Y-box-binding Protein YB-1 Mediates Transcriptional Repression of Human α2(I) Collagen Gene Expression by Interferon-γ*

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We have demonstrated previously that a proximal element within the human α2(I) collagen gene (COL1A2) promoter mediates transcriptional repression by interferon-γ (IFN-γ), and designated this region the IFN-γ response element (IgRE). Screening of a human fibroblast cDNA expression library with a radiolabeled IgRE probe exclusively yielded clones with a sequence identical to that of the transcription factor YB-1. Electrophoretic mobility shift assays (EMSA) using various IgRE-derived oligonucleotide probes containing serial two-base mutations showed that YB-1 protein was preferentially bound to the pyrimidine-rich sequence within the IgRE. This region is located immediately downstream of and partly overlaps the previously reported Sp1/Sp3 binding site. Overexpression of YB-1 in human dermal fibroblasts decreased steady state levels of COL1A2 mRNA and repressed COL1A2 promoter activity in a dose-dependent manner. This inhibitory effect of YB-1 on COL1A2 expression was abolished by mutations of the IgRE shown to prevent YB-1 binding in EMSA. In addition, these mutations also abolished the inhibitory effect of IFN-γ, suggesting that YB-1 mediates the inhibitory effect of IFN-γ on COL1A2 promoter through its binding to the IgRE. Also, overexpression of a deletion mutant YB-1, which lacks the carboxyl-terminal domain, abrogated the repression of COL1A2 transcription by IFN-γ. A functional correlation between IFN-γ and YB-1 was further supported by luciferase assays using four tandem repeats of the Y-box consensus oligonucleotide linked to a minimal promoter. EMSA and Western blot analysis using cytoplasmic and nuclear proteins implied that IFN-γ promotes the nuclear translocation of YB-1. Direct evidence for the nuclear translocation of YB-1 by IFN-γ was further provided by using a YB-1-green fluorescent protein expression plasmid transfected into human fibroblasts. Altogether, this study represents the definitive identification of the transcription factor responsible for IFN-γ-elicited inhibition of COL1A2 expression, namely YB-1.

These cytokines have been shown to control connective tissue cell recruitment and proliferation, as well as synthesis and degradation of the extracellular matrix components (2). Disruption of this equilibrium, leading to excessive collagen deposition, is the hallmark of interstitial fibrotic diseases. Type I collagen mRNA levels are increased in experimental and clinical fibrotic states (3, 4), which implies a regulation at the transcriptional level. The role of transforming growth factor-β (TGF-β) as the principal factor inducing collagen gene expression and leading to tissue fibrosis has been suggested by the observations that (a) TGF-β expression often parallels increased type I collagen gene expression (4, 5), and (b) a soluble TGF-β receptor prevents collagen accumulation in experimental fibrosis (6).

Several studies have previously indicated possible mechanisms by which tumor necrosis factor-α inhibits α2(I) collagen gene (COL1A2) expression at the transcriptional level (7–9). Tumor necrosis factor-α has also been demonstrated to antagonize the effects of TGF-β through induction of inhibitory Smad7 (10) or AP-1 components (11). In contrast, identification of the transcription factors involved in interferon-γ-elicited down-regulation of COL1A2 expression has been elusive, except that a cross-talk between TGF-β/Smad and IFN-γ/Stat1 signaling pathways has recently been implicated in antagonistic regulation of gene transcription (12). We have previously identified a proximal element within the human COL1A2 promoter, spanning nucleotide −161 to −150, that mediates transcriptional repression by IFN-γ. We designated this region the IFN-γ response element (IgRE) (13). UV cross-linking experiments using nuclear extracts prepared from IFN-γ-treated fibroblast cultures indicated that two DNA-protein complexes were formed with the IgRE (13). Interestingly, others have shown that Sp1 and Sp3 bind to a TCCCCC motif located between −164 and −159 (14), immediately upstream of and partly overlapping the IgRE.

In the present study, we attempted to characterize the human fibroblast nuclear protein that interacts with the IgRE by screening of a human fibroblast cDNA expression library. We have identified the transcription factor YB-1 as a component of the COL1A2 transrepressing complex bound to the IgRE. Co-transfection studies using a YB-1 expression vector and reporter constructs containing the wild-type and mutated

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1 The abbreviations used are: TGF-β, transforming growth factor-β; COL1A1, α1(I) collagen gene; COL1A2, α2(I) collagen gene; IFN-γ, interferon-γ; IgRE, IFN-γ response element; FCS, fetal calf serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assays; GFP, green fluorescence protein; CMV, cytomegalovirus; RT-PCR, reverse transcriptase PCR.

