Our studies indicate that the regulatory factor for X-box (RFX) family proteins repress collagen α2(I) gene (COL1A2) expression (Xu, Y., Wang, L., Buttice, G., Sengupta, P. K., and Smith, B. D. (2003) J. Biol. Chem. 278, 49134–49144; Xu, Y., Wang, L., Buttice, G., Sengupta, P. K., and Smith, B. D. (2004) J. Biol. Chem. 279, 41319–41332). In this study, we examined the mechanisms underlying the repression of collagen gene by RFX proteins. Two members of the RFX family, RFX1 and RFX5, associate with distinct sets of co-repressors on the collagen transcription start site in vitro. RFX3 specifically interacts with histone deacetylase 2 (HDAC2) and the mammalian transcriptional repressor (mSin3B), whereas RFX1 preferably interacts with HDAC1 and mSin3A. HDAC2 cooperates with RFX5 to down-regulate collagen promoter activity, whereas HDAC1 enhances inhibition of collagen promoter activity by RFX1. Interferon-γ promotes the recruitment of RFX5/HDAC2/mSin3B to the collagen transcription start site but decreases the occupancy by RFX1/mSin3A as manifested by chromatin immunoprecipitation assay. RFX1 binds to the methylated collagen sequence with much higher affinity than unmethylated sequence, recruiting more HDAC1 and mSin3A. The DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine, which inhibits DNA methylation, reduces RFX1/HDAC1 binding to the collagen transcription start site in chromatin immunoprecipitation assays. Finally, both RFX1 and RFX5 are acetylated in vivo. Trichostatin A stimulates the acetylation of RFX proteins and activates the collagen promoter activity. Collectively, our data strongly indicate two separate pathways for RFX proteins to repress collagen gene expression as follows: one for RFX5/HDAC2 in interferon-γ-mediated repression, and the other for RFX1/HDAC1 in methylation-mediated collagen silencing.

Collagen, currently consisting of more than 27 members, is a large family of extracellular matrix proteins that play vital structural and physiological roles maintaining the integrity and contributing to homeostasis of the human body (3). Because of their diverse structures and distributions as well as complex interactions with other components of the extracellular matrix, expression and regulation of collagen proteins are extremely complicated, yet critical processes, which occur at multiple levels as follows: transcriptional, post-transcriptional, and post-translational. Type I collagen, composed of two α1(I) (COL1A1) chains and one α2(I) (COL1A2) chain, is the most abundantly expressed member of the collagen genes, thereby playing a significant role in maintaining homeostasis. The transcription of these genes is important during development and repair of injury.

Transcription of eukaryotic genes is controlled by coordination of multiple complexes composed of co-activators/co-repressors or repressors/co-repressors, which function to alter histone-DNA structure in highly ordered chromatin. Histones are subjected to a number of post-translational modifications such as phosphorylation (4–7), acetylation (8–10), and methylation (11–14). These modifications, termed “histone code,” greatly impact the chromatin structure, which in turn leads to permissive or unfavorable access of transcription factors to a particular promoter causing the differential transcriptional activity of that promoter (12, 15). For example, a high level of acetylation on certain lysines on the tail of histones H3 and H4 is often associated with loose chromatin structure, favorable binding of activators/co-activators, and high rates of transcription, whereas a lower level of acetylation is usually accompanied by compact chromatin structure, denial of access of activators/co-activators to promoters, and low rates of transcription (16–19). It is not only the intensity of acetylation/deacetylation but also the specific sites becoming acetylated or deacetylated that dictate the overall transcriptional outcome.

Acetylation of histones is a dynamic and reversible process that is catalyzed by two groups of antagonistic enzymes called histone acetyltransferase and histone deacetylase (HDAC). So far, 18 different HDACs have been identified and categorized into three groups based on their homology to three yeast HDACs. Class I HDACs, containing HDAC1, -2, -3, and -8, are homologous to yeast γ-RPD3 and are expressed in most tissues (20–25). Class II HDACs, which share homology with yeast yHDAC1, consist of HDAC4, -5, -6, -7, -9, and -10 and are expressed in various tissues (26–30). HDAC11 has homology to both class I and II. Both class I and class II HDACs are sensitive to such inhibitors as trichostatin A (TSA) and sodium butyrate. Class III HDACs are mammalian homologs to yeast Sir2 protein that have distinct catalytic domains compared with both class I and II HDACs and are dependent on NAD for enzymatic activity (31, 32). Class III HDACs are insensitive to TSA treatment; instead, their activity can be inhibited by nicotinamide. Class I HDACs are invariably found in multimolecular complexes containing other repressor/co-repressor proteins. For example, HDAC1, -2, -3, and -8 associate with CBP/p300 (28, 29, 32), an enzyme involved in histone acetylation. Class II HDACs have been shown to bind to histone deacetylase interacting protein 1 (HDIP1) and histone deacetylase interacting protein 2 (HDIP2) (33). Class III HDACs associate with histone deacetylase interacting protein 3 (HDIP3) (34, 35).
ple, the NuRD complex is composed of HDAC1, HDAC2, and methyl group binding protein (33, 34). Another commonly found HDAC-containing complex is the Sin3 complex that consists of HDAC1 and HDAC2 in addition to Sin3-associated polypeptides SAP18 and SAP30 (35–37).

