binding sites are *boxed*. The transcription start site of the gene is shown by +1. The primer sequences used for ChIP analysis are *overlined with bracket*. *B*, EMSA was carried out using nuclear extracts prepared from COS-1 cells overexpressing C/EBPα or C/EBPδ, an oligonucleotide containing the C/EBP binding site present in the rat Mrp (multidrug resistance-associated protein) 3 gene proximal promoter region (−141 to −160 bp) as a probe, and oligonucleotides containing putative C/EBP binding sites as a competitor. Note that the gel for C/EBPδ was exposed to a phosphorimager screen twice longer than C/EBPα.

TABLE 1
EMSA probe and competitors

Putative C/EBP binding sites are shown by boldface letters or in italics with underline when two putative sites are overlapping. Mutated nucleotides are underlined.

Name	Sequence
Mrp3	5′-AGGCTGTGTTGCAATCGCTG-3′
COMP1	5′-GGATT**ACTAGAGGAC***ATTAGGAAAGG*TAACAG-3′
COMP1m1	5′-GGATTACTATGAAGTATTAGGAAAGGTAACAG-3′
COMP1m2	5′-GGATTACTAGAGGACAAAAGACTCGGTAACAG-3′
COMP2	5′-GTGAAT**ATTGATCCAT**CGTTAAG-3′
COMP2m1	5′-GTGAATCAAGATTTGGCGTTAAG-3′
COMP3	5′-CAAGTAATA**CAATTCTCCA**AACGTTAAATAAAC-3′
COMP4	5′-CAAACGT**TAAATAAACA**CAA**AACAAACTA**TAGGAAAGC-3′
COMP5	5′-CTTTAG**TGTTCTTCC***AGGGGGAAATCC*TTTGCCGTC-3′
COMP6	5′-GTCTGTC**ATCATTTCT**ATGCTAGGGCTTG-3′
COMP6m1	5′-GTCTGTCAAATCCTCTATGCTAGGGCTTG-3′
COMP7	5′-CTAGGGC**TTGGGAAAGA**ACTACTTGAGAC-3′
COMP7m1	5′-CTAGGGCATGGTCTCGAACTACTTGAGAC-3′
COMP8	5′-CTTCAG**AATGTTTAGTT**AG**GAAGATTGCCC**TGCATGC-3′
COMP9	5′-GCTCTTCTT**TGCTGGG***GTAAGTCAACA*GGCAGTG-3′
COMP9m1	5′-GCTCTTCTTTGCTGGGGTAAAAATACAGGCAGTG-3′
COMP9m2	5′-GCTCTTCTTTAAAAAAAATTGTCAACAGGCAGTG-3′
COMP10	5′-CAGGCAGTGGT**TGAAATTCAGG**TCTTCAGTGC-3′
COMP10m1	5′-CAGGCAGTGGTTGTCTCTCAGATCTTCAGTGC-3′
COMP11	5′-AGTGG**AAAACCCTT**CAGAATGTTT-3′
Unrelated	5′-CTGCCAAGAGGAAATCCTCAGGAC-3′

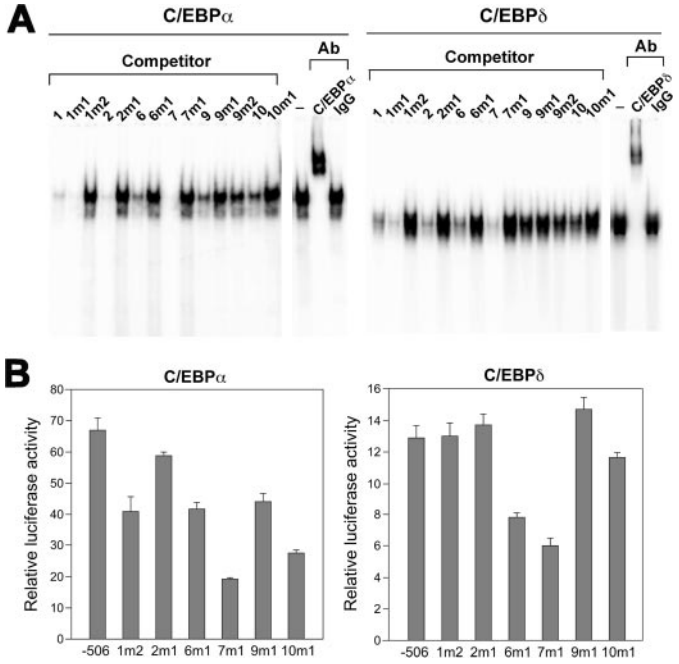

FIGURE 3. **Analysis of C/EBP binding site mutants.** *A*, EMSA was carried out using nuclear extracts prepared from COS-1 cells overexpressing C/EBPα or C/EBPδ, an oligonucleotide containing C/EBP binding site present in the rat Mrp (multidrug resistance-associated protein) 3 gene proximal promoter region (−141 to −160 bp) as a probe, and oligonucleotides containing mutated putative C/EBP binding sites as a competitor. The details of mutations are listed in Table 1. Antibody supershift (*Ab*) experiments were performed using antibody against C/EBPα or C/EBPδ, or IgG as a control. *B*, luciferase reporter analysis using −506 construct and those having C/EBP binding sites mutated. Based on the results obtained in *A*, only constructs having C/EBP binding sites to which C/EBPα or C/EBPδ can bind are used in this experiment. Relative luciferase activity determined from three independent experiments, each carried out in duplicate is shown as the mean ± S.D. based on the activity of control vector pGL4 as 1 (not indicated in the figure).