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COL1A2 IgRE have confirmed the role of YB-1 as a negative regulator of COL1A2 transcription mediating the effect of IFN-γ.

EXPERIMENTAL PROCEDURES

Screening of cDNA Expression Library—A Sgt 11 human dermal fibroblast cDNA library (Clontech) was screened by Southern blotting using the 32P-labeled three repeats of the IgRE oligonucleotide spanning −161 to −150 of the human COL1A2 promoter as described previously (15). The filters were subjected to a cycle of denaturation with 6 μg sodium hydroxide followed by renaturation before screening. After incubation with 5% skim milk in the binding buffer (10 mM Tris-HCl, pH 7.5, 75 mM NaCl, 1 mM MgCl2, 0.1% dithiothreitol) for 30 min at 4 °C, the filters were probed in the binding buffer containing 106 cpm/ml probe, 10 μg/ml denatured salmon sperm DNA, and 0.25% powdered milk. The positive clones were sequenced directly using Sgt 11 primers (Takara Biomedicals, Kyoto, Japan).

Plasmids—Point mutations were introduced into the YB-1 binding sites using mutational polymerase chain reaction (PCR) as described previously (16, 17). The constructs −161Mfuc and −161Mfuc containing two-base pair substitution mutations (5′-CCCCATCTGGCCTC-3′ to 5′-CGGATGCTGCTC-3′ and 5′-CCCATCGCCCCGC-3′, respectively) were prepared by inserting the mutated promoter sequences into a firefly luciferase gene vector, pGL3 basic vector (Promega, Madison, WI). Four copies of the Y-box consensus oligonucleotide (TCTATTGGGCTAA) linked to a minimal promoter containing only a TATA box were cloned into pGL3 basic vector. A YB-1 expression plasmid, YB1-RSV, was constructed by ligating the entire YB-1 coding sequence into the HindIII/XhoI sites of pRc/RSV vector (Invitrogen). A deletion mutant YB-1 expression plasmid, which lacks the carboxyl terminus, was constructed by ligating the corresponding sequence into the BamHI/XhoI sites of pcDNA3.1(+) vector (Invitrogen). The pCMX-YB1-1-FGP was prepared by ligating the YB-1 sequence into the HindIII/PseI sites of pCMX-hGR-GFP (kindly provided by Dr. H. Ogawa, Kyoto University, Kyoto, Japan) (18). The sequences of all plasmids were verified by automated sequencing (Applied Biosystems, Foster City, CA).

Cell Culture and Transient Transfection—Normal human dermal fibroblasts (Clontech, Palo Alto, CA) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS). Transient transfections were performed using the LipofectAMINE Plus reagent (Invitrogen), 100 units/ml IFN-γ was added into the culture medium and incubated for another 6 h. After removing the media, the cells were washed with phosphate-buffered saline and examined under a microscope (Nikon, Tokyo, Japan) equipped with a fluorescence isothiocyanate filter set for fluorescence detection (18).

Statistical Analysis—Values were expressed as mean ± S.D. Student’s t test was used to evaluate the statistical differences between groups, and a p value of less than 0.05 was considered significant.

RESULTS

Isolation of YB-1 by Southern Screening of a Human Dermal Fibroblast cDNA Expression Library—A human dermal fibroblast cDNA library cloned into Sgt 11 was screened using three tandem repeats of the IgRE (−161 to −150) as a probe. After screening −5 × 106 plaques, we obtained three positive clones. Direct sequence analyses of the three clones revealed overlapping subregions of the cDNA encoding YB-1, a member of the Y-box transcription factor family (data not shown). The COL1A2 IgRE sequence aligned in 7 of 12 bases with the consensus Y-box sequence (Fig. 1A).

