Interferon γ (IFN-γ) plays an important physiological role during inflammation by down-regulating collagen gene expression and activating major histocompatibility class II (MHC-II) complex. The activation of MHC-II by IFN-γ requires activation of a trimeric DNA binding transcriptional complex, RFX5 complex, containing RFXB (also called RFXANK or Tvl-1), RFXAP, as well as RFX5 protein. Previously, we demonstrated that RFX5 binds to the collagen transcription start site and represses collagen gene expression (24, 27). In this report, we have examined the role of RFXB and RFXAP proteins within the RFX5 complex to regulate collagen gene expression. The data show that all three RFX5 complex proteins are required for maximum repression. Expression of proteins with mutations known to be important for RFX5 complex formation does not repress collagen promoter activity. Two mutated forms of RFX5 act as dominant negative proteins activating collagen expression and reversing IFN-γ down-regulation of collagen expression in human lung fibroblasts. IFN-γ increases expression and nuclear translocation of RFX5. RFXB has a naturally occurring splice variant isoform (RFX SV). Interferon increases expression of the long form of RFXB and decreases expression of RFX SV with the same kinetics as collagen gene expression. Overexpression of the splice variant form reverses the IFN-γ-induced collagen repression in human lung fibroblasts. Finally, all three RFX5 complex proteins increase at the collagen transcription start site with IFN-γ treatment using chromatin immunoprecipitation analysis. Thus, these studies suggest an important role for RFX5 complex in collagen repression.

Interferon γ (IFN-γ)1 exerts a wide range of antiproliferative, immunomodulatory, and antibioretic effects. Cells respond to infection and inflammation by producing IFN-γ in T-cells (Th1) and NK cells. As an antibioretic agent, IFN-γ suppresses collagen production in a time- and dose-dependent manner primarily at the transcriptional level (1, 2). Both type I collagen chains are regulated coordinately by IFN-γ. In vivo studies in mice have demonstrated that IFN-γ inhibits collagen synthesis associated with the fibrotic response to an implanted foreign body, bleomycin-induced pulmonary fibrosis and the healing response to cutaneous thermal burns (3). Clinical trials have had some success in ameliorating certain fibrotic conditions such as interstitial lung fibrosis and systemic sclerosis (4). IFN-γ activates many genes through the Jak-Stat pathway (5). However, collagen repression is independent of Stat-promoter interactions (6–10). There is some indication that Y-box binding protein (YB-1) may be involved in DNA binding on the collagen promoter in response to IFN-γ (10) and that the co-activator p300/CBP may mediate the repression (11).

IFN-γ is a key cytokine that also induces the expression of major histocompatibility class II (MHC-II) complex proteins on the cell surface. MHC-II plays a central role by presenting antigens to CD4+ T cells, which serves as a crucial control of peripheral T-cell activation and thymic selection (12, 13). Failure to express MHC-II in patients with bare lymphocyte syndrome (BLS) is caused by mutations belonging to four complementation groups. The expression of MHC-II depends on the transcription factors encoded by the genes defined in these complementation groups. Supplementation group A is caused by mutations in Class II transcriptional activator (CIITA). This protein is considered a master regulator of MHC-II transcription and its mRNA levels are greatly stimulated by IFN-γ in many cells, including fibroblasts (14–16). CIITA is a histone acetyltransferase capable of acetylating histones and activating transcription through its interactions with the general transcription factors (TBP, TAF175, TAF170, TAF170B) (17–19) and other histone acetyltransferases (p300/CBP, pCAF) (20–22). It has been suggested that CIITA could repress collagen through interaction with p300/CBP (23).

The other three complementation groups are caused by mutations in three proteins, RFX5, RFXB (also called RFXANK or Tvl-1) and RFXAP that interact with each other forming a DNA binding complex, the RFX5 complex. The RFX5 complex binds to the X-box located in the MHC-II promoter (24, 25). DNA binding is primarily mediated by RFX5 (26) although RFXAP and RFXB may also contact DNA (27). RFX5 is the main protein that interacts with CIITA. RFXAP binds both RFX5 and RFXB through its C-terminal domain serving as a bridge between these two proteins (26). RFXB contains several ankyrin repeats that mediate protein-protein interactions. The RFXB gene gives rise to two differentially spliced mRNAs (28). The spliced variant (RFXB SV) has a substitution of amino acid 90 from aspartic acid to cysteine and is missing amino acids 91–112, exon 5, thereby eliminating its ability to induce expression of MHC-II genes (26). Interestingly, IFN-γ induces RFXB but down-regulates the splice variant isoform (29). All four
proteins, RFX5, RFXB, RFXAP, and CIITA are required for the MHC-II transcription activation but act in cooperation with other DNA binding factors such as CAAT binding factor/nuclear factor Y (CBF/NF-Y).

