Interferon γ (IFN-γ) plays an important role during inflammation by repressing collagen and activating major histocompatibility class II (MHC-II) expression. Activation of MHC-II by IFN-γ requires regulatory factor for X-box 5 (RFX5) complex as well as class II transactivator (CIITA). We have shown that the RFX family binds to the COL1A2 transcription start site (Sengupta, P. K., Fargo, J., and Smith, B. D. (2002) J. Biol. Chem. 277, 24936–24937), and the RFX5 complex represses COL1A2 gene expression during IFN-γ response (Xu, Y., Wang, L., Buttice, G., Sengupta, P. K., and Smith, B. D. (2003) J. Biol. Chem. 278, 49134–49144). In this report, we demonstrate that CIITA is a key mediator of COL1A2 repression by IFN-γ. IFN-γ up-regulates the expression of CIITA in a time-dependent manner in lung fibroblasts and promotes CIITA protein occupancy on COL1A2 transcription start site in vivo as judged by chromatin immunoprecipitation (ChIP) assays. There are coordinate decreases in the occupancy of RNA polymerase II on the collagen transcription start site with increasing CIITA occupancy during IFN-γ treatment. In addition, we are able to specifically knockdown the IFN-γ-stimulated expression of CIITA utilizing short hairpin interference RNA (shRNA) against CIITA. This leads to the alleviation of COL1A2 repression and MHC-II activation by IFN-γ. RFX5 recruits CIITA to the collagen site as evidenced by DNA affinity chromatography. The presence of RFX5 complex proteins enhances the collagen repression by CIITA reaching levels occurring during IFN-γ treatment. Co-expression of CIITA with deletion mutations and collagen promoter constructs demonstrates that CIITA represses collagen promoter mainly through its N-terminal region including the acidic domain and the proline/serine/threonine domain. Our data suggest that CIITA is a crucial member of a repressor complex responsible for mediating COL1A2 transcription repression by IFN-γ.

Disordering of the lung architecture through a variety of injuries often results in deposition of excess connective tissues leading to pulmonary fibrosis. Fibrosis is characterized by aberrant up-regulation of extracellular matrix production resulting in excess collagen deposition. Idiopathic pulmonary fibrosis is a progressive disease with no known etiology or effective treatment. Interferon γ (IFN-γ)3 and inducers of IFN-γ alleviate fibrosis in animal models identifying IFN-γ as an important anti-fibrotic cytokine (1, 2). Indeed, IFN-γ has long been reported to reduce collagen synthesis (3, 4). It suppresses collagen production in a time- and dose-dependent manner primarily at the transcriptional level. Both type I collagen chains, α1(I) and α2(I), are regulated coordinately by IFN-γ. Although the mechanism underlying this regulation remains largely unknown, IFN-γ has been tested in numerous clinical trials. Early results indicated that IFN-γ treatment leads to favorable reductions of collagen accumulation and cytokines that induce collagen synthesis (5, 6). However, recent data suggest that there are problems with increased inflammatory response (7).

In addition to its role as an anti-fibrotic agent, IFN-γ is also a key cytokine that induces the expression of major histocompatibility class II (MHC-II) complex (8, 9). MHC-II plays a central role during inflammation by presenting antigens to CD4+ T cells, which serves as a crucial control of peripheral T-cell activation and thymic selection (10, 11). Failure to express MHC-II in patients with bare lymphocyte syndrome is caused by mutations belonging to four complementary groups (12). The expression of MHC-II thus depends on the transcription factors encoded by the genes defined in these complementary groups.

Complementation group A is caused by mutations in Class II transactivator (CIITA). This protein is referred to as the master regulator of MHC-II transcription and is important for both constitutive expression of MHC-II in B-cells or dendritic cells as well as cytokine-induced expression of MHC-II in a variety of other cell types including fibroblasts and vascular endothelial cells (13, 14). CIITA expression is stimulated by IFN-γ even in cells such as fibroblasts through two of its four promoters, promoter III and IV (15, 16).

CIITA has several functional domains including the N-terminal acidic domain and proline-rich domains followed by a serine- and threonine-rich region (PST) (17). The acidic domain activates MHC-II transcription possibly through its interac-

3 The abbreviations used are: IFN-γ, interferon γ; MHC-II, major histocompatibility class II; CIITA, MHC-II transactivator; DME, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; ChIP, chromatin immunoprecipitation assay; RIPA, radioimmune precipitation assay buffer; IL, interleukin; ANOVA, analysis of variance; GFP, green fluorescent protein; TBP, TATA-binding protein; Pol II, RNA polymerase II; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; AD, acidic domain; RFX5, regulatory factor for X-box 5; TFIIIB, transcription factor IIB; WT, wild type.
tions with transcription machinery (TFIIB, TAFIIs) and co-activators (CREB-binding protein (CBP/p300) (18–20). The PSt region can be phosphorylated in vitro, which, in turn, facilitates oligomerization and accumulation of CIITA on the MHC-II promoter (21). The question of which kinase or combinations of kinases might phosphorylate CIITA to enhance its activity is still unanswered. CIITA also has a CTP binding domain with homology to the RAS superfamily that is important for self-association, nuclear localization, and activation of MHC-II (17, 22–24). In addition, there is a leucine-rich repeat (LRR) at the C terminus that is necessary for self-assembly, nuclear import, and dominant-negative function for MHC-II (25–27). Patients with group A bare lymphocyte syndrome are deficient in the LRR region of CIITA, thus losing the capacity to activate MHC-II expression (28, 29).

CIITA is considered a scaffolding protein forming cooperative interactions with several DNA binding proteins without being able to bind to DNA directly (30). CIITA requires RFX5 complex to activate MHC-II expression. The RFX5 complex contains three proteins (RFX5, RFXB, and RFXAP) and mutations in any of these proteins cause bare lymphocyte syndrome similar to CIITA mutants. Therefore, interactions between RFX5 and CIITA on the MHC-II promoter have been extensively studied. RFX5 complex forms cooperative interactions with several other proteins, including CBP/NF-Y and βzip proteins within an enhancer region located upstream of the MHC-II promoter. The formation of this enhancerosome allows the recruitment of CIITA to the MHC-II promoter to activate transcription.

Although CIITA was initially described as a master regulator of MHC-II transcription (28, 31), it is now clear that it both activates other genes (32) and suppresses transcription of several genes such as collagen (33, 34), IL-4, Fas ligand (35, 36), and thyroid-specific genes (9). The mechanism for repression is not clear and seems to be gene-and cell-type specific.