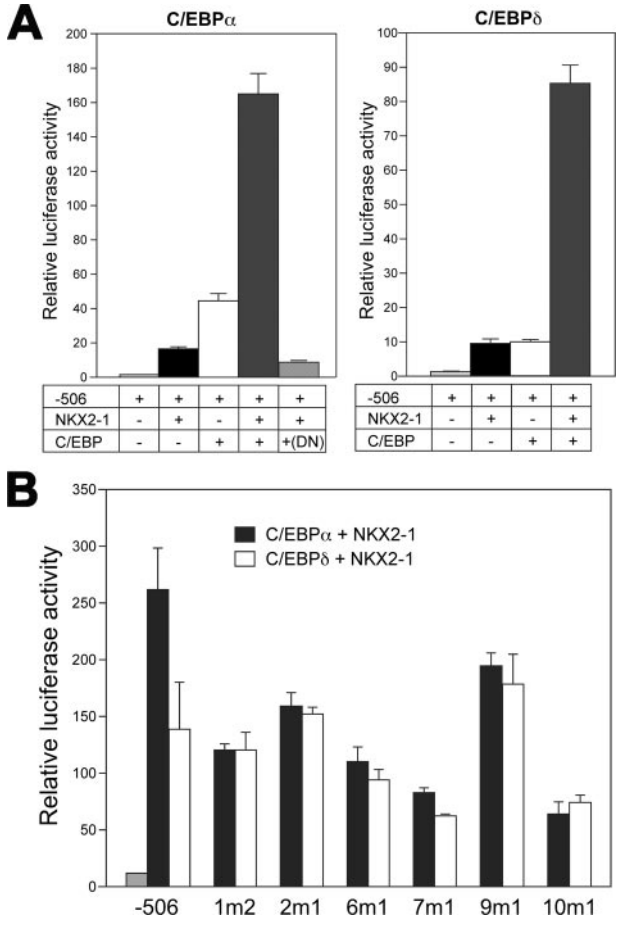

FIGURE 4. **Synergistic activation of mouse *Scgb3a2* gene transcription by NKX2-1 and C/EBPα or C/EBPδ.** *A*, construct −506 was co-transfected into COS-1 cells with NKX2-1 and C/EBPα or C/EBPδ expression plasmids singly or together, and luciferase activity was measured. For C/EBPα, the result with a dominant negative form of plasmid (DN) is also shown. *B*, effect of C/EBP binding site mutation on synergistic activity of *Scgb3a2* transcription. Construct −506 and various C/EBP site mutants were co-transfected with NKX2-1 and C/EBPα or C/EBPδ expression plasmids. The C/EBP mutant constructs are the same as those used in Fig. 3*B*. Relative luciferase activity is shown as the mean ± S.D. based on the activity of control vector pGL4 as 1 from three independent experiments, each carried out in duplicate.

were then carried out using −506 constructs with these individual C/EBP binding sites mutated and their relative luciferase activities compared (Fig. 3*B*). In the case of C/EBPα, mutant constructs of binding sites 1, 6, and 9 exhibited a similar reduction of ~40% in luciferase activity, whereas the activity of the binding site 10 mutant was reduced ~60% as compared with the parent construct −506 (Fig. 3*B*, *left panel*). Mutation of binding site 2 had very little effect on reporter activity. Among all sites, mutation of binding site 7 was the most effective in suppressing reporter activity. In the case of C/EBPδ, only mutations introduced into sites 6 and 7 demonstrated a significant reduction of reporter activities as compared with the construct −506, whereas other mutations were basically dispensable (Fig. 3*B*, *right panel*). In particular, the binding site 7 mutation showed the largest reduction of reporter activity. These results suggest that multiple C/EBP binding sites may be required for full activation of mouse *Scgb3a2* promoter, and although both C/EBPα and C/EBPδ can bind to the same binding sites, it appears that the promoter activity through their binding to the specific binding sites depends on which C/EBP binds to which binding site. Furthermore, mutations in C/EBP binding sites 1, 2, and 9 only affected C/EBPα, but not C/EBPδ co-transfected luciferase activity. This may account for the large difference in luciferase activity between C/EBPα and C/EBPδ with the construct −506 as compared with construct −387 observed in Fig. 1*A*. Overall, binding site 7 may be the most critical C/EBP binding site among those examined (1, 2, 6, 7, 9, and 10) for the activation of the mouse *Scgb3a2* promoter.