Previously we have demonstrated that the RFX family of transcription factors can bind to the α2(I) collagen gene (COL1A2) at the transcription start site (29, 30). In addition, RFXAP was shown to repress collagen gene expression in fibroblasts (31). This work continues these investigations by demonstrating that the other components of the RFX5 complex contribute to repression of collagen gene expression. Mutations of the expressed proteins indicate that the RFX5 complex is important for repression. Two mutant forms of RFX5 can activate collagen expression and reverse repression by IFN-γ. In the presence of IFN-γ, the RFXB isoform represses collagen promoter activity whereas the RFXBV SV isoform activates collagen promoter activity. All three proteins occupy the collagen transcription start site, and IFN-γ increased the occupation at that site, as judged by chromatin immunoprecipitation assays (ChIP). Finally, the levels of histone acetylation surrounding the collagen transcription start site decreased with time after IFN-γ treatment correlating with transcriptional repression.

MATERIALS AND METHODS

Cell Culture, Transfection, and Luciferase Assay—Human lung fibroblasts (IMR-90) (Coriell, NJ) and rat fibroblasts (FR) (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA). mouse collagen I (11003) subcultured with 10% fetal bovine serum (FBS, Hyclone). Prior to transfection, cells were plated in 6-well culture plates at densities of 3 × 105 (for human lung fibroblasts) or 6 × 105 cells (for rat fibroblasts cells) per well and incubated at 37°C with 5% CO2 for 24–24 h. Transfections were performed in serum-free DMEM using Lipofectamine reagent (Invitrogen) according to manufacturer’s protocol. Three hours after transfection, cells were switched to DMEM with 10% FBS and harvested 24 h later. Cells were then lysed in 1X reporter lysis buffer (Promega, Madison, WI), and luciferase activities were assayed using a luciferase reporter assay system (Promega, Madison, WI).

In several studies human lung fibroblasts were treated with IFN-γ. Human lung fibroblasts were plated in p35 tissue culture dishes at 4 × 105 cells/dish for mRNA studies or in p150 tissue culture dishes at 4 × 105 cells/dish for chromatin immunoprecipitation (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, 8, or 24 h). 

Plasmids—The COL1A2-luciferase construct (pH20) (32) contains sequences from –221 to +54 bp of mouse COL1A2 promoter fused to the luciferase reporter gene. Full-length Flag-RFX5 and Flag-RFXB/ANK constructs were kindly provided by Dr. Jenny Ting (33). His-RFX5 (245), and RFXB SV constructs were kindly provided by Dr. Jeremy Boss (26, 34). His-RFXAP was constructed by RT-PCR using forward primer: 5′-AAGTA-ACTGCTCTTACCC-3′ and reverse primer: 5′-TCCATATTGATGTC-3′, followed by cloning into pcDNA4/Max vector. Full-length His-RFX5 was constructed by RT-PCR using forward primer: 5′-AAGTA-ACTGCTCTTACCC-3′ and reverse primer: 5′-GATCCTTTGA-3′, followed by cloning into pcDNA4/Max vector.

Transfection of expression vectors into transfected cells was performed with Lipofectamine reagent (Invitrogen) according to manufacturer’s protocol. Real-time PCR reactions were performed on a ABI Prism 7700 sequence detection machine (Applied Biosystems, Foster City, CA) according to manufacturer’s protocol. The oligonucleotide forward and reverse PCR primers, and fluorescent probes are described in Table I. 

Immunofluorescence—Human lung fibroblasts were plated at 2 × 104 cells per 35-mm glass bottom culture dish and allowed to grow for 24 h in 0.4% serum and treated with IFN-γ (100 units/ml) for 6, 16, or 24 h before fixation. Cells were washed twice with phosphate-buffered saline and fixed in 4% paraformaldehyde for 20 min at room temperature. After 3 washes with phosphate-buffered saline, the cells were made permeable using 0.25% Triton X-100 in Tris-buffered saline (TBST) (0.25% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4). 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. The primary antibody, RFX5 (1:500) (Rockland), was incubated for 2 h at room temperature or overnight at 4°C in 4% bovine serum albumin in TBST. After washing four times, the slides were incubated with an appropriate secondary antibody conjugated with Cy3 (1:200) (Jackson Immunoresearch Laboratories). Fluorescence was examined using an Olympus IX70 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 analyses of each experiment were analyzed by picking pixels that covered the nucleus. The program computed the mean intensity in the nuclei and calculated the standard deviation. Statistical test of significance of the mean intensity difference between time points was calculated using ANOVA (analysis of variance) employing Scheffe’s post-hoc program.