Previously, we demonstrated that IFN-γ stimulates the expression of RFX5 complex proteins and promotes the assembly of RFX5 complex on the collagen start site suggesting that RFX5 proteins are potential mediators for collagen repression by IFN-γ (34, 37). In the present investigation, we report that IFN-γ promotes the recruitment of CIITA to the collagen promoter in part through binding to RFX5 complex. Abrogation of IFN-γ-induced expression of CIITA alleviates the activation of MHC-II and the repression of collagen. CIITA requires the presence of RFX5 complex for maximal repression of collagen transcription. Finally, we have identified two important CIITA domains responsible for collagen repression. Our results identify a critical role for CIITA in collagen IFN-γ repression.

**MATERIALS AND METHODS**

**Cell Culture, Transfection, and Luciferase Assay**—Human lung fibroblasts, IMR-90, (IMR) and rat fibroblasts, FR (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone). Prior to transfection, cells were plated in 6-well culture plates at a density of 3 × 10⁵ (for IMR-90) or 6–10⁵ cells (for FR cells) per well and incubated at 37 °C with 5% CO₂ for 16–24 h. Transfections were performed in serum-free DMEM using Lipofectamine reagent (Invitrogen) according to the manufacturer’s protocol. Three hours after transfection, cells were transferred to DMEM with 10% FBS and harvested 24 h later. Cells were then lysed in 1× reporter lysis buffer (Promega, Madison, WI), and luciferase activities were assayed using a luciferase reporter assay system (Promega). Experiments were routinely performed in duplicate plates. The ratio of experimental to control (reporter plasmid with empty vector) was calculated for each experiment with the control values set at 1.

**Value from three or more experiments were averaged and presented in this study.**

In several studies, IMR-90 cells were treated with IFN-γ. IMR-90 fibroblasts were plated in p35 tissue culture dishes at 4 × 10⁵ cells/dish for mRNA studies or in p150 tissue culture dishes at 4 × 10⁴ cells/dish for CHIP studies and maintained in DMEM with 10% FBS for 16–24 h. Cells were pretreated in DMEM with 0.4% FBS for 16 h prior to IFN-γ treatment (100 units/ml in 0.4% DMEM for 0, 2, 4, 6, 8, 16, or 24 h).

**Plasmids**—The COL1A2-luciferase construct (pH20) (38) contains sequences from −221 to +54 bp of mouse COL1A2 promoter fused to the luciferase reporter gene. A larger COL1A2-luciferase construct (pGL3-Col-Luc) containing sequences from −357 to +55 bp of mouse COL1A2 promoter fused to the luciferase reporter gene was a gift from Dr. Jenny Ting (33). Full-length FLAG-RFX5, FLAG-RFXB/ANK, FLAG-CIITA, and FLAG-CIITA-(613–221) constructs were kindly provided by Dr. Jenny Ting (FLAG-CIITA-(613–1130), FLAG-CIITA-(1–410), and FLAG-CIITA-(253–410)) constructs were kindly provided by Dr. Matija Peterlin (21). Full-length His-RFXAP was constructed in this laboratory as previously described (37).

**RNA Isolation and Real-time PCR**—Cells were harvested, and RNA was extracted using an RNeasy RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Reverse transcription reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen) according to the manufacturer’s protocol. Real-time PCR reactions were performed on ABI Prism 7700 sequence detection PCR machine (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. The oligonucleotide primers (forward and reverse) and Taqman probes are described in Table I.

**Western Blots**—For detection of expressed proteins, fibroblasts were extracted using RIPA buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with freshly added phenylmethylsulfonyl fluoride (100 μg/ml RIPA), and protease inhibitor tablet (Roche Applied Science, Mannheim, Germany). For certain experiments, to examine the effects of IFN-γ treatment on CIITA localization, nuclear and cytoplasmic protein was separated as previously described (39). The protein concentration was determined by the Bradford protein assay (Bio-Rad). Proteins in extracts were separated by 8 or 10% polyacrylamide gel electrophoresis with prestained markers (Bio-Rad) for estimating molecular weight and efficiency of transfer to blots. The proteins were transferred to nitrocellulose membranes (Bio-Rad) in a Mini-Trans-Blot Cell (Bio-Rad). The membranes were blocked with 5% milk powder in Tris-buffered saline (TBST) (0.05% Tween 20, 150 mM NaCl, 100 mM Tris-HCl, pH 7.4) buffer at 4 °C overnight and incubated for 3 h to monoclonal antibody anti-CIITA (7–1H, 1:100) (Santa Cruz.
Biotinylated, monoclonal anti-β actin (1:1000) (Sigma), monoclonal anti-FLAG (1:1000) (Sigma), polyclonal anti-RFX5 (194/41, Jackson ImmunoResearch, West Grove, PA), or polyclonal anti-RFX1 (1:100) (1-19) (Santa Cruz Biotechnology) antibodies. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies, either anti-goat IgG (Sigma), mouse-anti IgG, or anti-rabbit IgG (Amersham Biosciences) conjugated to horseradish peroxidase, for another 1 h at room temperature. The bound proteins were visualized using ECL reagent (PerkinElmer Life Sciences) on a Kodak image station (PerkinElmer Life Sciences).

**Immunofluorescence**—Human lung fibroblasts were plated at 200,000 cells per 35-mm glass bottom culture dish and allowed to grow for 24 h in 10% serum before pretreatment in 0.4% serum for 16 h. Cells were pretreated with IFN-γ (100 units/ml) or in 24 h before the next incubation. The Cells were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde for 20 min at room temperature. After three washes with phosphate-buffered saline, the cells were made permeable using 0.25% Triton X-100 in TBST. After four more washes with phosphate-buffered saline, the slides were incubated in blocking buffer with 5% bovine serum albumin in phosphate-buffered saline for 2 h at room temperature and incubated with polyclonal anti-CIITA (1:400) (N-20) (Santa Cruz Biotechnology) for 2 h at room temperature or overnight at 4 °C with 4% bovine serum albumin in TBST. After four washes with TBST, the slides were incubated with an anti-goat secondary antibody conjugated with Cy3 (1:200) (Jackson ImmunoResearch Laboratories). Fluorescence signals were observed by an Olympus 1X70 inverted fluorescent microscope equipped with a Kodak professional EOS.DCS digital camera. The intensity of fluorescence was analyzed using ImagePro Plus software. Three digitized images from three different experiments were analyzed by picking pixels that covered the nucleus. The program computed the mean intensity in the nuclei and the standard deviation. Statistical test of significance of the mean intensity difference between time points was performed using ANOVA (analysis of variance) employing Scheffe’s post-hoc procedure.

**DNA Affinity Pull-down Assay**—The collagen sequence (COL1A2 -25/-30, Genbank accession number AF048457) with a HindIII overhang was synthesized as complementary strands and annealed as previously described (40). Double-stranded collagen DNA was biotinylated by incubating with Klenow fragment (NE Biolabs) and biotin-14-dATP (Invitrogen) supplemented with regular dCTP, dTTP, and dGTP at room temperature for 30 min. The reaction mixture was phenol/chloroform-extracted and alcohol-precipitated to remove unincorporated biotin.