The major focus of research at this laboratory has been toward understanding the transcriptional events occurring at the transcription start site of type I collagen genes. Earlier investigations have led to the discovery of a binding site for the regulatory factor for X-box (RFX) at the transcription start site of both α1(I) and α2(I) genes, COL1A1 and COL1A2 (38, 39). Binding of two members of RFX proteins, RFX1 and RFX5, to the start sites of the type I collagen genes represses their expression. RFX1 is able to form dimers with itself as well as with two other RFX members, RFX2 and RFX3, with which RFX1 shares significant homology, including the dimerization domain that mediates the complex formation. On the other hand, RFX5, lacking the dimerization domain, is less homologous to other family members and forms a trimeric complex with two other proteins, RXB and RXAP1 (1). RFX5 is also responsible for recruiting class II transactivator (CIITA), the master regulator for major histocompatibility II (MHC II) expression, to the collagen transcription start site during IFN-γ response (2). Although both RFX1 and RFX5 down-regulate collagen expression, their distinct association with other transcription factors suggests that they are involved in different physiological and pathophysiological events leading to the repression of collagen synthesis. Our results presented here demonstrate that RFX1 and RFX5 differentially interact with class I HDACs, underlying the different pathways when repressing collagen synthesis.

MATERIALS AND METHODS

Cell Culture Maintenance and Treatment Protocols—Human lung fibroblasts, IMR-90 (IMR, NJ), human kidney cells 293FT (Invitrogen), and human fibrosarcoma cells HT1080 (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen). IMR-90 fibroblasts were plated in p150 tissue culture dishes at 4 × 104 cells/dish 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, 16, or 24 h) and/or TSA treatment (0.5–2 μM for 24 h where applicable). IFN-γ and TSA was added together.

In other studies, HT1080 cells were plated at a density of 5 × 104 cells per p35 tissue culture dish and treated with 5-aza-2′-deoxycytidine (aza-dC) (50 nM and 1 μM) for 3 days in DMEM adding fresh aza-dC each day. In some studies HT1080 cells were treated with TSA (300 nM) for 24 h.

DNA Affinity Pull-down Assay—The collagen sequence (COL1A2 −25/+30, GenBank™ accession number AF004877) with a HindIII overhang was synthesized as complementary strands and annealed as described previously (40). Double-stranded collagen DNA was biotin-labeled by incubating with Klenow fragment (New England Biolabs, Beverly, MA) 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 from IMR-90 cells were obtained as described previously using 450 mM sodium chloride (1, 41). The streptavidin beads (Promega, Madison, WI) were washed three times with ice-cold phosphate-buffered saline supplemented with 1 mM PMSF. Nuclear proteins (100–200 μg) were pre-cleared by incubating with the washed beads for 30 min at 4 °C on a shaking platform as described previously (2). Pre-cleared nuclear proteins were prepared by capturing the beads on a magnetic stand and removing the supernatant. The supernatant was then incubated with biotin-labeled collagen DNA probe (−25/+30) for 1 h at room temperature in binding buffer (60 mM NaCl, 20 mM HEPES, pH 7.9, 0.1 mM EDTA, 4% glycerol, 2 mM dithiothreitol) supplemented with bovine serum albumin, poly(dI-dC), and sonicated salmon sperm DNA to remove nonspecific binding. DNA-protein complex formed was captured by the magnetic beads and washed extensively with binding buffer supplemented with 0.01% Triton X 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-polyacrylamide gels.