ChIP—After IFN-γ treatment, cells were fixed with 1% formaldehyde for 8 min at room temperature, sequentially washed with phosphate-buffered saline, Solution I (10 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM EGTA, 0.75% Triton X-100), and Solution II (10 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). 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) and DNA was fragmented into ~500-bp pieces by using a B Clarence 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-RFX5 (194, Rockland), anti-RFXB (C-18, Santa Cruz Biotechnology, Santa Cruz, CA), anti-RFXAP (Abcam, Cambridge, UK), anti-acetylated histone H4 and anti-acetylated histone H4 (Hich grade, Upstate Biotechnology, Lake Placid, NY) antibodies followed by adsorption to Protein A/G plus-agarose. Precipitated DNA-protein complexes were washed with RIPA buffer (50 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, pH8.0, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), LiCl buffer (50 mM Tris, pH8.0, 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. The 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. Dried 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 are found in Table II.

RESULTS

RFXB, RFXAP, and RFX5 Repress Collagen Transcription in Rat Fibroblasts—Previously, we demonstrated that co-transfection of RFX5 represses COL1A2 promoter-reporter plasmids (31). Since the RFX5 complex contains three proteins, we continued these experiments by examining the role of the two additional subunits of the RFX5 complex, RFXB, and RFXAP, on collagen gene transcription. Rat fibroblasts were used for these experiments because these fibroblasts do not exhibit an RFX5 binding activity or demonstrable RFX5 protein by Western analysis (30).
Next, each subunit was co-transfected alone or in combinations of two or three. All three RFX5 proteins significantly down-regulated COLIA2 promoter activity (**, p < 0.05) as compared with empty vector control group. In a separate, but parallel experiment, expression of RFXB (A, inset) in rat fibroblast cells increased with increasing doses and was detected by anti-FLAG antibody (IB:Flag). RFXB represses collagen promoter activity in a dose-responsive manner. B, RFXAP represses collagen promoter activity in a dose-responsive manner. At highest concentration, RFXAP significantly down-regulated COLIA2 promoter activity (**, p < 0.05) as compared with empty vector control group. Expression of RFXAP (B, inset) in rat fibroblasts increased with increasing doses and was detected by anti-His antibody (IB:His). C, RFXB, RFXAP, and RFX5 were transfected at low dose (0.25 μg) singly or in combinations of two or three. All three RFX5 proteins significantly down-regulated COLIA2 promoter activity (***, p < 0.001) as compared with the empty vector control group. RFX5 complex represses collagen promoter activity better than single proteins or combinations of two proteins.

Expression of Truncated Forms of RFX5 Identifies Two Domains That Are Necessary for Collagen Repression—Next, RFX5 deletion mutant constructs (Fig. 2A) (26) (kindly provided by Dr. Jeremy Boss) were expressed (Fig. 2B) and tested for collagen gene repression activity in combination with other members of the RFX5 complex (Fig. 2C). RFX5 is the only protein in the complex that has a clearly defined DNA binding domain. The RFX5 mutant, named RFX5Δ1, does not have this DNA binding domain, but it does contain a proline-rich region that interacts with CIITA and a region that interacts with the other subunits (26) (Fig 2A). As expected, when this mutant was co-transfected with RFXB and RFXAP, the collagen promoter expression equaled that in cells transfected only with RFXB and RFXAP. The RFX5Δ5 mutant does not have the CIITA interaction domain, but it can interact with RFXAP and RFXB (26). The RFX5Δ5 mutant also did not repress collagen transcription. However, the mutant RFX5Δ6 with domains that interact with CIITA and DNA retained most of the collagen repression activity.

To determine if these mutants could compete for wild-type reporter (pH20) was co-transfected with different amounts of full-length cDNAs expressing RFXB and RFXAP (Fig. 1, A and B). Reporter gene activity decreased in a dose-responsive manner. The RFXB dose response curve (Fig. 1A) was similar to the previously published dose response using RFX5 expression constructs (31). Protein extracted from cells transfected with full-length cDNA constructs of RFXB and RFXAP were compared with protein extracted from cells transfected with pcDNA3 empty vector. Since the expressed RFXB and RFXAP contain an epitope tag (FLAG and His, respectively), Western analysis using commercially available antibodies were used to indicate that the constructs are expressed in a dose-dependent manner (Fig. 1, left panels).

Expression of Collagen Promoter Activity in RFXAP Transfected Cells

To further analyze the role of RFXAP in collagen repression, the reporter construct was co-transfected with RFXB, RFXAP, and RFX5 (Fig. 1C). This repression by all three subunits was more than any individual subunit at the same dose (0.75 μg).
Interferon γ-induced Collagen Repression Mediated by RFX5

RFX5 and act as dominant negative mutants, each mutant was co-transfected at different doses with the complete complex containing wild-type RFX5 in addition to RFXB and RFXAP. As indicated in Fig. 2D, RFX5Δ1 and RFX5Δ5, but not RFX5Δ6 reversed the repression of the RFX5 complex in a dose-dependent manner reaching the level of the control transfected with RFXB and RFXAP only. Thus, two mutant constructs act as dominant negative constructs. This suggests that both the DNA binding domain and the proline-rich region may be necessary for collagen repression.