Nuclear protein extracts were obtained as previously described (37). 100–200 μg of nuclear protein was used per DNA affinity pull-down assay. The streptavidin beads (Promega) were washed three times with ice-cold phosphate-buffered saline supplemented with 1 mM phenylmethylsulfonyl fluoride. Nuclear proteins were precleared by incubating with the washed beads for 30 min at 4 °C on a shaking platform. Protein-A/G beads were captured by the magnetic stand and removing the supernatant. The supernatant was precipitated with biotin-labeled collagen DNA probe (−25/−30) for 1 h at room temperature in binding buffer (20 mM HEPES pH 7.9, 0.1 mM EDTA, 4% glycerol, 2 mM dithiothreitol) supplemented with bovine serum albumin, poly-dIdC, and sonicated salmon sperm DNA to remove nonspecific binding. The DNA-protein complex formed was captured by the magnetic beads and washed extensively with binding buffer supplemented with 0.01% Triton X-100 and 100 mM KCl. The bound proteins were eluted with 1× electrophoresis sample buffer by incubating at 90 °C for 10 min and analyzed by SDS-PAGE gels. In certain experiments, nuclear protein was incubated with anti-RFX5 antibody (194, Roche Molecular Biochemicals), or anti-RFX1 antibody (Genzyme, Cambridge, MA) overnight to deplete RFX5 protein.

**CHIP Assays**—Chromatin in control and IFN-γ-treated cells were cross-linked with 1% formaldehyde for 8 min at room temperature, sequentially washed with phosphate-buffered saline, Solution 1 (10 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM EGTA, 0.75% Triton X-100), and Solution 2 (10 mM HEPES, pH 7.5, 75 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM EDTA). Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor cocktail (Roche Applied Science) and phenylmethylsulfonyl fluoride. DNA was fragmented into ~500-bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were analyzed by immunoprecipitation with an antibody (seriously provided by Dr. Jeremy Boss), anti-Pol II (N-20, Santa Cruz Biotechnology), anti-TBP (SI-1, Santa Cruz Biotechnology), and anti-TFIIB (SI-1, Santa Cruz Biotechnology) antibodies followed by precipitation with protein A/G plus-agarose beads. Precipitated DNA-protein complexes were washed sequentially with RIPA buffer (25 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), high salt buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), LiCl buffer (50 mM tris, pH 8.0, 250 mM LiCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), and TE buffer (10 mM tris, 1 mM EDTA, pH 8.0, respectively). DNA-protein cross-link was reversed by heating the samples to 65 °C overnight. Proteins were digested with proteinase K (Sigma), and DNA was phenol/chloroform-extracted and precipitated by 100% ethanol. DNA was dissolved in 50 μl of deionized distilled water, and 10 μl was used for each real-time PCR reaction. The primers for real-time PCR have been described previously (37).

**PCR Shagging-based RNA Interference (RNAi)**—Three different reverse primers for RNAi were designed by RNAi oligo retriever program on the Cold Spring Harbor Web site (kodakdn.ohsu.edu/RNAi/html/rna.html) and are shown in Table I. A pGEMI-U6 vector was used as the template. PCR reactions were performed by 95 °C 3 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min followed by one cycle of 72 °C for 10 min. PCR products were separated on 1% agarose gel, sliced from the gel, purified by a Qiagen gel extraction kit, and ligated into pLenti6-V5-TOPO vector using a Viralpower directional cloning kit. Ligation products were transformed into TOP10 competent cells, and positive clones were screened by restriction digestion and direct sequencing. Viral packaging cells (293FT, Invitrogen) were maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 50 μg/mL gentamycin (Invitrogen). Three shRNA clones were transfected into 293FT cells using Lipofectamine 2000 reagent according to the manufacturer’s protocol (Invitrogen). Viruses were harvested 48 h post-transfection and used to infect IMR-90 cells. 48 h after infection, IMR-90 cells were treated with 100 units/ml IFN-γ or left untreated for additional 24 h before harvesting. Proteins were extracted using RIPA buffer (1× phosphate-buffered saline, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) and analyzed by SDS-PAGE gel followed by Western blot. RNAs were extracted and analyzed as described before.

**Statistical Analysis**—One-way ANOVA analysis with post-hoc Scheffe analysis, one-sample Student’s t test against a population mean with a Bonferroni correction, and linear regression analysis were performed using an SPSS software package (SPSS Inc., Chicago, IL).

**RESULTS**

IFN-γ Up-regulates CIITA Expression in Human Lung Fibroblast Cells—Previously, we demonstrated that the expression of RFX5 complex proteins can be stimulated by IFN-γ treatment in human lung fibroblasts (37). In this study, we examine the expression of CIITA in response to IFN-γ stimulation in human lung fibroblasts with time. First, CIITA mRNA was assayed by real-time PCR following IFN-γ treatment (Fig. 1A). There was a minimal amount of CIITA mRNA present (<0.01 pg/0.1 μg of total RNA) before IFN-γ treatment with a dramatic increase at 8 h after IFN-γ treatment. There was a significant increase (*, p < 0.05) in CIITA mRNA steady state levels at 8, 16, and 24 h as indicated by ANOVA employing Scheffe’s post-hoc procedure (Fig. 1A). Unlike levels of RXF5 mRNA (37), CIITA mRNA steady state levels remain stable between 8 and 24 h.

Next, accumulation of CIITA protein was examined by Western blot and immunohistochemistry. Nuclear and cytoplasmic proteins from human lung fibroblasts were analyzed separately by Western blot (Fig. 1B). CIITA protein could be detected both in the cytoplasm and in the nucleus at 6 h after IFN-γ treatment. The protein levels continued to increase at 16 h and were either stabilized or slightly reduced at 24 h. The immunofluorescent staining of CIITA (Fig. 1 in Supplemental Materials) clearly indicates that both cytoplasmic and nuclear localization of CIITA increase at 6 h. When nuclear CIITA staining intensity was measured, there was a small increase in staining between 6 and 24 h. Therefore, CIITA protein expression is not detectable in human lung fibroblasts without IFN-γ induction but can be markedly stimulated with IFN-γ.

**IFN-γ Promotes the Occupation of CIITA on the COL1A2 Promoter While Blocking the Recruitment of Preinitiation Complex to the Same Site**—The next question is whether CIITA
Induced by IFN-γ is activated to interact with the collagen gene at the transcription start site. ChIP was performed following IFN-γ treatment at different time points to address this question. Conditions and primers for this assay are the same as previously described (37). In the earlier studies, we found that RFX5 is present at the collagen transcription start site without IFN-γ (1.6 ± 0.2 ng of total genomic DNA) and is stimulated 3-fold 24 h after IFN-γ treatment. CIITA occupancy on the collagen transcription start site was minimal before IFN-γ treatment (0.45 ± 0.2 ng of total genomic DNA), because there is very little CIITA protein being made without IFN-γ in fibroblasts as shown above. The binding of CIITA to collagen sequence was greatly enhanced by more than 5-fold 8 h after IFN-γ treatment (Fig. 2A). The increase continued up to 16 h and remained constant at 24 h, when the occupancy was upregulated by more than 10-fold.