Plasmids, Transfections, and Luciferase Assays—The COL1A2-luciferase construct (pH2O) (42) contains sequences from −221 to +54 bp of mouse COL1A2 promoter fused to the luciferase reporter gene. Full-length class I HDAC expression constructs (HDAC1, HDAC2, and HDAC3) in pcDNA3 and silencing HDACs (43) were kindly provided by Dr. Edward Seto. Full-length FLAG-RFX5 were kindly provided by Dr. Jenny Ting. RFX1 cDNA was excised from pHisB-RFX-1 plasmid, respectively, and inserted into the pIRE5-hrGFP-2A construct (Stratagene) that has green fluorescent protein-coding sequence. RFX1 plasmid was digested with NotI and XhoI to produce the 5-kb vector along with 3-kb fragment. The RFX1 fragment was inserted into the EcoRI/XhoI site of the pIRE5-hrGFP-2A plasmid. This bicistronic plasmid can encode the protein along with the green fluorescent protein when expressed in mammalian cell lines.

Cells were plated at the density of 3 × 105 cells/well in 6-well tissue culture dishes (for IMR-90 cells) or 5 × 106 cells per p100 tissue culture dish (for 293FT cells). Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were harvested 48 h post-transfection, and luciferase assays were performed with a luciferase reporter assay system (Promega).

Immunoprecipitations—To investigate whether factors interact in vivo, co-immunoprecipitations were performed. Whole cell lysates (IMR-90 or 293FT with transfected constructs as indicated where applicable) were obtained by resuspending cell pellets in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor (Roche Applied Science) and PMSF (100 μg/ml RIPA). Anti-RFX5 (194, Rockland) or anti-RFX1 (I-19; Santa Cruz Biotechnology) antibody was added to and incubated with IMR-90 cell lysate overnight before being absorbed by protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). Precipitated immune complex was released by boiling with 1× SDS electrophoresis sample buffer. Alternatively, FLAG-conjugated beads (M2, Sigma) were added to and incubated with 293FT cell lysate overnight. Precipitated immune complex was eluted with 3% FLAG peptide (Sigma).

Westerns—Proteins were separated by 8 or 10% polyacrylamide gel electrophoresis with pre-stained markers (Bio-Rad) for estimating molecular weight and efficiency of transfer to blots. 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 buffer (TBST; 0.05% Tween 20, 150 mM NaCl, 100 mM Tris-HCl, pH 7.4) at 4 °C overnight and incubated for 3 h to monoclonal anti-FLAG (1:1000) (Sigma), polyclonal anti-RFX5 (194, 1:1000) (Rockland), polyclonal antilmSin3A (K-20, 1:200) (Santa Cruz Biotechnology), polyclonal anti-mSin3B (AK-12, 1:200) (Santa Cruz Biotechnology), anti-HDAC1 (H-51, 1:200) (Santa Cruz Biotechnology), polyclonal anti-HDAC2 (C-19, 1:200) (Santa...
Cruz Biotechnology), polyclonal anti-HDAC3 (H-99, 1:200) (Santa Cruz Biotechnology), polyclonal N-acetyl-l-lysine (1:2000) (Cell Signaling), and polyclonal anti-RFX1 (I-19, 1:100) (Santa Cruz Biotechnology) antibodies at room temperature. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies, either anti-goat IgG (Sigma), anti-mouse IgG, or anti-rabbit IgG (Amersham Biosciences) conjugated to horseradish peroxidase, for another 1 h at room temperature. Then protein blots were visualized using ECL reagent (PerkinElmer Life Sciences) on a Kodak image station (PerkinElmer Life Sciences).

Chromatin Immunoprecipitation (ChIP)—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 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) supplemented with protease inhibitor tablet (Roche Applied Science) and PMSF. DNA was fragmented into ~500-bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-RFX5 (194; Rockland), anti-RFX1 (I-19; Santa Cruz Biotechnology), anti-mSin3A (K-20; Santa Cruz Biotechnology), anti-mSin3B (AK-12; Santa Cruz Biotechnology), anti-HDAC1 (H-51; Santa Cruz Biotechnology), anti-HDAC2 (C-19; Santa Cruz Biotechnology), and anti-HDAC3 (H-99; Santa Cruz Biotechnology) antibodies followed by adsorption to protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). Precipitated DNA-protein complexes were washed sequentially 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, 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 protease 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. The primers surrounding the collagen start site for real time PCR have been described previously (1).

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 transcriptase reactions were performed using a SuperScript first-strand synthesis system (Invitrogen) according to the manufacturer’s protocol. Real time PCRs were performed on a ABI Prism 7700 sequence detection PCR 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 1.