Recombinant YB-1 Binds to the IgRE—Although YB-1 was identified originally as a transcription factor bound specifically to the Y-box sequence containing a CCAAT motif (25), it has also been shown to bind to pyrimidine-rich oligonucleotides that can adopt an intramolecular triplex, single-stranded structure (26, 27). The IgRE within the human COL1A2 promoter possesses both the Y-box-like element and the pyrimidine-rich sequence (Fig. 1A). To examine the binding specificity of YB-1 to the IgRE, a (His)6-YB-1 fusion protein was expressed in E. coli and used for gel mobility shift assays. Various double-stranded oligonucleotides containing serial two-base mutations or single-stranded oligonucleotides were used as probes to determine the recognition targets of YB-1 (Fig. 1B). As shown in Fig. 1C, recombinant YB-1 effectively bound to both the double-stranded (ds WT) and the sense single-stranded (ss WT) IgRE, whereas complex formation was not observed between YB-1 and the antisense single-stranded (ss AS WT) IgRE. To further characterize DNA sequences involved in complex formation between YB-1 and double-stranded IgRE, a series of oligonucleotides containing substitution mutations were used as probes in EMSA. As shown in Fig. 1C, M1 and M5 oligonucleotide probes completely failed to form the YB-1-DNA complex, and M3 and M4 probes showed markedly diminished complex formation. In contrast, introduction of mutations into A160C and A160T (M2) had no effect on the binding of YB-1 to the IgRE. These results suggest that both −160C−159C and −156T−155C bases were most essential for YB-1 binding to the IgRE.

Expression and Purification of Recombinant Protein—Recombinant YB-1 protein was produced using the pET system (Novagen, Madison, WI) as described previously (19). Briefly, after transformation of Escherichia coli TOP10 strain (Invitrogen) with pET15b-YB1, the 1525C and 1525T single-stranded oligonucleotide (ss-2) was added into LB medium at 0.5 A600 followed by incubation for another 3 h. The induced cells were collected and sonicated until no longer viscous. The supernatant was applied to a nickel-nitrilotriacetic acid-agarose column (Qiagen, Hilden, Germany), and recombinant (His)6-YB-1 was eluted with the buffer containing 200 mM imidazole. Purity of the expressed YB-1 fusion product was ascertained by analytic SDS polyacrylamide gel electrophoresis (PAGE).

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear and cytoplasmic extracts were prepared according to the method of Andrews and Faller (20). For EMSA, the probes (−50,000 cpm) were incubated with recombinant YB-1 protein or nuclear extracts for 30 min on ice in 2 μl of binding reaction buffer as described previously (21). For competition experiments, 20–500-fold molar excess of unlabeled IgRE or consensus sequences for YB-1 (22) and Sp1/Sp3 (23) were added to the binding reaction. In some experiments, antibody interference assays were performed by preincubating nuclear extracts with 2 μg of anti-YB-1 polyclonal antibody prepared against the 15-amino acid peptide residues (299–313) of YB-1 as previously reported (24) or with anti-Sp1 and anti-Sp3 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Western Blot Analysis—Twenty micrograms of nuclear proteins or 40 μg of cytoplasmic proteins were separated on a 7.5% SDS-polyacrylamide gel, electroblotted, and incubated with anti-YB-1 antibodies. ECL detection system (Amersham Biosciences) was used to detect immunoreactive proteins.