Since both RFX5 complex and CIITA bind at the COL1A2 transcription start site and IFN-γ enhances the binding in conjunction with the down-regulation of COL1A2 transcription, it is reasonable to hypothesize that RFX5/CIITA complex might block the formation of the preinitiation complex. To test this idea, ChIP assays were performed to determine whether there is any change in occupancy of the preinitiation complex on COL1A2 transcription start site in response to IFN-γ treatment. Three critical components of the preinitiation complex, TATA-binding protein (TBP), RNA polymerase II (Pol II), and transcription factor for Pol II (TFIIB), were chosen. Indeed, recruitment of TBP, TFIIB, and Pol II are all decreased in a temporal manner during a 24 h period in response to IFN-γ treatment (Fig. 2B). There are, however, distinct differences in the kinetics of occupancy of these proteins with time after IFN-γ treatment. Blockage of Pol II to the COL1A2 promoter appears as early as 8 h after IFN-γ treatment when there is no significant change in either TBP or TFIIB binding. By 24 h, occupancy of both TBP and TFIIB decreases by 50% in contrast to 90% for Pol II.

Binding of Pol II seems to occur much more rapidly than the recruitment of RFX5 complex. Since the binding of CIITA on COL1A2 promoter also increased at a much faster pace than the RFX5 complex, we decided to take a closer look at the kinetics of occupancies of both Pol II and CIITA. Briefly, human lung fibroblasts were treated with IFN-γ for 0, 2, 4, 6, 8, 16, and 24 h and the binding of Pol II and CIITA on the collagen transcription start sites was examined by ChIP analysis. Indeed, the removal of Pol II from the collagen site correlates very well with the recruitment of CIITA on the same site (Fig. 2C), identifying CIITA as a potential modulator of Pol II on the collagen promoter.

CIITA Is Indispensable for IFN-γ-mediated Collagen Repression—The next question is whether CIITA is a critical collagen repressor induced and activated in fibroblasts during IFN-γ treatment. Therefore, it is of interest to investigate whether cells will respond to IFN-γ by repressing endogenous collagen transcription in the absence of CIITA. To this end, RNA interference (RNAi) was utilized to knockdown the IFN-γ stimulated expression of CIITA in human lung fibroblasts. Briefly, short hairpin RNA (shRNA) sequences against different regions of CIITA were cloned into a lentiviral vector. Three viral stocks were generated using lentiviral vectors containing different CIITA shRNA sequences to infect human lung fibroblasts (Table II). Cells were transduced and treated 48 h later with IFN-γ for an additional 24 h before harvesting. Two clones, named pLenti6-CSH4 and pLenti6-CSH6, respectively, were able to inhibit IFN-γ-induced expression of CIITA at both mRNA and protein levels (Fig. 3A). Moreover, they were capable of alleviating the IFN-γ-mediated down-regulation of COL1A2 mature mRNA (Fig. 3B) and heterogeneous nuclei RNA levels (data not shown) suggesting that CIITA is required for IFN-γ-mediated collagen transcription repression. The relative mRNA levels of collagen decreased when CIITA protein levels increased as demonstrated by the linear regression plot of four experiments performed in duplicate (Fig. 2A in Supplementary Materials). Expression of certain MHC-II molecule, HLA-DRA, was also examined. CIITA shRNA could specifically block the stimulation of HLA-DRA expression by IFN-γ (Fig. 3C), confirming that CIITA is also the key regulator of MHC-II expression. Up-regulation of RFX5 by IFN-γ, on the other hand, was not affected by any of the three shRNA clones (data not shown), suggesting that CIITA specifically mediates IFN-γ effect on MHC-II and collagen expression.

DNA Affinity Pull-down Assays Indicate That CIITA Can Interact Through RFX5 on the Collagen Transcription Start Site—Next, we determined whether CIITA could be recruited to the collagen promoter through RFX5 proteins using DNA affinity pull-down assays when human fibroblasts were treated with IFN-γ (100 units/ml). A probe containing COL1A2 sequence spanning the transcription start site (~25 to +30) was labeled with biotin and incubated with nuclear proteins ex-
Fig. 2. The in vivo occupancy of CIITA increases while Pol II, TBP, and transcription factor IIB (TFIIB) decrease with time after IFN-γ treatment. A, CIITA occupancy on the COL1A2 transcription start site increases dramatically after IFN-γ treatment. IMR-90 cells were treated with 100 units/ml IFN-γ for 0, 8, 16, or 24 h. ChIP assays were performed using anti-CIITA antibodies as described under “Materials and Methods.” Data were expressed as fold changes of CIITA occupancy compared with 0-h time point. Each experiment was performed in duplicate at least three times, and values represent the mean ± S.D. CIITA occupancy increased significantly as early as 8 h after IFN-γ treatment (**, p < 0.01 one-sample Student’s t test). The increase continued up to 16 h and remained constant at 24 h (*, p < 0.05 one-sample Student’s t test). B, Pol II decreases faster than TBP or TFIIB from COL1A2 transcription start site after IFN-γ treatment. IMR-90 cells were treated with 100 units/ml IFN-γ for 0, 8, 16, or 24 h. ChIP assays were performed using anti-Pol II, anti-TBP, and anti-TFIIB antibodies as described under “Materials and Methods.” Data were expressed as relative changes of occupancies compared with 0-h time point. Each experiment was repeated at least three times, and values represent mean ± S.D. All three components of the pre-initiation complex decreased significantly (*, p < 0.05; **, p < 0.01 one-sample T test) on the collagen site 24 h after IFN-γ treatment whereas RNA Pol II was significantly decreased at 8 h. C, increase in CIITA correlates with a decrease in Pol II occupancy from COL1A2 transcription start site after IFN-γ treatment. IMR-90 cells were treated with 100 units/ml IFN-γ for 0, 2, 4, 6, 8, 16, or 24 h. ChIP assays were performed using anti-Pol II and anti-CIITA antibodies as described under “Materials and Methods.” Data were expressed as nanograms of DNA precipitated by indicated antibody per microgram of total genomic DNA calculated from a standard curve using IMR-90 genomic DNA. Each experiment was repeated at least three times, and a representative figure is shown.