RESULTS

RFX1 and RFX5 Differentially Interact with Class I HDACs on COL1A2 Transcription Start Site—Previously, we reported that two members of the RFX family, RFX1 and RFX5, bind to the transcription start site of the COL1A2 gene and repress its expression (39). During IFN-γ treatment, when RFX5 occupies the COL1A2 transcription start site, there is a coordinate decrease in acetylation of histones (1). Because transcriptional repression is usually associated with the recruitment of HDACs, we examined whether RFX1 and/or RFX5 is responsible for recruiting the HDACs to the collagen site. To this end, DNA affinity pull-down experiments were performed, as described previously (2), with nuclear proteins extracted from human IMR-90 cells and a biotin-labeled double-stranded DNA probe that spans from −25 to +30 of the collagen promoter containing the RFX-binding site. Different DNA oligonucleotides were also used as competitors for RFX binding to test the specificity of the interactions. Streptavidin-conjugated magnetic beads were used to sequester the biotinylated probe with bound nuclear proteins.

After extensive washing, bound proteins were eluted with SDS electrophoresis buffer, separated on SDS-10% PAGE, and transferred to membranes for Western analysis using specific antibodies for RFX and HDAC family members. No proteins were present in the eluates without a DNA probe (Fig. 1, A, lane 2, and B, lane 3). In the presence of the DNA probe, RFX1 is detected in the eluates along with HDAC1 (Fig. 1, A, lane 3, and B, lane 4). When a methylated sequence, which acts as a
Interactions between RFX Proteins and HDACs Are DNA-independent—Next, co-immunoprecipitation experiments were performed between RFX proteins and HDACs to determine whether interactions depend on DNA. The first immunoprecipitation experiments were performed using epitope-tagged RFX5 and HDAC-expressed proteins in 293FT cells because they are fast-growing human kidney cells that stably express the large T antigen of SV40 allowing increased amounts of overexpressed protein. FLAG-conjugated beads were used to precipitate protein extracts from 293FT cells transfected with either FLAG-tagged RXF5 or HDAC2. Eluates were separated by SDS-polyacrylamide gels and examined for endogenous proteins that might co-precipitate with FLAG-tagged proteins. As depicted in Fig. 2A, FLAG-RFX5 co-precipitates with endogenous HDAC2 (left panel) and vice versa (right panel), indicative of a reciprocal interaction. In both cases, mSin3B is present in the immune complex.

Next, nuclear proteins from IMR-90 cells treated with or without IFN-γ were precipitated using an anti-RFX5 or anti-HDAC2 antibody to examine endogenous interactions. Eluates were separated by SDS-polyacrylamide gels and examined for endogenous proteins that might co-precipitate with FLAG-tagged proteins. As depicted in Fig. 2A, FLAG-RFX5 co-precipitates with endogenous HDAC2 (left panel) and vice versa (right panel), indicative of a reciprocal interaction. In both cases, mSin3B is present in the immune complex.

Finally, to examine endogenous interactions, RFX1 was immunoprecipitated from IMR-90 nuclear extracts with an anti-RFX1 antibody. Proteins in the supernatant and precipitate were separated by SDS-polyacrylamide gels and examined for endogenous proteins that remain in the supernatant or co-precipitate. A considerable fraction of HDAC1 co-precipitates with RFX1 (Fig. 2C). In contrast to RFX5, RFX1 did not co-precipitate with HDAC2 (Fig. 2C). These data suggest that RFX proteins probably associate with HDAC before recruiting them to the collagen transcription start site.
RFX Proteins Interact with HDACs

Class I HDACs Display Distinct Functions Regulating COL1A2 Promoter Activity—Because there were interactions between RFX proteins and HDACs, we examined whether these interactions bear any functional significance. Transient transfections were performed in IMR-90 cells with different HDAC expression constructs with or without RFX expression plasmids. As shown in Fig. 3, both HDAC1 and HDAC2 repress the collagen promoter activity, but HDAC3 does not significantly alter COL1A2 promoter activity, which is in accordance with our binding data (Fig. 1). RFX5 represses better with HDAC2 (Fig. 3A). Similarly, RFX1 represses better with HDAC1 (Fig. 3B).

Next, sHDACs (43) were transfected with or without RFX expression constructs (Fig. 3, C and D). Silencing either HDAC1 or HDAC2, but not HDAC3, activated collagen promoter activity. Surprisingly, without these HDAC enzymes, RFX5 also further activated the collagen promoter (Fig. 3C). RFX1 remained a repressor with sHDAC2 and sHDAC3 but lost its ability to repress collagen promoter without HDAC1. This further demonstrates the different functional interactions between the RFX family members.