Specific Domains of RFXB and RFXAP Are Necessary for the Repression Activity of RFX5 Complex on Collagen Transcription—RFXB has a natural splice variant form, RFXB SV, with a deleted exon 5 that alters amino acid 90 (D to C) followed by a deletion of amino acids from 91 to 113 (26, 28, 29) (Fig. 3A). RFXB SV does not activate MHC-II transcription (26). The deleted region may interact with DNA although there is no clearly defined DNA binding region (26). Alternatively, this deleted region may contribute to the stability of the RFX5 complex and improve its DNA binding activity (26, 29). The splice variant of RFXB maintains its PEST domain and several ankyrin repeats allowing interactions with other proteins. The splice variant does not repress collagen promoter as well as the full-length form in the RFX5 complex (Fig. 3B) suggesting that the full-length form in the complex is important for repression.

The RFXAP subunit contains regions rich in acidic and basic amino acids as well as a glutamine-rich domain (Fig. 4A). The glutamine region is required for association with RFX5 and RFXB (26). This region was also critical for activation of MHC-II. An RFXAP mutant without the glutamine region did not contribute to repression of collagen promoter activity (Fig. 4B). Taken together, the data suggest that all three RFX5 subunits are necessary for optimal repression of the COL1A2 promoter.

IFN-γ Increases Gene Expression of RFX5 Proteins in Human Lung Fibroblasts—Human lung fibroblasts contain high levels of RFX5 complex by gel shift analysis and respond to IFN-γ by down-regulating collagen promoter activity (31). In addition, IFN-γ alters the amount of the RFXB splice variant (29) and activates CIITA expression (37). Therefore, the amount of RFX5, RFXB, and RFXAP mRNA as a function of time of IFN-γ treatment was examined using real-time PCR. Splice variant specific probes were prepared to analyze any shift in ratio of the two splice variant forms (Table I). First, there was a continual increase in both RFX5 and RFXAP mRNA levels with time of IFN-γ treatment (Fig. 5A). At 24 h after IFN-γ treatment, RFX5 mRNA increased more than 3-fold while RFXAP mRNA was stimulated by more than 2.5-fold. In addition, there

30 μg of whole cell extract was loaded into each lane. The proteins were detected by anti-HIS antibody (IB: HIS). RFX5 mutant constructs are expressed at comparable levels in rat, C. construct pH20 (0.5 μg) was co-transfected with various combinations of RFXB (0.25 μg), RFXAP (0.25 μg) and either wild-type or mutated RFX5 (0.25 μg) constructs as indicated. Luciferase activities were measured and statistically analyzed as described in Fig. 1. While RFXB complexes and either RFXBΔ5 or RFX5Δ6 complex displayed significant down-regulation when compared with RFX5Δ1 and RFX5Δ5 complex showed no significant difference when compared with wild-type RFX5 and RFXAP-only group (p > 0.1). D, reporter construct pH20 (0.5 μg) was co-transfected with 0.25 μg each of wild-type RFX5, RFXB, and RFXAP constructs along with increasing amounts of either RFX5Δ1, RFX5Δ5, or RFXAPΔ6 as indicated. Luciferase activities were measured and statistically analyzed as described in Fig. 1. At highest concentration, both RFX5Δ1 and RFX5Δ5 significantly up-regulated the promoter activity to the level of RFX5 and RFXAP-only group (p< 0.01, RFX5Δ5, p< 0.05). RFX5Δ6 did not alter the promoter activity by RFX5 complex. Note that RFX5Δ1 and RFX5Δ5 both competed with wild-type RFX5 to reverse the inhibition exerted by RFX5 complex on COL1A2 in rat fibroblasts. RFX5Δ6 did not compete.
was a smaller but significant increase in the long form of RFXB at 24 h with a loss in splice variant form (Fig. 5B). The decrease in the splice variant followed the same time course as the decrease in collagen mRNA levels. The ratio of RFXB to RFXB SV increased 2.5-fold at 24 h with a loss in splice variant form (Fig. 5B).

We then determined if the RFX5 protein levels in the nucleus increased using both immuno-fluorescence and Western blot analysis. The cells clearly contained more RFX5 protein in 6 h increasing at 24 h whereas little change was observed with RFXAP staining (data not shown).