Table II
Sequences cloned into lentiviral vectors

| Lentivirus | Location in CIITA | Sequences |
|------------|------------------|-----------|
| CSH4       | 266–294          | 5'-AAAAAAAATTTATCGATAGCTCCCTATGCTTAGCTAGGCGCTCCCAAGGACCCAGGGAGGCTTATGCCAATATCCGGTGTTTCGTCCTTTCCACAA-3' |
| CSH5       | 2231–2259        | 5'-AAAAAAAATTTATCGATAGCTCCCTATGCTTAGCTAGGCGCTCCCAAGGACCCAGGGAGGCTTATGCCAATATCCGGTGTTTCGTCCTTTCCACAA-3' |
| CSH6       | 1522–1550        | 5'-AAAAAAAATTTATCGATAGCTCCCTATGCTTAGCTAGGCGCTCCCAAGGACCCAGGGAGGCTTATGCCAATATCCGGTGTTTCGTCCTTTCCACAA-3' |

* Accession number is NM000246.

* The sequence in bold is the short hairpin sequence. The sequence in italics is the reverse complement of the CIITA sequence.
Interferon-γ-induced Collagen Repression Mediated by CIITA

![Collagen Repression by CIITA](https://via.placeholder.com/150)

**A**. CIITA shRNA abrogates IFN-γ-induced CIITA expression thereby blocking repression of collagen mRNA expression and activation of MHC II mRNA. A. CIITA protein levels (top panel) and mRNA levels (lower panel) are decreased by CSH4 and CSH6 shRNA. RNAi experiments were performed as described under “Materials and Methods.” Each experiment was repeated at least three times and a representative figure of CIITA Western and mRNA each was shown. B, collagen (COL1A2) mRNA levels are not repressed by IFN-γ in the presence of CSH4 and CSH6 shRNA. RNAi experiments were performed as described under “Materials and Methods.” (See Supplemental Materials, Fig. 2A, for the correlation of expression of collagen and CIITA.) C, MHC II mRNA levels are less activated by IFN-γ in the presence of CSH4 and CSH6 shRNA. RNAi experiments were performed as described under “Materials and Methods.” (See Supplemental Materials, Fig. 2B, for the correlation of expression of MHC II and CIITA.)

were performed as described under “Materials and Methods.” (See Supplemental Materials, Fig. 2A, for the correlation of expression of collagen and CIITA.) C, MHC II mRNA levels are less activated by IFN-γ in the presence of CSH4 and CSH6 shRNA. RNAi experiments were performed as described under “Materials and Methods.” (See Supplemental Materials, Fig. 2B, for the correlation of expression of MHC II and CIITA.)
confirm whether or not CIITA repression is enhanced when exogenous RFX5 complex proteins are expressed. A COL1A2 promoter-reporter construct (0.5 μg) (pH20, −221 to +54) was co-transfected with CIITA expression construct (0.1–0.5 μg) plus or minus RFX5 proteins (0.25 μg each) into rat fibroblasts (Fig. 5A). In the presence of RFX5 proteins, the full-length CIITA (0.5 μg) could repress the COL1A2 promoter-reporter activity by more than 80% reaching the level of inhibition observed in human lung fibroblasts. Both RFX5 proteins and CIITA significantly repressed collagen transcription compared with empty vector control using one-sample Student’s t test (Fig. 5). At each dose tested, the presence of RFX5 complex significantly (p < 0.01) enhanced the repression by CIITA by ANOVA employing Scheffe’s post-hoc procedure.

CIITA is a complex molecule with multiple functional and structural domains diagrammed in Fig. 5A (41). The interaction domains of CIITA with RFX5 complex proteins have been mapped by co-immunoprecipitation (30) within the first 612 amino acids and by glutathione S-transferase pull-down assays further downstream between amino acids 319 and 730 (41). In order to determine whether RFX5 recruits CIITA, mutant CIITA proteins were co-expressed with collagen promoter construct in rat fibroblasts with and without RFX5 complex proteins. The N-terminal region of CIITA from 1 to 335 (designated CIITA-(1–335)) repressed collagen gene expression in a similar manner to wild-type CIITA (Fig. 5B) confirming earlier data (33). At each dose tested, CIITA-(1–335) significantly (p < 0.01 by Student’s t test) down-regulated COL1A2 promoter activity, whereas there was no significant difference between wild-type CIITA and CIITA-(1–335) in terms of collagen repression both in the presence and absence of RFX5 complex (determined by ANOVA).

The C-terminal portion of the CIITA molecule from 336 to 1130 (CIITA-(336–1130)) did not repress collagen transcription (Fig. 5C). However, the presence of transfected RFX5 proteins reduces collagen promoter activity. Most important, the expression of CIITA-(336–1130) with RFX5 proteins partially relieves the RFX5 repression (p < 0.05 by ANOVA). This effect was not responsive to different doses of transfected CIITA (Fig. 5C). This construct contains a region within CIITA-(335–612) that interacts with RFX5 (30). Expression of the mutant without this region (CIITA-(613–1130)), does not relieve the RFX5 repression (Fig. 5D). Taken together, these data suggest that RFX5 complex is co-operating with CIITA to down-regulate collagen transcription.

The PST Domain of CIITA Is Both Necessary and Sufficient for Basal Level Collagen Transcription Repression in Human Lung Fibroblasts—To further elucidate the mechanism of CIITA repression of collagen, efforts were taken to explore the domains within CIITA that modulate collagen transcription in human fibroblasts using different lengths of collagen promoter constructs as well as different mutant CIITA expression constructs. In fibroblasts producing RFX5 proteins, CIITA is a potent repressor of collagen transcription, repressing the COL1A2 promoter activity by more than 80% using a short collagen promoter-luciferase construct (pH20) containing the TFID, NFY, and YB1 binding sites (Fig. 6, construct 1). A previous study (33) suggests that a 36 amino acid region within the acidic domain that interacts with a co-activator, CBP/p300, is mainly responsible for collagen down-regulation. When this 36 amino acid region is deleted from the CIITA expression construct (CIITA-(Δ59–94)) (Fig. 1B, construct 2) and co-transfected into human lung fibroblasts with the short collagen α2(I) promoter construct, collagen promoter activity was repressed almost to the same level achieved by wild-type CIITA, indicating that this AD region interaction with CBP/p300 is not necessary for repression of collagen transcription using the short construct.

On the other hand, others (21) have suggested that phosphorylation of the proline-serine/threonine (PST) domain is important for oligimerization and enhancing activation of MHC-II promoter. This PST domain alone is a potent repressor of collagen transcription (Fig. 6, A and B, construct 3, light gray). Strikingly, when this domain is deleted from CIITA (CIITA-(Δ253–410)), the shorter protein lost the capability of down-regulating the basal promoter activity (Fig. 6B, construct 4). In these experiments, CIITA constructs were transfected at the same molar ratio as the reporter construct and were expressed.