RFX5/HDAC2/mSin3B Are Involved in IFN-γ-mediated Collagen Transcriptional Repression—Because there is differential association between RFX proteins and HDACs, both physically and functionally, we hypothesized that RFX5/HDAC2 and RFX1/HDAC1 might be involved in different mechanisms responsible for collagen repression. Our previous reports suggest that RFX5 complex might be responsible for mediating IFN-γ repression of collagen transcription (1, 2). Because certain co-repressor proteins were associated with RFX5 on the collagen start site in vitro in DNA affinity pull-down assays (Fig. 1) and the association could be increased by IFN-γ (Fig. 1B), it was hypothesized that these repressors might be recruited to the collagen site by RFX5 during IFN-γ response in vivo to repress collagen expression. Chromatin immuno-precipitation (ChIP) assays were performed with anti-RFX5, RFX1, HDAC1, HDAC2, HDAC3, mSin3A as well as mSin3B antibodies in IMR-90 cells treated with IFN-γ for 0, 8, 16, or 24 h.

Similar amounts of genomic DNA surrounding the COL1A2 transcription start site is precipitated by either RFX5 or RFX1 antibody (Fig. 4A). During IFN-γ treatment, however, binding of RFX5 to the collagen sequence is greatly enhanced, but RFX1 binding is gradually and slightly decreased, suggesting that RFX5, but not RFX1, is involved in IFN-γ-mediated collagen repression.

More DNAs are precipitated by mSin3A binding to the collagen start site than mSin3B before IFN-γ is added (Fig. 4B, compare 0 h mSin3A to 0 h mSin3B). Although binding of mSin3B increases with time during IFN-γ treatment, mSin3A occupancy is continuously decreased. By 24 h of IFN-γ treatment, mSin3A binding is decreased to the level of mSin3B binding before IFN-γ treatment, whereas binding of mSin3B is stimulated to the level of mSin3A binding at the beginning of IFN-γ treatment. In other words, it appears as though IFN-γ induces an exchange of mSin3A for mSin3B on the collagen transcription start site.

Meanwhile, when the occupancy of HDACs was examined, it was discovered that HDAC2 was the predominant form of histone deacetyl-
lase present on the collagen transcription start site before IFN-γ treatment. Binding of HDAC1 and HDAC3 was minimal compared with the binding of HDAC2, although the amount of DNA precipitated by either anti-HDAC1 or anti-HDAC3 antibody was significantly above control levels (Fig. 4C).

RFX1/HDAC1/mSin3A Are Involved in Methylation-mediated Collagen Transcriptional Repression—Our recent studies indicate that collagen expression is greatly decreased when the gene is methylated (44) and that RFX1 binding to the collagen site is methylation-sensitive (38, 40), raising the possibility that RFX1 might be involved in methylation-mediated collagen repression by recruiting co-repressors to the collagen transcription start site. To examine the validity of this hypothesis, DNA affinity pull-down experiments were performed using either a regular biotinylated COL1A2 DNA probe (−25 to +30) or the same probe that was methylated at the +7 site that enhances the binding of RFX1 to the collagen transcription start site (40). As depicted in Fig. 5A, binding of RFX1 was much more robust on the methylated probe than on the unmethylated probe as expected. Interestingly, both HDAC1 and mSin3A bind to the methylated probe more strongly just like RFX1, supporting the notion that during methylation of collagen DNA more RFX1 is able to bind to the transcription start site and recruit more co-repressor complexes to repress collagen transcription. No RFX5 binding is detectable under these conditions.
The inhibitor, aza-dC, a compound that inhibits DNA methyltransferases, dramatically (40-fold) increases collagen gene expression in a fibrosarcoma cell line, HT1080, in which 50% of collagen genomic DNA is methylated (38, 44). Therefore, we examined whether the effect of aza-dC is mediated through diminished binding of RFX1 as well co-repressors to the collagen transcription start site because of demethylation. ChIP assays were performed with HT1080 cells treated with different concentrations of aza-dC for 72 h using anti-RFX1, HDAC1, as well as HDAC2 antibodies. RFX1 binding is decreased with aza-dC treatment in a dose-response manner by up to 60% (Fig. 5B). Interestingly, HDAC1 binds to the partially methylated collagen transcription start site in vivo much more strongly than HDAC2 (Fig. 5C). Binding of HDAC1 is also greatly decreased by aza-dC treatment similar to RFX1, further confirming that RFX1/HDAC1 might be involved in DNA methylation-mediated collagen transcriptional repression.