**Mutant Forms of RFX5 Can Activate Collagen Expression in Human Lung Fibroblasts**—In order to determine the role of RFX5 in IFN-γ-induced collagen repression, several RFX5 mutants were transfected into human lung fibroblasts with and without IFN-γ treatment. In the absence of IFN-γ, RFX5 repressed collagen promoter activity only slightly (Fig. 7A). However, both RFX5Δ5- and RFX5Δ1-activated collagen expression, behaving like dominant negative constructs. RFX5Δ6 mutant repressed similar to wild-type and the RFX5Δ3, a mutant without DNA binding domains or CIITA interaction domains (amino acids 410–616) (26), had no repression or enhancing activity (data not shown). In the presence of IFN-γ, RFX5 expression had no effect, as might be expected if IFN-γ treatment induced high levels of RFX5 expression. The activation by RFX5Δ5 and RFX5Δ1 also occurred with or without IFN-γ treatment. This data suggest an important role of RFX5 in repression of collagen basal promoter activity in these cells. Two regions of the molecule, the DNA binding domain and the proline-rich CIITA interaction domain may both be important for repression of collagen.

**The Natural Splice Variant of RFXB Activates in the Presence of IFN-γ**—Next, a similar experiment was performed with the two natural isoforms of RFXB transfected into human lung fibroblasts. Luciferase activities were assayed and statistically analyzed as described in Fig. 1. Both RFXAP complex and RFXAPΔ3 complex showed significant difference from RFX5 and RFXB-only group (column 2, p < 0.01) but there is no significant difference between the RFXAPΔ3 complex and RFX5/RFXB-only group.
fibroblasts with and without IFN-\(\gamma\) treatment. RFXB and RFXF SV had little effect on collagen transcription without IFN-\(\gamma\) (Fig. 7B). However, in the presence of IFN-\(\gamma\), the splice variant activated rather than repressed collagen promoter activity. This data suggest that IFN-\(\gamma\) alters the splicing of RFXB thereby increasing the long form and decreasing the shorter protein, which leads to a nuclear RFX5 complex that represses collagen transcription.

**IFN-\(\gamma\) Induces RFX5 Complex Protein Binding at the Collagen Transcription Start Site**—Commercially available antibodies were first tested to determine if they could immunoprecipitate endogenous RFX5 complex proteins from human lung fibroblast extracts. All three antibodies were able to precipitate the correct proteins from cell extracts (data not shown). Next, in order to determine if all three RFX proteins assemble at the collagen transcription start site in vivo, chromatin immunoprecipitation (ChIP) was performed following IFN-\(\gamma\) treatment. Proteins and DNA were cross-linked with formaldehyde after 0, 8, 16, or 24 h of IFN-\(\gamma\) treatment. The sheared chromatin DNA complexes were immunoprecipitated with antibodies to RFX5 complex proteins or the acetylated histones H3 and H4. The DNA was then amplified with primers surrounding the collagen start site (Table II). All three RFX antibodies precipitated increasing amounts of protein on the collagen transcription start site with time of IFN-\(\gamma\) treatment (Fig. 8A). Primers (Table II) within the coding region of the collagen gene were used as a negative control. Since this region did not contain an RFX5 binding site, there was no detectable DNA precipitated by any of the RFX5 protein antibodies with no change in occupancy during IFN-\(\gamma\) treatment (data not shown).

Several investigators have demonstrated that RFX5 complex proteins bind to the MHC-II promoter at the X-box site in B-cells (24, 38) and that more RFX5 proteins bind with time during IFN-\(\gamma\) treatment (39). Therefore, in certain experiments, the same primers (Table II) surrounding MHC-II promoter X-box were used as a positive control and compared with collagen primers. Similar amounts of DNA (1–4 ng) were amplified using MHC-II X-box or collagen start site primers as determined by genomic DNA standard curves. Most importantly, there was a similar fold increase in RFX5 complex proteins on the collagen start site as on the MHC-II promoter during IFN-\(\gamma\) treatment (Fig. 8B).

**IFN-\(\gamma\) Reduces Histone Acetylation on the Collagen Transcription Start Site**—Decreased acetylation is often associated

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**Table I**

| Gene       | Amplicon location | Exon | Sequences                                             |
|------------|------------------|------|-------------------------------------------------------|
| RFX5       | 245-331          | Exon 2 | 5'-TCAGAGGCTCCGAGGTACCA-3'                            |
| Forward primer | Exon 2/3        |      | 5'-TCGTTTCTGAAAATTTCTGGCATCTTG-3'                    |
| Tagman probe | Exon 3           |      | 5'-TCGCTTGTAGAGGATGTTGT-3'                           |
| Reverse primer | Exon 4          |      | 5'-TGCGGCCACCACTCTTAGA-3'                            |
| RFXB/Tv1-s  | 416-523          | Exon 4 | 5'-GCACTCACCACACTCTAGAA-3'                           |
| Forward primer | Exon 5           |      | 5'-GCCCACGCGGAGAGA-3'                                 |
| Tagman probe | Exon 6           |      | 5'-GAAAGGAGCAGGGAATGTA-3'                            |
| Reverse primer | Exon 6          |      | 5'-AGGTAGTGGTGGGAAGAGA-3'                             |
| COLIA2     | 346-436          | Exon 6 | 5'-GTAACCTTACCTGATGTTTG-3'                           |
| Forward primer | Exon 7          |      | 5'-GCCACAGGCCTGAACTG-3'                               |
| Tagman probe | Exon 8           |      | 5'-ATGCACTCTCCGTGGTG-3'                               |
| Reverse primer | Exon 1/2        |      | 5'-ATCGACTCTTCCGTGGTG-3'                              |