C/EBP and NKX2-1 Synergistically Activate Mouse Scgb3a2 Gene Promoter—Previously, we reported that two NKX2-1 binding sites located at −120 to −125 and −182 to −187 bp of the mouse *Scgb3a2* promoter are responsible for transcriptional activation of the gene (2). When C/EBP and NKX2-1 expression plasmids were co-transfected together with the −506 *Scgb3a2* reporter construct, a robust synergistic effect was observed for both C/EBPα and C/EBPδ (Fig. 4*A*). The synergism was stronger with C/EBPδ and NKX2-1 than with C/EBPα and NKX2-1, whereas the final synergistic reporter activity appeared to be higher for C/EBPα than C/EBPδ. This could be due to an apparent higher expression level of C/EBPα than C/EBPδ in this experimental system (see Fig. 1*C*). When a dominant-negative form of C/EBPα (34) was used instead of C/EBPα, together with the NKX2-1 expression plasmid and the −506 *Scgb3a2* reporter construct, *Scgb3a2* expression was

about a one-half of the activity obtained with the NKX2-1 expression plasmid alone, suggesting some degree of interaction between NKX2-1 and C/EBPα dominant-negative. To understand which C/EBP binding site(s) is important for the synergistic effect, various C/EBP binding site mutants as described in the legend to Fig. 3 were subjected to co-transfection with C/EBP and NKX2-1 expression plasmids (Fig. 4*B*). A pattern of synergistic reporter activity among various binding site mutants was somewhat similar to that obtained by co-transfection of the C/EBP expression plasmid alone for both C/EBPα and C/EBPδ (compare Fig. 3*B versus* 4*B*). The transfection analysis revealed that mutations in C/EBP binding sites 7 and 10 appear to be similarly critical for C/EBP and NKX2-1 synergistic regulation of mouse *Scgb3a2* gene expression. Of interest is that C/EBP binding site 7 is juxtaposed to the distal NKX2-1 binding site (−182 bp, see Fig. 2*A*).

The effect of mutations in the NKX2-1 binding sites on *Scgb3a2* promoter activity was next examined using the −506 construct and those having mutations at −182 (site 1) or −120 bp (site 2) of NKX2-1 binding sites (group of constructs −506, Fig. 5*A*). When C/EBPα or C/EBPδ and NKX2-1 expression plasmids were co-transfected, synergistic luciferase activity was markedly reduced by mutation of either of the NKX2-1 binding sites, in which the degree of reduction was greater with the site 1 mutation than site 2. Both mutations together almost abolished the synergistic reporter activity, suggesting that NKX2-1 binding site 1 may be slightly more responsible for the synergistic activation of the *Scgb3a2* gene promoter by C/EBP and NKX2-1, however, both NKX2-1 binding sites are required for full synergism. The similar effect of the NKX2-1 binding site mutation on luciferase activity was also observed with constructs having an additional mutation at C/EBP binding sites 7 (group of −506-7M) or 10 (group of −506-10M), although the actual activities were lower than those of a group of −506 constructs as expected (Fig. 5, *B* and *C*). In all three groups of constructs, the degree of synergism between C/EBPα and NKX2-1 appeared to be more affected by NKX2-1 binding site 1 mutation as compared with C/EBPδ and NKX2-1. In addition, activation of reporter activity by C/EBP alone seems to be affected by the NKX2-1 binding site 1 mutation (see 2nd *versus* 1st and 3rd rows in Fig. 5, *A* and *C*). However, this was not seen with C/EBP binding site 7 mutants (see Fig. 5*B*). These results suggest the importance of interaction between the binding site of C/EBP site 7 and interacting of NKX2-1 with binding site 1 for efficient *Scgb3a2* gene expression. When both C/EBP binding sites 7 and 10 were mutated, luciferase activity was markedly reduced, which was further suppressed to almost zero by the additional mutation in the NKX2-1 binding sites (Fig. 5*D*). These results suggest that sites 7 and 10 may be the most critical C/EBP binding sites, and that the binding of C/EBP to these sites and their interaction with NKX2-1 bound at sites 1 and 2 may be responsible for full expression of the mouse *Scgb3a2* gene.