TSA Has Differential Cell-specific Effects on Steady State mRNA Levels—Because HDACs are clearly involved in the repression of collagen transcription, we postulated that inhibition of overall HDAC activity would increase collagen transcription. TSA is a general inhibitor for class I and II HDACs that alters expression of many genes presumably through histone deacetylation. First, HT1080 cells were treated with several doses of TSA (50, 100, 500, and 1000 nM) for 24 h. There was no change in collagen mRNA levels at low doses. At higher doses of TSA there was a 5- (500 nM) or 10-fold (1000 nM) increase in collagen mRNA (Fig. 6A).

On the other hand, when IMR-90 cells were treated with TSA (500 nM), there was no significant increase in collagen mRNA levels (Fig. 7B). IFN-γ repressed collagen mRNA levels by 50% in 24 h. TSA partially reversed the repression of collagen mRNA expression.

The mRNA steady state levels are a combination of transcription and degradation or processing of mRNA. In order to examine early tran-
scription, primers within the second intron and second exon (Table 1) were used to measure heterogeneous RNA transcripts. Total RNA was DNase-treated, and no reverse transcriptase was used as controls to be sure that RNA, not DNA, was measured by these primers. TSA treatment increased transcription of heterogeneous collagen mRNA more than steady state mRNA in the IMR-90 cells (Fig. 7C), suggesting that TSA increases transcription and degradation or processing of mRNA.

IFN-γ repressed collagen heterogeneous RNA to the same extent as steady state mRNA.

TSA Stimulates Collagen Promoter Activity and Partially Blocks Repression by RFX Proteins—Even though TSA effects on collagen mRNA were low, TSA activated collagen promoter-luciferase activity in IMR-90 cells (Fig. 8A). Overexpression of RFX1 and RFX5 repressed collagen synthesis, and TSA treatment was able to partially block this repression (Fig. 8B). RFX5 seemed to be more sensitive to TSA than RFX1 because TSA (500 nM) almost completely blocked repression by RFX1 overexpression.

RFX Proteins Are Acetylated by TSA Treatment—Although HDACs are active on histones, several acetylated transcription factors are deacetylated by HDACs. To determine whether RFX proteins are acetylated, IMR-90 cells were treated with 1 μM TSA, and the acetylation of either RFX5 or RFX1 was examined by immunoprecipitation followed by Western blot with a specific anti-lysine antibody. Most intriguingly, both RFX5 (Fig. 7C) and RFX1 (Fig. 7D) are acetylated in vivo, and their acetylation is dramatically stimulated by TSA treatment within 24 h.

DISCUSSION

Previously, we demonstrated that there is a binding site for the RFX family of transcription factors within the first exon (−1 to +20) of the COLIA2 gene (40). Two members of the RFX family, RFX5 and RFX1, bind to this site and repress collagen expression (39). RFX1 homodimers and RFX1/RFX2 heterodimers bind to the collagen gene with higher affinity especially when it is methylated on the coding strand (38–40, 44, 45). In this study, it is clear that RFX1 interacts on the collagen start site with HDAC1 by DNA affinity precipitation (Fig. 1). This interaction of RFX1 and HDAC1 with collagen DNA is increased when the CpG site within the RFX consensus sequence is methylated (Fig. 5A). In normal fibroblasts, there is a small but measurable amount of RFX1 binding within chromatin as judged by ChIP assays (Fig. 4A). Collagen type I genes are methylated in DNA from certain cancer lines and in colorectal tumors (44). The collagen gene in HT1080 cells is 50% methylated at the CpG within the RFX1-binding site (44, 46). RFX1 occupies the site 2-fold more in HT1080 cells than in IMR-90 cells (data not shown). In HCT116 cells that have higher methylation status of the collagen gene, RFX1 occupancy is further increased (data not shown).
shown), suggesting that RFX1 does interact more with the collagen start site when the gene is methylated. Aza-dC, which decreases methylation by inhibiting DNA methyltransferases, also decreases the occupancy of RFX1 as well as HDAC1 on the collagen start site (Fig. 5). These data again suggest that RFX1 and HDAC1 interact at the DNA within chromatin. In fact, RFX1 also co-immunoprecipitates with HDAC1, but not HDAC2 or HDAC3, suggesting that these protein-protein interactions occur independent of RFX1-DNA interactions.