* GenBank™ accession numbers used for location: NM_000449 = RFX5, AF105427 = RFXB/Tv1-1, AF105428 = RFXB SV/Tv1-s. Genomic sequences: AF004877 = collagen, NT_024524 = RFXAP.
with transcriptional repression. The acetylated histones, H3 and H4, surrounding the collagen start site decreased after IFN-γ treatment, correlating with collagen transcriptional repression. There was an increase in acetylated histones on the MHC-II X-box region with time after IFN-γ treatment, whereas there was no change within the collagen gene (Fig. 8C). The levels of amplified collagen promoter/first exon DNA were highest in acetylated histone precipitates from untreated cells (45–70 ng). These levels decreased and reached the MHC-II X-box site levels (20–30 ng) after 24 h of IFN-γ treatment. There is a low level (10 ng) of collagen gene DNA precipitated by acetylated histone with minimal changes in the amount of acetylated histones during IFN-γ treatment. Therefore, this data demonstrate that RFX5 complex forms in vivo with time during IFN-γ stimulation on both the collagen and the MHC-II promoter. However, RFX5 complex formation is accompanied by lowered histone acetylation and repressed collagen gene expression, whereas the complex formation on the X-box of the MHC-II promoter is accompanied by increased histone acetylation.

DISCUSSION

Our previous data demonstrate that several members of the RFX family interact with the collagen start site and repress transcription (30, 31). The RFX5 complex in human fibroblast nuclear extracts interacts with the collagen transcription start site as judged by gel shift migration patterns and blocking of binding by RFX5 and RFXB antibodies. This was the first demonstration that RFX5 complex can form on DNA other than the X-box site in the MHC-II or MHC-I promoters.

Interestingly, unlike human fibroblast, a rat fibroblast cell line did not contain RFX5 binding activity or detectable levels of RFX5 protein by Western blot analysis. In these cells, RFX5 co-transfected with a collagen promoter-luciferase construct, reversed collagen transcription in a dose-dependent manner. RFX5 complex formation is also an essential component in the activation of MHC-II proteins (40). RFX5 forms a trimeric
complex with two other proteins RFXB (also called RFXANK or Tvl-1) and RFXAP. This report continues this line of investigation to elucidate the role of the RFX5 complex, including RFXB and RFXAP, in collagen repression. First, we demonstrated that in co-transfection studies, both RFXB (Fig. 1A) and RFXAP (Fig. 1B) repress collagen promoter activity in a dose-responsive manner. All three components of the RFX5 complex repress collagen activity more effectively than any one or combinations of two proteins (Fig. 1C).

RFX5 complex assembly is critical for activation of MHC-II (26, 41). Using mutational studies to examine protein-protein interactions and DNA-protein interactions, it was demonstrated that RFXB and RFXAP nucleate the RFX5 complex without DNA. RFX5 is required for transactivation of MHC-II through its proline-rich domain that interacts with the transactivator CIITA (Fig. 2A). Although RFX5 has a clearly defined helix-turn-helix DNA binding domain, it does not bind MHC-II DNA per se unless RFXB and RFXAP are present. RFX5 contacts these two proteins via two separate regions that surround its DNA binding domain (26). It was of interest to determine if these regions of the molecule are necessary for repression of collagen promoter activity as well as activation of MHC-II. It is clear that deletion of either the RFX5 DNA binding domain or its transactivation domain eliminates the repression of collagen (Fig. 2C). The C-terminal mutant (RFX5Δ6) containing both domains maintained the repressive properties of wild-type RFX5, while the mutant lacking both domains did not repress collagen promoter activity (data not shown). In order to test whether the mutants can compete with wild-type RFX5 and reverse the repression, each deletion mutant was transfected in increasing concentrations in the presence of RFX5 complex (Fig. 2D). The mutant lacking the N-terminal DNA binding domain (RFX5Δ1) could compete with wild-type RFX5 and abrogate the repression. This mutant, most likely, competes with wild-type RFX5 and forms a mutant RFX5 complex, which cannot bind to collagen DNA.