C/EBP and NKX2-1 Directly Interact—To further understand the synergistic effect of C/EBP and NKX2-1 on mouse *Scgb3a2* gene expression, EMSA was carried out using nuclear extracts prepared from COS-1 cells overexpressing C/EBPα, C/EBPδ, or NKX2-1 and an oligonucleotide containing C/EBP binding site 7 as a probe (Fig. 6*A*). EMSA revealed that in addition to an individual specific DNA-protein band for NKX2-1 and C/EBPα (Fig. 6*A*, *lanes 2* and *4*), which was supershifted by the addition of a specific antibody to each transcription factor, a higher molecular weight band appeared by co-incubation of nuclear extracts expressing C/EBPα and NKX2-1 together (Fig. 6*A*, *lane 6*). This band was completely abolished by the addition of either C/EBPα or NKX2-1-specific antibody (Fig. 6*A*, *lanes 7* and

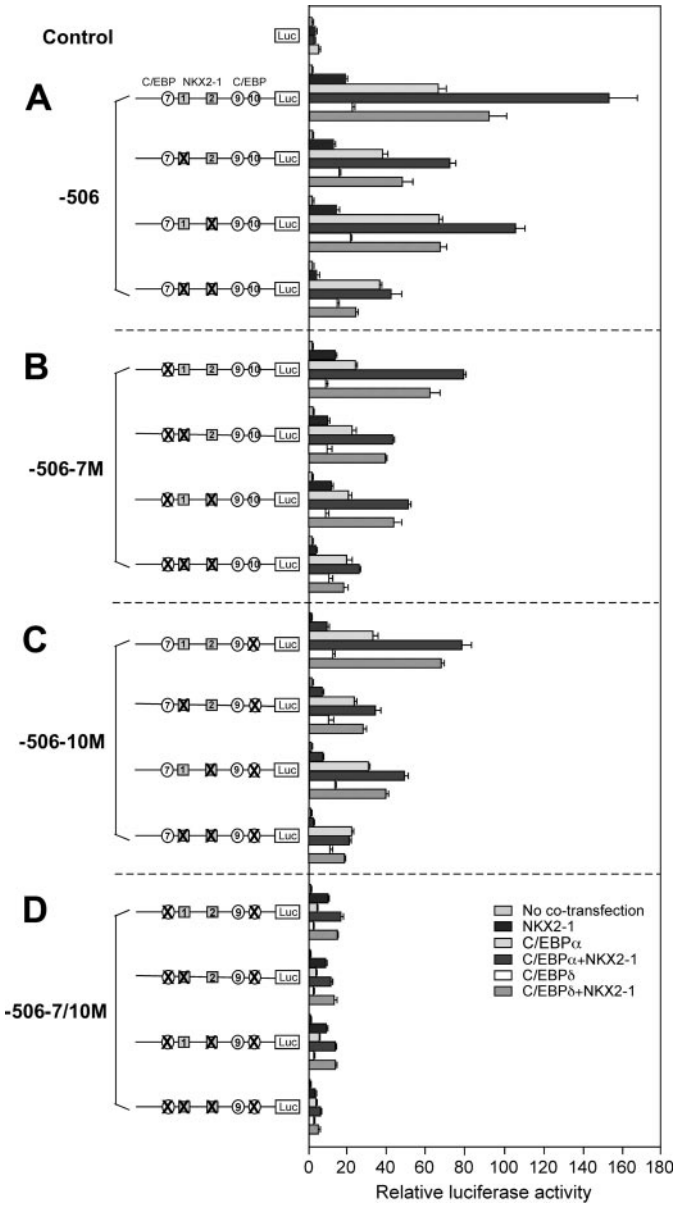

FIGURE 5. **Effect of NKX2-1 and C/EBP binding sites mutations on mouse *Scgb3a2* gene transcription.** *A*, −506 construct and those having NKX2-1 binding sites 1 and 2 singly or doubly mutated were co-transfected into COS-1 cells with NKX2-1, C/EBPα, and/or C/EBPδ expression plasmids as indicated. *B*, −506 construct having C/EBP binding site 7 mutated (*−506–7M*) and those having additionally NKX2-1 binding sites 1 and 2 singly or doubly mutated were used for co-transfection. *C*, −506 construct having C/EBP binding site 10 mutated (*−506-10M*) and those having additionally NKX2-1 binding sites 1 and 2 singly or doubly mutated were used for co-transfection. *D*, −506 construct having C/EBP binding site 7 and 10 mutated (*−506-7/10M*) and those having additional NKX2-1 binding sites 1 and 2 singly or doubly mutated were used for co-transfection. Relative luciferase activity determined from three independent experiments, each carried out in duplicate is shown as the mean ± S.D. based on the activity of control vector without any co-transfection as 1.