On the other hand, RFX5 interacts with two other RFX proteins, RFXB and RFXAP, as well as CIITA to repress collagen during IFN-γ/H9253 stimulation (1, 2). This study demonstrates that RFX5 occupies the collagen gene, and RFX5 occupancy increases with IFN-γ treatment as RFX1 occupancy decreases (Fig. 4A). HDAC2, but not HDAC1 or HDAC3, also increases on the collagen gene with time of IFN-γ treatment (Fig. 4B) suggesting that HDAC2 may be responsible for methylation, whereas RFX5 may be responsible for IFN-γ-mediated collagen down-regulation.

CIITA is also recruited to the collagen transcription start site by RFX5 with time of IFN-γ treatment (2). Earlier studies demonstrate that CIITA interacts with HDAC1 and mSin3A to terminate MHC II activation (47, 48). However, this investigation suggests that HDAC2 and Sin3B occupy the collagen transcription start site during IFN-γ treatment (Fig. 4B). Our preliminary results suggest that CIITA can complex with HDAC2 and Sin3B (data not shown). Although our effort so far has been concentrated on RFX5 and CIITA, recent evidence has been published that the other two members of the RFX complex, RFXB and RFXAP, are both capable of interacting with certain histone-modifying proteins.

**TABLE 1**

| Gene           | Amplicon location | Exon       | Sequences                                      |
|----------------|-------------------|------------|------------------------------------------------|
| COLIA2 mRNA*  | 7568–7650/8929–8965 | Exon 5     | 5’-GCCGCCAGGCCAGADA-3’                         |
| TaqMan probe   |                   | Exon 5/6   | 6FAM-CCTTGTCCTGGTGGGAACTTGTGCTG-TAMRA*         |
| Reverse primer |                   | Exon 6     | 5’-CCAACCTTTTTCCATCTGACTGA-3’                  |
| COLIA2 hnRNA*  | 2468–2552         | Exon 1     | 5’-CTGAGTAACCTTAGGCACCA-3’                     |
| Forward primer |                   | Exon 1/Intron 1 | 6FAM-CATGCAGATTAGTGCTACGTTGTT-TAMRA*         |
| TaqMan probe   |                   | Intron 1   | 5’-CCCATCTAACTCTTACCCAGTCT-3’                  |

* GenBank accession number AF004877.

The TaqMan probes contained a fluorescent dye (FAM) and a quencher (TAMRA).

**FIGURE 8. Model for RFX family repression of collagen gene expression.** A schematic representation illustrating two distinct pathways for collagen repression by RFX1 and RFX5. RFX1 is in a complex with HDAC1 and Sin3A. Methylation of the collagen gene at CpG +7 activates RFX1 interaction with the collagen gene bringing HDAC1 to the site. RFX5 differentially interacts with HDAC2 and Sin3B. IFN-γ treatment increases expression of RFX5 complex proteins and CIITA allowing increased interaction with HDAC2/Sin3B as well. Once the RFX5 complex is assembled, it interacts with CIITA which may also interact with HDACs. The drawing at the bottom represents some possible interactions showing proteins such as TBD, TIFF, and NFI that can interact with CIITA. The collagen promoter cis-acting consensus sites are designated (TATA, TATA box, or TFIID-binding site, CCAAT, CBF/NF-Y-binding site (78)).
RFX Proteins Interact with HDACs

Y. Xu, P. K. Sengupta, E. Seto, and B. D. Smith, unpublished results.

The actual increased acetylation of RFX1 and RFX5 proteins with TSA treatment suggests that the mechanism for repression of collagen through the RFX family may require active deacetylase activity of HDAC. The role of acetylation of RFX family needs to be further analyzed. Protein acetylation of transcription factors has been implicated in multiple cellular processes, including differentiation, metabolism, proliferation, and survival against stress (59–62). NF-κB, c-Myc, Sp3, p53, FOXO, GATA, and SMAD7 have all been identified as targets for acetylation (61, 63–66). For example, acetylation of SMAD7 is involved with stabilizing the protein (67). On the other hand, acetylation of p53 is required for efficient recruitment of co-activators to promoter regions as well as activation of target genes (68–70). Transcriptional output mediated by FOXO is controlled via a two-tiered mechanism of phosphorylation and acetylation (71). FOXO and RFX family have similar winged helix DNA binding domains (72) so they could be regulated in a similar manner by acetylation. The acetylation may alter the interaction of RFX1 with co-repressors or with DNA. The overall consequence of acetylation is not well understood and seems to vary from one factor to another and to depend on specific circumstances.