![Fig. 4. RFX5 and CIITA interact at COLIA2 transcription start site in DNA affinity pull-down assays.](image)
Fig. 5. CIITA represses collagen promoter activity better in the presence of RFX5 complex. A, COLIA2 promoter construct (pH20, −220/+54) (0.5 µg) was co-transfected into duplicate flasks of rat fibroblasts with GFP construct (0.1 µg) and increasing amounts of wild-type CIITA construct as indicated either in the presence of RFX5 complex (striped bars) or not (black bars). RFX5 (gray bar) or empty plasmids (white bar) were transfected with no CIITA. Luciferase activities were normalized by both protein concentration and GFP fluorescence. Relative luciferase is expressed as a ratio compared to empty vector control (white bar) that is set at 1. Each experiment was repeated at least three times, and values represent mean ± S.D. The data were evaluated for significance by one-sample Student’s t test compared to control considered as a population mean (*, p < 0.05; **, p < 0.01). Comparisons were also performed without control using ANOVA followed by Sheffe’s post-hoc analysis. At each dose tested, the presence of RFX5 complex significantly (p < 0.01) enhanced the repression by CIITA.

B, COLIA2 promoter construct (pH20, −220/+54) (0.5 µg) was co-transfected into duplicate flasks of rat fibroblasts with GFP (0.1 µg) and either wild-type CIITA (0.5 µg) (black bar) or increasing amount of CIITA-(1–335) construct (dark gray bar) in the presence of RFX5 complex (striped bar) or not (dark gray) as indicated. RFX5 (gray bar) or empty plasmids (white bar) were transfected with no CIITA. Luciferase activities were normalized by both protein concentration and GFP fluorescence. Relative luciferase is expressed as a ratio compared to empty vector control. Each experiment was repeated at least three times, and values represent mean ± S.D. The data were evaluated for significance by one-sample Student’s t test compared to control considered as a population mean (*, p < 0.05; **, p < 0.01). At each dose tested, CIITA-(1–335) significantly down-regulated COLIA2 promoter activity whereas there is no significant difference between wild-type CIITA and CIITA-(1–335) in terms of collagen repression both in the presence and absence of RFX5 complex (by ANOVA).

C, COLIA2 promoter construct (pH20, −220/+54) (0.5 µg) was co-transfected into duplicate flasks of rat fibroblasts with GFP (0.1 µg) and either wild-type CIITA (0.5 µg) (black bar) or increasing amount of CIITA-(335–1130) construct (striped bars) or not (dark gray bars) as indicated. RFX5 (gray bar) or empty plasmids (white bar) were transfected with no CIITA. Luciferase activities were normalized by both protein concentration and GFP fluorescence. Relative luciferase is expressed as a ratio compared to empty vector control. Each experiment was repeated at least three times, and values represent mean ± S.D. The data were evaluated for significance by one-sample Student’s t test compared to control considered as a population mean (*, p < 0.05; **, p < 0.01). Comparisons were also performed without control using ANOVA followed by Sheffe’s post-hoc analysis. At each
at similar levels as detected by epitope FLAG antibody (Fig. 3A see Supplemental Materials). In addition, there are two protein bands when the PST domain is expressed. The higher molecular weight band is sensitive to phosphatase digestion (data not shown) suggesting that this region is phosphorylated in human lung fibroblasts. These data suggest that the PST domain, dose tested, CIITA-(335–1130) significantly (p < 0.05) partially reversed the repression by RFX5 complex (comparing RFX5 with and without CIITA-(335–1130)). D, COL1A2 promoter construct (pH20, −220/54) (0.5 µg) was co-transfected into duplicate flasks of rat fibroblasts with GFP (0.1 µg) and either wild-type CIITA (0.5 µg) (black bar) or increasing amount of CIITA-(613–1130) construct in the presence of RFX5 complex (striped bar) or not (dark gray bar) as indicated. Luciferase activities were normalized by both protein concentration and GFP fluorescence. Relative luciferase is expressed as a ratio compared to empty vector control. Each experiment was repeated at least three times, and values represent mean of three experiments ± S.D. The data were evaluated for significance by one-sample Student’s t test compared to control considered as a population mean (**, p < 0.01). Comparisons were also performed without control using ANOVA followed by Sheffe’s post-hoc analysis. At each dose tested, CIITA-(613–1130) did not significantly reverse the repression by RFX5 complex.
which mediates the phosphorylation and oligomerization of CIITA, is both necessary and sufficient for repression of a short promoter in lung fibroblasts.

Both the AD and PST Domains of CIITA May Be Required to Achieve Maximum Inhibition of Collagen Transcription by Larger Promoters—In order to determine whether the AD domain of CIITA might interact with proteins binding upstream in the collagen promoter, we used in co-transfection assay a longer collagen promoter-luciferase construct (−357 to +55), which contains an enhancer-like structure. Interestingly, whereas wild-type CIITA remains a competent repressor, CIITA lacking the activation domain (CIITA-(Δ59–94)) only down-regulates the promoter activity slightly (Fig. 7A). On the other hand, the PST domain itself is not as repressive on the larger promoter as it was on the shorter promoter and the mutant CIITA with deleted PST region (CIITA-(Δ253–410)) retains some repression activity. These results imply that CIITA represses collagen through more than one region of the promoter. CIITA repression activity or recruitment may be influenced by proteins binding upstream between −221 and −357 of the COL1A2 promoter.

Multiple N-terminal deletions and two C-terminal deletions were also co-transfected with both collagen-promoter constructs into human lung fibroblasts in order to define the collagen repression domains in CIITA (Fig. 7B). When the N-terminal deletions do not affect the integrity of the PST domain (CIITA-(253–1130)), CIITA mutants retain all of the repression activity on both sized collagen promoters (Fig. 7, B and C). As deletions go further into the PST domain (CIITA-(335–1130)), CIITA-(300–1130), and CIITA-(613–1130)). However, CIITA mutants start to lose most or all the repression activity. Again, the PST domain is less active as a repressor of the longer promoter comparing two N-terminal deletions (CIITA-(253–1130) and CIITA-(300–1130)). CIITA molecules containing the PST and AD domains significantly (p < 0.01 by Student’s t test) repress the collagen promoters. Two C-terminal deletion mutants, which contain most or the entire PST domain, greatly repress the promoter activity. These studies clearly indicate that the N-terminal portion through the PST domain of the CIITA molecule is essential for collagen promoter repression in human lung fibroblasts.

DISCUSSION

CIITA is a master regulator of MHC-II gene expression (28, 31). This protein also enhances the transcription of the invariant chains coordinately regulated with classical MHC-II, suggesting that it could be an important protein in antigen presentation (42, 43). Recently, it has been established using microarray analysis that multiple genes are both activated and suppressed by CIITA (32). COL1A2 is one of the genes repressed by CIITA (33, 34) along with IL-4 (20, 44) cathepsin E (45), and Fas ligand (35). This study examines the mechanism
for CIITA repression of collagen transcription.