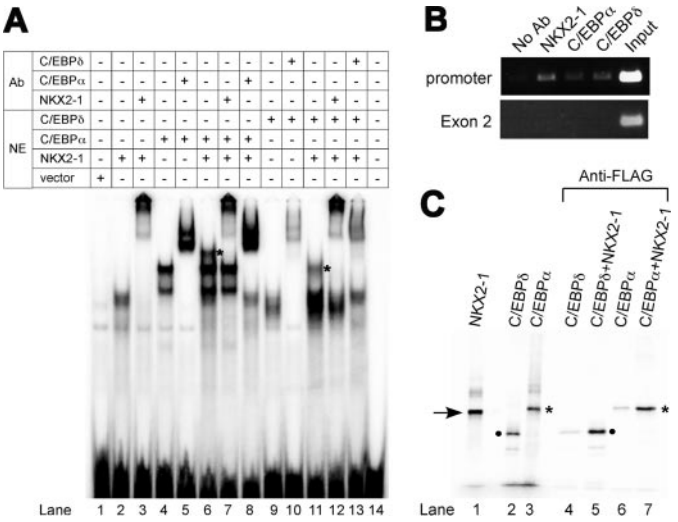

FIGURE 6. **Direct interaction between C/EBP and NKX2-1.** *A*, EMSA was carried out using nuclear extracts prepared from COS-1 cells overexpressing C/EBPα, C/EBPδ, or NKX2-1 and an oligonucleotide containing C/EBP binding site 7 as a probe in various combinations in the absence or presence of antibody specific to each transcription factor. A newly formed complex between C/EBPα and NKX2-1 (*lane 6*) or C/EBPδ and NKX2-1 (*lane 11*, both indicated by an *asterisk*) is seen as a slower mobility band. *B*, ChIP analysis was carried out using adult mouse lung with a primer pair flanking C/EBP binding sites 7 and 9 and two NKX2-1 binding sites in the mouse *Scgb3a2* promoter (see Fig. 2*A*). A primer pair located in *Scgb3a2* gene exon 2 was used as a control. *C*, co-IP experiments using transcription-translation products of FLAG-tagged NKX2-1 (*lane 1*, shown by an *arrow*), C/EBPδ (*lanes 2* and *5*, shown by a *black dot*), and C/EBPα (*lanes 3* and *7*, shown by an *asterisk*). A specific band corresponds to C/EBPδ (*lane 5*) or C/EBPα (*lane 7*) after incubation with FLAG-tagged NKX2-1, followed by immunoprecipitation with FLAG antibody.

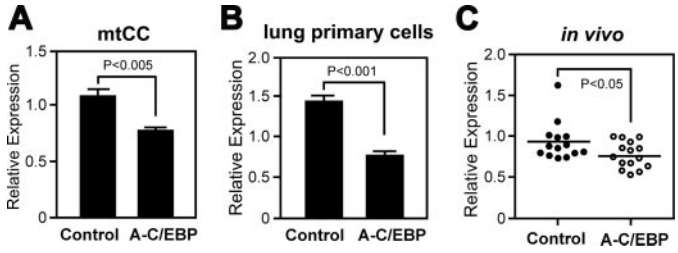

FIGURE 7. **Effect of a dominant negative A-C/EBP on the expression level of SCGB3A2.** SCGB3A2 mRNA level was determined by quantitative RT-PCR in mtCC cells (*A*) and embryonic lung primary (*B*) cells after transfection of control and A-C/EBP expression plasmids, and individual lungs (*C*) of *CCSP-rtTA;TetO-A-C/EBP* (A-C/EBP) and *CCSP-rtTA* (control) mice. SCGB3A2 level was determined in triplicate after normalization to 18 S and is expressed as a relative level. Student's *t* test was used for statistical analysis. $p < 0.05$ is considered as significantly different.

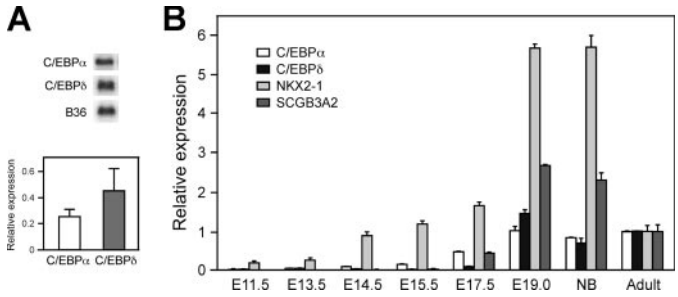

FIGURE 8. **Expression of C/EBPα, C/EBPδ, NKX2-1 and SCGB3A2 in embryonic and adult lungs.** *A*, Northern blotting analysis of C/EBPα and C/EBPδ expression. Duplicate filters containing 3 μg of lung total RNAs were hybridized with C/EBPα or C/EBPδ probes, followed by B36 as a control. Quantitated band intensity was plotted using the mean ± S.D. from three independent experiments (*lower panel*). *B*, quantitative RT-PCR was employed to determine the level of C/EBPα, C/EBPδ, NKX2-1, and SCGB3A2 in lungs of various gestational stages and adult as shown. All expression levels are shown relative to those of adult, which were given a value of 1. Note that the relative expression level among four genes does not represent the relative levels of actual expression because quantitative RT-PCR was carried out using SYBR Green and the ΔC_T method.

8). The same phenomenon was also observed when C/EBPδ was replaced for C/EBPα (Fig. 6*A*, *lanes 9–13*). These results suggest that the two transcription factors can simultaneously bind to the same oligonucleotide and/or a direct protein-protein interaction may exist between C/EBPα or C/EBPδ and NKX2-1.