The roles of HDACs in several diseases, especially in lung inflammation (73, 74) and in cancer (75), have been recently outlined. HDAC2 specifically may decrease in chronic obstructive pulmonary disease patients causing corticosteroid resistance (74, 76). HDAC1 has been associated with certain types of breast cancer (77). These studies point out the biological significance of acetylation and deacetylation and the need to investigate the mechanism of action on individual genes associated with these diseases.

In summary, the RFX family represses collagen transcription at the transcription start site through either methylation-specific binding by RFX1–3 family members or through IFN-γ-stimulated binding during inflammation by the RFX5–CIITA complex (Fig. 8). In each case, a specific co-repressor complex most likely contains RFX family members. RFX1 interacts best with HDAC1 and mSin3A, whereas RFX5/CIITA interacts with HDAC2 and mSin3B on the collagen transcription start site. During IFN-γ treatment, RFX5 synthesis, translocation to the nucleus, and complex formation are increased (1). CIITA is expressed and recruited to the collagen start site with RFX5 (2). Most likely RFX5 becomes deacetylated by HDAC2, which increases complex interactions and repressor activity of RFX5. If the collagen gene is methylated, RFX1 interacts with the collagen gene and represses transcription through HDAC1 deacetylation of chromatin. This study reveals that different members of the same family of transcription factors repress collagen transcription through similar but distinct mechanisms.

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A new role for RFX family proteins in transcriptional control has been revealed. RFX Proteins Interact with HDACs (BRG1), an ATPase-dependent chromatin remodeling molecule, to regulate MHC II expression (49). RFXANK is a binding partner for HDAC4, although the biological significance of this interaction remains unclear (50). Therefore, there might be extensive interactions between histone-modifying proteins and RFX-associated transcription factors. This may explain why silencing of any one of the class I HDACs still caused an increase in collagen promoter activity. Certainly other classes of HDACs, such as HDAC4 discussed above, could be involved in collagen repression through the RFX complex.

Co-repressor complexes have been isolated containing several HDACs with individual transcription factors have been noted especially in the Sp1/kruppel zinc finger family (51–54). HDACs interact with Sp1 protein without activation of the enzyme activity by blocking access of the Sp1 protein to the DNA (53). Collagen is an Sp1-activated promoter, and therefore, TSA cannot activate a gene silenced by complete methylation. However, one of the cell lines, PLC/PfR, had no detectable collagen expression. Our investigations on this cell line indicate that the collagen gene is very highly methylated, and therefore, TSA cannot activate a gene silenced by complete methylation. On the other hand, TSA can induce collagen gene expression in a variety of cancer cell lines with partially methylated collagen genes such as breast cancer cell line, MCF7, or colorectal cancer cell line, HCT116.

Our studies also indicate that in fibroblasts TSA produces no significant changes in steady state mRNA levels. However, this seems to be caused by a combination of increased transcription with accompanying increases in degradation and/or processing. Others have demonstrated that TSA suppresses collagen synthesis in dermal fibroblasts and hepatic stellate cells (56–58). Although steady state mRNA and protein levels of collagen decrease after TSA treatment, nuclear run-on studies indicated that transcription of type I collagen actually was activated thus supporting our results with heterogeneous nuclear RNA (58). The decrease in collagen found by these investigators is most likely due to indirect mechanisms involving increased degradation or processing of collagen mRNA and protein because of multiple changes in gene expression from TSA inhibition of HDACs.

The actual increased acetylation of RFX1 and RFX5 proteins with TSA treatment suggests that the mechanism for repression of collagen through the RFX family may require active deacetylase activity of HDAC. The role of acetylation of RFX family needs to be further analyzed. Protein acetylation of transcription factors has been implicated in multiple cellular processes, including differentiation, metabolism, proliferation, and survival against stress (59–62). NF-κB, c-Myc, Sp3, p53, FOXO, GATA, and SMAD7 have all been identified as targets for acetylation (61, 63–66). For example, acetylation of SMAD7 is involved with stabilizing the protein (67). On the other hand, acetylation of p53 is required for efficient recruitment of co-activators to promoter regions
Regulatory Factor for X-box Family Proteins Differentially Interact with Histone Deacetylases to Repress Collagen \( \alpha2(I) \) Gene (COLIA2) Expression

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