In addition, deletion of the MHC-II transactivation region (RFX5Δ5) in RFX5 that interacts with CIITA through a proline-rich domain (26) also reverses repression in rat fibroblasts (Fig. 2D). Most importantly, both mutant forms of RFX5 could activate collagen promoter activity in human lung fibroblasts and reverse the IFN-γ repression thereby acting as dominant-negative activators (Fig. 7A). The RFX5 mutant without the proline-rich-interacting domain may compete with wild-type RFX5 and form a complex with RFXB and RFXAP that can interact with DNA. However, because of the deleted domain, this complex may be inactive without interaction with CIITA and/or other proteins. Because we (31) and others (23) have demonstrated that CIITA represses collagen expression, the proline-rich domain of RFX5 may be recruiting CIITA to the collagen promoter similar to the MHC-II promoter.

The binding of the RFX5 complex to DNA is enhanced through the central region of RFXB, although this region is not a clearly defined DNA binding domain (Fig. 3A). There is a naturally occurring splice variant with an in-frame deletion of exon 5 (amino acids 91–112) that forms a less stable RFX5 complex (29) with diminished ability to bind to DNA (26, 29). The ankyrin repeats within RFXB mediate interactions between RFXAP, RFX5, and CIITA. The RFXB splice variant did not repress collagen as well as wild-type RFXB (Fig. 3A). Most interestingly, this RFXB-spliced variant activates collagen promoter activity only in the presence of IFN-γ, and neither form effects collagen promoter activity in human fibroblasts without IFN-γ (Fig. 7B). RFXB (also called Tvl-1) has multiple functions in the cytoplasm as well as in the nucleus (29, 42, 43). The RFXB protein in the cytoplasm is a substrate and regulator of Raf-1 (42). It also induces apoptosis in a complex with caspase-9 and Tpl-2 (43). It is not clear whether a significant proportion of RFXB is actually complexed with RFX5 and RFXAP without IFN-γ stimulation.

The C-terminal amino acids within RFXAP contain a glutamine rich region that is necessary for complex formation and activation of the MHC-II promoter (Fig. 4A) (26). This region may be critical for nucleation of the RFX5 complex (41). This region is also important within the RFX5 complex for collagen promoter repression. Taken together, all the regions necessary for RFX5 complex assembly are required for repression of collagen suggesting an important role for the RFX5 complex.

If RFX5 proteins are involved with IFN-γ repression, it might be expected that their production, localization and/or activity will change with IFN-γ treatment. Therefore, mRNA levels were first examined for all three RFX5 complex proteins with time of IFN-γ treatment. Earlier work suggested that RFX5 was present with or without IFN-γ whereas CIITA mRNA levels were dramatically increased with IFN-γ treatment (25). Our results indicate that all three RFX5 mRNAs increase during IFN-γ treatment (Fig. 5). In addition, while the RFXB mRNA level increases, the RFXB splice variant decreases with the same kinetics as collagen mRNA. Therefore, the IFN-γ treatment seems to maximize the formation of a transcriptionally active RFX5 complex as suggested by others (29). Our data confirm that IFN-γ induces dramatic increases of CIITA mRNA within 6 h of IFN-γ treatment (data not shown). Since protein levels and localization are important for understanding function, the protein levels of RFX5 were measured during IFN-γ treatment. Although there is RFX5 protein present without IFN-γ, RFX5 clearly accumulates in the nucleus as early as 2–6 h (Fig. 6) as judged by both Western blot analysis and immunofluorescence. There was a small increase in nuclear RFXB protein at 16 h (data not shown).
antibody recognized both natural forms of the protein. However, the changes in RFXB localization may be more significant with more specific antibodies that only detect one form or the other. On the other hand, the RFXAP antibody did not react well with RFXAP by Western blot analysis or immunofluorescence. Therefore, RFXAP may be in small limiting amounts in human lung fibroblasts.

Most importantly, all three RFX5 complex proteins interact with the collagen transcription start site in increasing amounts with time of IFN-γ treatment as judged by ChIP assays (Fig. 8, A and B). The increased occupancy of the RFX5 complex on the collagen-transcription start site during IFN-γ treatment occurred with similar kinetics to the increased occupancy on the MHC-II promoter X-box. This is an important in vivo verification that RFX5 complex binds to the collagen transcriptional start site with the same kinetics as it binds to MHC-II. Clearly, IFN-γ may cause coordinate antigen presentation along with decreased collagen synthesis through RFX5/CIITA interactions.