CIITA is dramatically induced in human lung fibroblasts by IFN-γ as judged by mRNA and protein levels. Others have also demonstrated increased CIITA expression in fibroblasts (8, 46, 47). Most importantly, IFN-γ-induced CIITA protein occupies the collagen start site with faster kinetics than RFX5 complex proteins or the deacetylation of histones (37). RFX5 is already in the nucleus and present at the collagen transcription start site in unstimulated nuclei. This is in keeping with MHC-II activation, where the activators are present along with certain basal transcription proteins on the promoter prior to IFN-γ treatment (48), but is in contrast to other genes where activators are recruited with the transcription machinery during transcriptional activation (49, 50). RFX5 proteins at the collagen start site are continuously increased 2–3-fold during 24 h of IFN-γ treatment (37). Whereas, once CIITA is induced, it rapidly (within 8 h) occupies the collagen start site, suggesting a major role of CIITA in transcriptional regulation of collagen.

Interestingly, RNA Pol II occupancy decreases on COL1A2 transcription start site rapidly with similar kinetics to increased CIITA occupancy. Other transcription factors that are part of basic transcription machinery, TBP and TFIIB, decrease with similar kinetics to increases in RFX5 occupancy on collagen start site (37). Possibly, while the collagen gene is being repressed by IFN-γ, certain transcriptional activators remain "poised" on the collagen gene, ready for reactivation. The correlation of increasing CIITA with decreasing RNA Pol II occupancy suggests possible mechanisms for the CIITA down-regulation of collagen expression. First, since CIITA is recruited to the collagen start site, there could be spatial blockage or competition for binding at the start site between CIITA and RNA Pol II. In the case of the collagen gene, the CIITA oligomers may be blocking re-entry of RNA polymerase II. Second, since CIITA is an acetyltransferase (51), once recruited to the gene, the enzyme could be activated and modify RNA Pol II or proteins in the holoenzyme complex. The C-terminal domain of RNA Pol II is a substrate for various modifications, which alter preinitiation complex formation, promoter clearance, elongation and splicing (52–56). Recent evidence suggests that CIITA recruitment to MHC-II promoter correlates with phosphorylation of serine 5 within the C-terminal domain of RNA Pol II (48). A similar scenario could occur on the collagen promoter whereby CIITA, through its interaction with certain kinases or phosphatases, may modulate the phosphorylation status of the C-terminal domain and alter RNA Pol II occupancy.

RNA Pol II occupancy on a gene may also be regulated by nucleosome positioning and chromatin remodeling (57, 58). Indeed, CIITA can interact with co-repressor complexes, Sin3A/HDAC1 proteins that deacetylase histones and cause compact chromatin structures (59). During MHC-II activation, the histones surrounding the MHC-II gene become acetylated during IFN-γ treatment (37, 60–62). The interaction of CIITA with Sin3A/HDAC1 disrupts MHC II activation (59). Possibly
CIITA interacts with co-repressors at the collagen gene to alter histone acetylation. In fact, decreased histone acetylation occurs at the collagen start site during IFN-γ treatment (37). However, the deacetylation of the histones surrounding the collagen start site follows the same kinetics as the changes in RFX5, not the more rapid CIITA kinetics. Finally, the repression process may not occur on all alleles or in all cells in culture. Most likely, several of these mechanisms operate during IFN-γ-induced collagen repression. Additional data are necessary to establish how temporal interactions occur between proteins during transcription repression.

In order to determine whether CIITA is an essential protein in the IFN-γ-induced collagen repression, RNAi was used to decrease CIITA expression. In order to examine endogenous levels of collagen mRNA, we used shRNA in a lentiviral vector, which has a high transfection efficiency in human cell strains, to abrogate the induction of CIITA during IFN-γ treatment. The transactivation of MHC-II and the repression of collagen were decreased when CIITA levels were low. Others have noted that IFN-γ suppression of the collagen promoter activity, and mRNA level occurs in the wild-type but not in a CIITA mutant fibrosarcoma cell line. In addition, suppression is restored when CIITA is introduced (33). These data suggest that IFN-γ-induced expression of CIITA is indeed critical for collagen gene repression.

CIITA is not a DNA-binding protein, but rather exerts its functions through interaction with other proteins. Several mechanisms have been suggested for CIITA function including interaction of CIITA with DNA-bound transcription factors, sequestration of factors important for regulation of a gene, activation of a transcription factor that regulates a gene, and induction of genes involved in chromatin modulation. CIITA interacts with several transcription factors including RFX5 complex, NFY complex, and CREB to activate transcription of MHC II (30, 41, 48). CIITA inhibits IL-4 gene transcription by competing with a transcription factor, NF-AT, to bind the co-activator CBP/p300 (20). A similar sequestration of CBP from promoter/enhancers was suggested as the mechanism for down-regulation of collagen (33). However, we have demonstrated that RFX5 complex proteins, the major transcription binding complex on MHC-II, can also bind to COLIA2 gene during IFN-γ treatment and can repress collagen transcription (34, 37). Several different lines of evidence in this report using DNA affinity pull-down assays and transfections in cells without RFX5 suggest that CIITA repression of collagen depends, in part, on the binding of CIITA to RFX5 complex proteins and other DNA-binding proteins.

Our DNA affinity pull-down assays indicate that CIITA can be recruited to the collagen start site through RFX5. Two approaches examine the specificity of RFX5 recruitment of CIITA to the collagen transcription start site: 1) oligonucleotides were used to compete for RFX5 protein and 2) nuclear extracts, immune-depleted with RFX5 antibody, were compared with mock-depleted nuclear extracts. The DNA used in these experiments (−25 to +30) does not contain other sites for DNA binding proteins that might also recruit CIITA such as TATA box, NFY, or an AP1 site. These studies examine only interactions between RFX5 and endogenous IFN-γ-induced CIITA. CIITA is present only in IFN-γ-treated nuclear extract on the collagen gene in the presence of RFX5 proteins. Therefore, RFX5 proteins recruit CIITA to the collagen start site.

In addition to DNA affinity studies, we also examined CIITA repression activity in a rat fibroblast line that produces no detectable RFX5 proteins or RFX5 complex formation (34, 37). Our results indicate that CIITA represses collagen transcription better in the presence of RFX5 proteins. Expression of the N-terminal region of CIITA through the PST domain represses collagen expression similar to the wild-type CIITA. This expressed protein has most of the PST domain (1–335) that interacts with NFYB, NFYC, and RFXAP on the HLA-DRA promoter (30, 41). The C-terminal region (334–1130) without this domain has little collagen repression activity confirming previous data (33). However, the C-terminal region of CIITA (334–1130) partially relieves the repression by RFX5 possibly because it contains the RFX5/RFXB interaction region (335–615) (30, 41). The C-terminal mutant (613–1130) has no repression activity. Therefore, RFX5 is important for CIITA repression activity, although it may not be the only protein recruiting CIITA to the collagen promoter.