Chromatin immunoprecipitation analysis using antibodies against each transcription factor and primer pairs flanking two NKX2-1 binding sites and C/EBP binding sites 7 and 10 revealed that NKX2-1, C/EBPα, and/or C/EBPδ are present in the mouse *Scgb3a2* promoter *in vivo* (Fig. 6*B*, primer sequences in Fig. 2*A*). Co-immunoprecipitation experiments were then carried out using *in vitro* transcription-translation products of C/EBPα, C/EBPδ, or FLAG-tagged NKX2-1 (Fig. 6*C*). When FLAG-tagged NKX2-1 was incubated with ^{35}S-labeled C/EBPα or C/EBPδ, followed by immunoprecipitation with anti-FLAG antibody, the intensity of a specific band to C/EBPα or C/EBPδ was markedly increased (Fig. 6*B*, *lanes 4*, *6 versus 5*, *7*). These results further suggest that NKX2-1 directly interacts with C/EBPα or C/EBPδ.

C/EBPα and/or C/EBPδ Are Responsible for Mouse Scgb3a2 Gene Expression in Vivo—Previously, we demonstrated that SCGB3A2 is a direct downstream target for NKX2-1 using *Nkx2-1*-null mouse studies and *in vitro* transfection analysis (2). To demonstrate that C/EBPα and/or C/EBPδ play a role in mouse *Scgb3a2* gene expression *in vivo*, the endogenous SCGB3A2 mRNA level was determined using quantitative RT-PCR in the absence and presence of a dominant negative A-C/EBP that inhibits the DNA binding of all C/EBP family members (35). SCGB3A2 expression was decreased to ~70 and 50% in mtCC and embryonic lung primary cells, respectively, after transfection of A-C/EBP plasmid (Fig. 7, *A* and *B*). Furthermore, a tetracycline-regulated A-C/EBP transgenic mouse (*TetO-A-C/EBP*) (36) was crossed with a transgenic mouse expressing the rtTA under the CCSP (SCGB1A1) promoter (37). After treatment with doxycyclin in the feed for 1 month, the *CCSP-rtTA;TetO-A-C/EBP* double transgenic mouse had statistically significantly decreased levels of SCGB3A2 in their lungs as compared with *CCSP-rtTA* transgenic mice (Fig. 7*C*). Interestingly, *C/EBP*δ knock-out mice (38) did not have any differences in SCGB3A2 expression levels in their lungs as compared with wild-type (data not shown). This suggests that in the absence of C/EBPδ, C/EBPα was sufficient to compensate in regulating transcription of the *Scgb3a2* gene. These results altogether demonstrate that C/EBPα and/or C/EBPδ are responsible for *Scgb3a2* expression *in vivo*.

Expression of C/EBPα and C/EBPδ in Embryonic and Adult Lungs—To understand how SCGB3A2 expression correlates with expression of C/EBPα and C/EBPδ in lung *in vivo*, Northern blotting and/or quantitative RT-PCR were carried out using lung RNAs isolated from embryos at various gestational stages and adult (Fig. 8). Northern blotting results demonstrated that C/EBPδ is expressed in adult lung at the level approximately twice that of C/EBPα (Fig. 8*A*). Using quantitative RT-PCR,

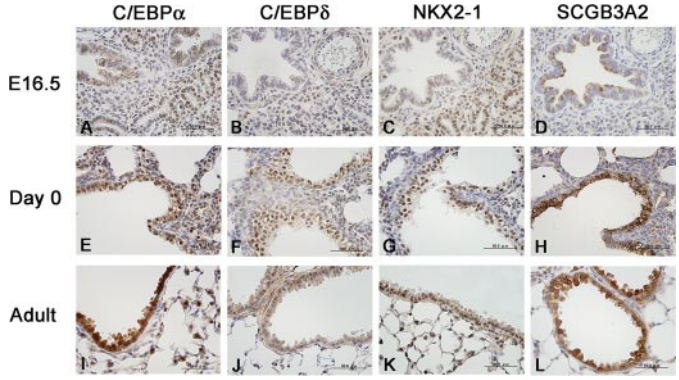

FIGURE 9. **Immunohistochemistry for C/EBPα, C/EBPδ, NKX2-1, and SCGB3A2 in E16.5, postnatal day 0, and adult lungs.** Positive staining (seen in *brown*) for C/EBPα, C/EBPδ, and NKX2-1 is observed in nuclei, whereas SCGB3A2 positive staining is found in the cytoplasm. In the case of adult lung, positive staining for C/EBPα and C/EBPδ is also found in the cytoplasm of epithelial cells; particularly the latter is profound. Magnifications: ×400.