Histone acetylation is correlated with activation of transcription. We have confirmed earlier results demonstrating that during IFN-γ treatment, the histones surrounding the MHC-II X-box site become acetylated (19, 44, 45). However, when RFX5 proteins occupy the collagen transcription start site, the histones near this DNA become less acetylated. This correlates with repression of transcription. Recently, it has been demonstrated that CIITA can interact with histone deacetylases found in co-repressors containing histone deacetylases (HDAC) (45). Therefore, RFX5 may be recruiting CIITA with histone deacetylases at the collagen transcription start site.

RFX5 represses collagen but activates MHC-II indicating that the complex has differential effects on two different genes. Most likely, this is a result of interactions with different proteins on the two promoters and placement of the complex in relationship to the transcription start site. Since the RFX5 complex is interacting with the collagen start site and repressing (Fig. 9), it may be blocking RNA polymerase II. RFX5 complex proteins bind cooperatively with other proteins on the MHC-II promoter, especially nuclear factor-Y (NF-Y) (46, 47). It is clear that NF-Y and RFX5 complex can interact through RFXB (48). It has been suggested that these proteins form a complex before interaction with the MHC-II promoter. Collagen has a clearly defined NF-Y binding site (46) suggesting that NF-Y may cooperate with RFX5 during interaction with DNA. The NF-Y complex along with RFX5 complex interacts strongly with CIITA (49). Indeed, NF-Y proteins co-transfected with CIITA repress, rather than activate, collagen promoter activity, further suggesting that NF-Y may participate in recruitment of CIITA (23). CIITA interacts with other important general transcription factors such as TFIIB, TBP, and certain TAF proteins (17, 18) and may replace TAFII250 on some promoters (50, 51). Since collagen has a TATA box, which can interact with TBP (52), it is possible that these proteins interact with CIITA at the collagen promoter. Since the RFX5 binding site is at the collagen transcription start site, the RFX5 complex may block RNA Pol II recruitment. CIITA on the MHC-II promoter interacts with several other proteins including AP1, CREB and X2BP (27, 33). Similarly, CIITA also interacts with CREB-binding protein (CBP) (21, 23, 53, 54). In

FIG. 8. IFN-γ increases RFX5 complex binding on COL1A2 transcription start site while decreasing acetylated histone H3 and H4 binding in vivo. A, ChIP assays were performed as described under “Materials and Methods.” The real-time PCR reactions were performed using the primers/probe set flanking COL1A2 transcription start site (Table II). Each experiment was repeated three times, and data represent the average ± S.D. After IFN-γ treatment for 24 h, RFX5 complex binding to the COL1A2 transcription start site was significantly increased. (RFX5: p < 0.05, RFXB: p < 0.01, RFXAP: p < 0.05). B, ChIP assays were performed as described in “Materials and Methods.” The real time PCR reactions were performed using the primers/probe set flanking X-box sequences on the MHC II promoter (Table II). Data shown represent the average of two independent experiments. C, IFN-γ decreases histones H3 and H4 acetylation on collagen transcription start site while increases it on X-box of MHC II promoter. Data are shown as averages of three (collagen start site) or two (MHC-II X-box) experiments. After 24 h of IFN-γ treatment, significantly less of both acetylated histones H3 and H4 were precipitated from the COL1A2 transcription start site (p < 0.01).
fact, it has been suggested that CBP may be “squelched” by CIITA when CIITA represses promoter activity (21, 23). In addition, an IFN-γ response element has been described in the COL1A2 promoter (9) and YB-1 has been suggested as the active protein in the binding complex with Sp1/Sp3 (9). It is possible that this complex cooperates with CIITA on the collagen promoter. Recruitment of RFX5 on collagen gene may or may not include cooperative interactions with NF-Y, basic transcription factors and/or YB-1. Therefore, our model (Fig. 9) includes other proteins that bind to collagen such as NF-Y and TAFs that may also interact with CIITA and/or be involved in a cooperative manner to recruit CIITA to the collagen promoter. We hypothesize that RFX5 recruits CIITA to the transcription COL1A2 start site with histone deacetylation. The histone deacetylase then changes the histones to a less transcriptionally active state, possibly causing condensation of the histones to repress collagen gene expression.

In summary (Fig. 9), we have demonstrated that IFN-γ increases the synthesis of RFX5 complex proteins. RFX5 translocation into the nucleus increases with time of IFN-γ treatment. RFXB is produced in a long form, which is more conducive to collagen repression. Most importantly, IFN-γ increases RFX5 complex assembly on both MHC-II promoter and the collagen transcription start site. Therefore, during inflammation when IFN-γ is produced, certain fibroblasts respond by increased levels of RFX5 as well as CIITA, which both activate antigen presentation on membranes and reduce collagen gene expression. This may be a mechanism to decrease scar or fibrosis.

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Interferon γ Repression of Collagen (COL1A2) Transcription Is Mediated by the RFX5 Complex
Yong Xu, Lin Wang, Giovanna Butticè, Pritam K. Sengupta and Barbara D. Smith

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