Since CIITA can repress collagen transcription in rat fibroblasts that have no measurable amounts of RFX5 proteins, there may be interactions with other proteins binding within the collagen promoter or gene. The collagen promoter has a clearly defined CBF/NFY site, and CBF/NFY complex proteins activate collagen transcription (63, 64). It has been demonstrated that CBF/NFY proteins do not reverse the CIITA-mediated repression of collagen transcription (30). However, NFY may stabilize recruitment of CIITA when RFX5 interacts with the collagen gene. In addition, there are two inhibitory binding sites close to the CBF/NFY site for cKrox (64, 65) and Y-box-binding protein (YB-1) (66, 67). The YB-1 protein has been implicated in mediating IFN-γ repression of collagen transcription (66, 67). Possibly, YB-1 accounts for increased RFX5 binding during IFN-γ stimulation. On the MHC-II promoter Y-box is a binding site for CBF/NFY, which is critical for RFX5 recruitment (41). In addition, YB-1 protein, which represses MHC II expression, also interacts at this site and induces single stranded regions in the promoter (68, 69). An additional CREB binding site (X2 Box) is also important in CIITA activation and recruitment to the MHC II promoter (48, 70, 71). However, there is no data that there is a consensus CREB binding site in the collagen promoter. In addition, the region of the CIITA molecule that interacts with CREB (70, 71) is not necessary for repression of collagen transcription making it less of a possibility that CREB might be involved in CIITA recruitment to the collagen start site. Finally, there are specific spacing requirements between binding sites that are necessary to recruit RFX5 rather than RFX1 (72). Therefore, the spacing of similar sites on the collagen promoter needs to be explored more carefully to determine which proteins are recruiting CIITA. Taken together, CIITA most likely interacts with RFX5 at the collagen transcription start site along with other proteins binding to the promoter.

We have confirmed results of others (33) that expression of N-terminal region (1–335) of CIITA is required for repression of collagen promoter. This region alone represses collagen transcription, cathepsin E (45), and IL-4 repression (20), but does not activate MHC-II transcription (33). CIITA is a complex protein with several defined domains based on activation of MHC-II and interactions with other molecules. The first 160 amino acids are referred to as an activation domain (19, 27, 74) followed by an acetyltransferase catalytic domain (AT) (51). The acidic amino acid domain interacts with components of the transcriptional machinery (18, 19, 75, 76) and co-activators such as CBP (71, 75). The PST domain (160–410) has multiple phosphorylation sites that directs oligomerization, accumulation, and increased activity of CIITA on the MHC-II promoter (33). Our co-transfection results in human lung fibroblasts indicate that the PST domain in CIITA is essential for collagen repression on a short promoter (−220 to +54). The collagen promoter with binding sites is diagrammed in Fig. 8. Deletion...
of the PST domain eliminated repression of the short promoter whereas deletion of a 36-amino acid sequence in the acidic activation domain did not abrogate repression. The PST domain was partially phosphorylated in the human lung fibroblasts. The data suggest that oligomerization and accumulation of CIITA at the collagen start site might be important for blocking RNA polymerase II. Recently, it was reported that this domain is also important for cathepsin E repression (45).

An earlier publication defined a 36-amino acid region within the acidic domain of CIITA as an IFN-γ mediated suppression domain that sequesters CBP (33). These investigators used a larger collagen promoter construct in either 3T3 cells or fibrosarcoma cells derived from an HT1080 cell line. Our results with the larger collagen promoter in human lung fibroblasts indicate that both AD and PST regions of the CIITA molecule are important for repression of collagen. The longer collagen promoter has an enhancer-like region containing binding sites for multiple transcription factors including Smad, Sp1, and Ets (Fig. 8). Most of the proteins binding to this enhancer region of collagen gene interact with CBP; therefore, CIITA could be repressing collagen through its AD by blocking interaction with CBP on the enhancer regions in addition to forming oligomers at the start site.

The mechanism involved in CIITA repression may be cell specific. The published data using mutated HT1080 fibrosarcoma cell lines demonstrate an interaction between CIITA and CBP that was important for collagen repression (33). The HT1080 transformed cells produce small amounts of collagen because the collagen gene is partially methylated making a higher affinity binding site for RFX1 (34, 77). Possibly, in that case, the RFX5 proteins are not able to bind as readily on the methylated COL1A2 gene and, therefore, CIITA may not be recruited to the start site. This could make the enhancer interactions more prominent in these cells. The human lung fibroblast cell line used in this report has an unmethylated collagen gene (40). Therefore, RFX5 might bind better and recruit CIITA to the start site to repress collagen transcription.

Finally, there are other parameters that could affect the recruitment of CIITA to a particular promoter and be responsible for different cell specific responses. CIITA is subjected to various post-translational modifications including acetylation, ubiquitination, and phosphorylation (21, 78–80). These modifications might play important roles in determining the enlistment of CIITA to different sites. Indeed, ubiquitination enhanced the association of CIITA with MHC class II transcription factors and, therefore, its association with the MHC class II promoter, resulting in an increase in transcriptional function and in the expression of MHC class II mRNA (81). Now that we have shown in this report that the PST domain, a highly phosphorylated region of CIITA, is critical for collagen repression (Figs. 6 and 7), it remains as a good possibility that binding of CIITA to the COL1A2 promoter could be guided by its phosphorylation status.

In summary (Fig. 8), we have demonstrated here and in the previous publication (37) that IFN-γ increases the synthesis of CIITA dramatically along with a more modest increase in RFX5 complex proteins. CIITA is located both in the cytoplasm and the nucleus. Translocation of RFX5 and CIITA into the nucleus increases with time of IFN-γ treatment. IFN-γ increases RFX5 complex assembly on both MHC-II promoter and the collagen transcription start site. This is accompanied by a decrease in histone acetylation on the collagen gene. CIITA is more rapidly recruited to the collagen transcription start site at the same time when RNA Pol II occupancy is decreased. Finally, the presence of CIITA is critical for IFN-γ repression of collagen transcription. Therefore, during inflammation when IFN-γ is produced, certain fibroblasts respond by increasing levels of RFX5 as well as CIITA, which both activate antigen presentation on membranes and reduce collagen gene expression. This may be a mechanism that decreases scar or fibrosis.

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Major Histocompatibility Class II Transactivator (CIITA) Mediates Repression of Collagen (COLIA2) Transcription by Interferon γ (IFN-γ)

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