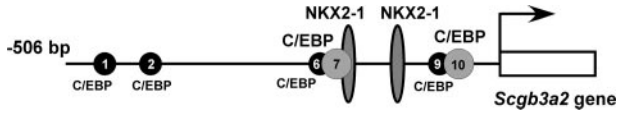

FIGURE 10. **Schematic diagram of multiple binding sites for C/EBP and NKX2-1 in the mouse *Scgb3a2* gene promoter.** Within −506 bp of the promoter, six C/EBP binding sites (*circled* with *1*, *2*, *6*, *7*, *9*, and *10*; see Fig. 2*A*) are responsible for transcription of the *Scgb3a2* gene. C/EBP binding sites 7 and 10 (*larger circle in gray*) are particularly critical for synergistic activation of the gene with NKX2-1 bound to the NKX2-1 specific binding sites; one located juxtaposed to C/EBP binding site 7 and the other between 7 and 9.

during embryonic development, a very small increase of C/EBPα expression was detected at E14.5, which markedly increased by E17.5 to the level approximately one-half of adult (Fig. 8*B*). The expression was further increased just before birth, and stayed at similar levels throughout adulthood. On the other hand, C/EBPδ expression remained low during early embryogenic stages, which slightly increased by E17.5. A dramatic increase was found just before birth (E19.0), followed by a decrease to about two-thirds of the peak levels at newborn and throughout adulthood. Quantitative RT-PCR further demonstrated that NKX2-1 expression was detected at E11.5, which was markedly increased around E14.5 and peaked just before and after birth. Adult NKX2-1 expression was at approximately one-fifth of the peak level. The expression of SCGB3A2 was detectable at E11.5 by quantitative RT-PCR, whereas a marked increase was found at E17.5. An additional increase in expression was found toward the end of gestation and in newborns. In adult, the expression was at approximately one-half the peak levels. These results suggest that the sharp increase in SCGB3A2 expression toward the end of gestation may be the results of increased expression of both C/EBPs and NKX2-1.

Immunohistochemistry was next carried out using antibodies against C/EBPα, C/EBPδ, NKX2-1, and SCGB3A2. At E16.5, embryonic lung expression of C/EBPα (Fig. 9*A*) and C/EBPδ (Fig. 9*B*) was mainly found in the epithelial cells of the airways with C/EBPδ expression being very weak and much lower than C/EBPα. At this stage, expression of NKX2-1 in the epithelia of bronchus was weak, whereas the expression in the bronchioles was intense (Fig. 9*C*) (39). SCGB3A2 expression was mainly found in the bronchial epithelial cells (Fig. 9*D*). At postnatal day 0, airway expression of C/EBPα (Fig. 9*E*), C/EBPδ (Fig. 9*F*), and SCGB3A2 (Fig. 9*H*) became stronger, particularly in bronchial epithelial cells. NKX2-1 expression was also found in the epithelial cells of bronchus (Fig. 9*G*). In adult lungs, strong C/EBPα and C/EBPδ expression was found in the airway epithelial cells, particularly bronchus, and type II cells (Fig. 9, *I* and *J*, respectively), whereas NKX2-1 expression was high in type II cells and was also found in bronchial epithelial cells (Fig. 9*K*). In adult lung, high expression of SCGB3A2 was observed in bronchial epithelial cells (Fig. 9*L*). Expression of NKX2-1 was always found in the nucleus, whereas SCGB3A2 expression was in the cytoplasm. C/EBPα and C/EBPδ expression was observed in the nucleus of epithelial cells of the bronchus and bronchioles in E16.5 and postnatal day 0 lungs, whereas the expression was also found in the cytoplasm of bronchial epithelial cells of adult lung. These results demonstrate that all three transcription factors, C/EBPα, C/EBPδ, and NKX2-1, are simultaneously expressed in the airway epithelia, particularly bronchial cells where strong SCGB3A2 expression is found, supporting their role in synergistic activation of SCGB3A2 expression.

DISCUSSION

We demonstrate here that C/EBPα and C/EBPδ interact with NKX2-1 to synergistically regulate mouse *Scgb3a2* gene transcription. Among 11 putative C/EBP binding sites present within −506 bp of the promoter of mouse *Scgb3a2* gene, six binding sites appear to play a role in transcription of the gene, of which two binding sites located at −44 to −54 bp (site 10) and −192 to −201 bp (site 7) may be the most critical for the synergistic activation of *Scgb3a2* gene promoter by C/EBPs and NKX2-1 (Fig. 10).

The involvement of C/EBP in *Scgb3a2* regulation was found after examining 44 individual NKX2-1 interacting proteins; 17 identified by yeast two-hybrid, 12 by anti-FLAG-IP followed by mass spectrometric analysis, and 15 based on literature that are known to be involved in transcription of thyroid- or lung-specific (enriched) genes (see Table S1). One protein known to interact with NKX2-1 is PARP-1 (poly(ADP-ribose) polymerase-1), which activates the mouse *Sftpb* (SP-B) gene promoter in MLE15 cells (40). We found the same protein, also called ADPRT (ADP-ribosyltransferase), by anti-FLAG-IP-MS/MS. Interestingly, ADPRT did not have any effect on transcription of the *Scgb3a2* gene in transient co-transfection analysis in four different cell lines examined including MLE15. This implies that the role of NKX2-1 interacting proteins in transcription of thyroid or lung-specific genes may be gene-dependent. This further suggests that the NXK2-1 interacting proteins we identified might play a role in transcription of other thyroid or lung-specific genes than *Scgb3a2*.

All three transcription factors, C/EBPα, C/EBPδ, and NKX2-1, are bound *in vivo* to the promoter region of *Scgb3a2* flanking C/EBP binding sites 7 and 10, and two NKX2-1 binding sites as demonstrated by ChIP assays. Of note is that the PCR bands produced by ChIP analysis were relatively weak, suggesting the low abundance of these transcription factors and/or a low specificity of the antibodies used. In this regard, we tried co-IP studies using whole cell lysates as well as nuclear fractions from mtCC and mouse lung cells instead of overexpressed pro-

teins to demonstrate the interaction between C/EBPs and NKX2-1; however, we were not able to obtain successful results. This could be due to low expression levels of the three transcription factors in mtCC cells and mouse lung cells because we did not detect any bands corresponding to these transcription factors by Western blotting analysis (data not shown).

Previously we demonstrated that NXK2-1 regulates SCGB3A2 expression by transient transfection analysis (2). This was expected because SCGB3A2 was originally identified as a downstream target for NKX2-1 using suppressive subtractive hybridization between normal and *Nkx2-1*-null embryo lungs (2). In the current study, C/EBPα and C/EBPδ were identified as transcription factors that also regulate *Scgb3a2* transcription. The physiological importance of C/EBPs in the expression of *Scgb3a2* was demonstrated using a dominant negative A-C/EBP by transfection analysis and *in vivo* using transgenic mouse expressing a dominant negative A-C/EBP in lung. A-C/EBP provides a means to demonstrate the role of C/EBPs in transcriptional regulation of a gene in a tissue where multiple C/EBPs are expressed. That A-C/EBP did not completely abolish *Scgb3a2* expression is not surprising and is in good agreement with the fact that *Scgb3a2* transcription also requires NKX2-1 (2).

The role of C/EBPα and C/EBPδ and their synergistic interaction with NKX2-1 have been well documented for transcription of the *Scgb1a1* (*Ccsp*) gene (10, 13, 19). SCGB1A1 is the prototypical protein of the SCGB gene superfamily (1), and its immunomodulatory and anti-inflammatory function in lung have been extensively studied (41, 42). SCGB1A1 is specifically expressed in the Clara cells of the bronchiolar epithelium (41). The expression robustly increases after E16–17, which coincides with the marked increase of C/EBPα expression (10, 19). It was therefore suggested that the strong synergism between C/EBPα and NKX2-1, and the onset of C/EBPα expression in developing lung are the key to high levels of SCGB1A1 expression (19). In previous (2) and current studies, a similar robust increase in the expression of SCGB3A2 was observed around E16.5–17.5. This may also be due to the marked increase in C/EBPα and C/EBPδ expression around this stage of development. Thus, two genes in the same gene superfamily are regulated by C/EBPs in a similar fashion. However, the *Scgb1a1* gene is synergistically transactivated by NKX2-1 and C/EBPα, but not C/EBPδ (19). This is in sharp contrast to *Scgb3a2,* in which both C/EBPα and C/EBPδ can synergistically activate the gene with NKX2-1.

SCGB3A2 expression drastically increases toward the end of gestation and peaks around birth at a level approximately twice as high as adult. All three transcription factors C/EBPα, C/EBPδ, and NKX2-1 markedly increase their expression toward the end of gestation, and the expression stays relatively high throughout adulthood. These transcription factors are simultaneously expressed in the bronchial epithelial cells where high expression of SCGB3A2 is found as demonstrated by immunohistochemistry. Thus, the expression pattern of SCGB3A2 during gestation through adulthood may be the result of cooperative interaction of these three transcription factors. In particular, C/EBPδ expression in the bronchial epithelial cells became markedly intense at postnatal day 0 lungs as compared with E16.5 lungs, which was in good agreement with the marked increase in C/EBPδ expression toward the end of gestation as determined by quantitative RT-PCR. These results suggest that the marked increase in C/EBPδ in the bronchial epithelial cells may be largely responsible for the marked increase in expression of SCGB3A2 in these cells toward the end of gestation. Several genes are known to display increased expression toward the end of gestation such as SP-A, -B, and -D (43, 44), aquaporin 1 (45), leptin receptor (46), and SCGB1A1 (10, 19). SCGB3A2 may be added to the list of genes that increase their expression toward the end of gestation. The role for this increase in SCGB3A2 toward the end of gestation needs to be understood.

In conclusion, we demonstrated that C/EBPα and C/EBPδ interact with NKX2-1, and synergistically activate transcription of the mouse *Scgb3a2* gene through binding to specific binding sites located in the promoter of *Scgb3a2* gene. These three transcription factors are simultaneously expressed in bronchial epithelial cells, which may allow their synergistic interaction to activate mouse *Scgb3a2* gene transcription in lung.

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