Fluorescence Microscopy—Thirty-six hours after transfection with 1 μg of pCMV-YB1-GFP using the LipofectAMINE Plus reagent (Invitrogen), 100 units/ml IFN-γ was added into the culture medium and incubated for another 6 h. After removing the media, the cells were washed with phosphate-buffered saline and examined under a microscope (Nikon, Tokyo, Japan) equipped with a fluorescein isothiocyanate filter set for fluorescence detection (18).
binding of Endogenous YB-1 and Sp1/Sp3 to the IgRE—To determine whether endogenous YB-1 binds to the IgRE, we next performed EMSA using nuclear extracts prepared from human fibroblasts. As shown in Fig. 2A, incubation of nuclear extracts with double-stranded IgRE probe yielded at least four retarded bands (arrows 1–4), all of which were diminished by the addition of increasing amounts of unlabeled double-stranded competitor. Interestingly, although formation of the three slowly migrating complexes (arrows 1–3) was completely abolished by adding a 20-fold molar excess of unlabeled double-stranded competitor, the faster migrating complex (arrow 4) was still observed even after adding a 500-fold molar excess of the competitor. These results suggested that the binding affinities of those nuclear proteins to the IgRE are different from each other. In addition, unlabeled sense single-stranded IgRE interfered with the formation of complex 4 in a dose-dependent manner (Fig. 2A), whereas it did not affect complexes 1–3. On the other hand, antisense single-stranded IgRE as a competitor failed to diminish the formation of complex 4 (data not shown). These results clearly demonstrated that the nuclear factor(s) forming complex 4 preferentially bind to both the double- and the sense single-stranded IgRE. Considering the results shown in Fig. 1C, we then tested the possibility that YB-1 interacts with the IgRE to form complex 4. For this purpose, we generated anti-YB-1 antibodies against the carboxyl terminus of YB-1, which inhibited the binding of recombinant YB-1 to the IgRE (Fig. 2B). Preincubation of nuclear extracts with anti-YB-1 antibodies resulted in significant but partial inhibition of the complex 4 formation (Fig. 2B). With regard to the slowly migrating complexes 1–3, a previous study indicated the binding of Sp1/Sp3 to the −164 to −159 COL1A2 sequence, immediately upstream of and partly overlapping the IgRE (23). Consistent with their results, the formation of complex 1 was interfered with by anti-Sp1 antibodies and that of complexes 2 and 3 was abolished by adding anti-Sp3 antibodies (Fig. 2B). Furthermore, in agreement with the results of Fig. 1C, M1 and M5 oligonucleotides as a probe hardly formed the YB-1-IgRE complex (Fig. 2C). Interestingly, interruption of YB-1 binding to the IgRE augmented the binding of Sp1 and Sp3 to the IgRE (Fig. 2C).

YB-1 Suppresses COL1A2 Promoter Activity via the IgRE—To confirm the transcriptional repression of COL1A2 expression by YB-1, we first performed real-time RT-PCR assay using Taqman probes. As shown in Fig. 3A, expression of YB-1 suppressed endogenous COL1A2 mRNA levels in a dose-dependent manner. We then examined whether the binding of YB-1 to the IgRE is necessary for the regulation of COL1A2 promoter activity. To this end, we transfected human dermal fibroblasts with either the wild-type −161WT or with mutated −161M1 and −161M5 reporter plasmids together with a YB-1 expression plasmid. The basal transcription level of −161M1 was significantly higher than that of −161WT, whereas the basal transcription level of −161M5 was not statistically different from that of −161WT (Fig. 3B). Overexpression of YB-1 significantly decreased transcription of −161WT construct by about 50% in human dermal fibroblasts. In contrast, the promoter activities of both the −161M1 and −161M5 constructs, which lack the YB-1 binding sequence within the IgRE, were not affected by overexpression of YB-1 (Fig. 3B). Taken together, these results suggest that YB-1 is able to suppress COL1A2 gene expression and that the inhibitory action is exerted via a promoter region identified previously as the IgRE (13).

YB-1 Mediates IFN-γ-elicted Repression of COL1A2 Transcription—The results described above led us to investigate the role of YB-1 in mediating the inhibitory effect of IFN-γ on COL1A2 promoter activity. We first performed real-time RT-PCR assays to confirm the transcriptional repression of COL1A2 by IFN-γ. As shown in Fig. 3C, IFN-γ significantly suppressed endogenous COL1A2 mRNA levels in a dose-dependent manner. On the other hand, the levels of endogenous YB-1 mRNA remained unchanged, indicating that IFN-γ-elicted inhibition of COL1A2 transcription is independent of de novo synthesis of YB-1. Then, the mutated constructs −161M5 and −161M6, as well as their wild-type counterpart −161WT, were used in transient transfection experiments. Consistent with the results of our previous study (13), IFN-γ exerted a significant transcriptional inhibition of the wild-type −161WT construct by about 50%. In contrast, the point mutations introduced into both −160C−159C nucleotides (−161M6) and −155T−154C nucleotides (−161M5) prevented the COL1A2 response to IFN-γ (Fig. 3D). These results clearly demonstrate that the YB-1 binding site within the 12-base-pair IgRE is essential for IFN-γ responsiveness. To further confirm that YB-1 is involved in COL1A2 repression by IFN-γ, we generated a deletion mutant YB-1 expression plasmid, which lacks the
carboxyl-terminal region compared with the full-length YB-1 expression plasmid. As shown in Fig. 3E, transcriptional repression of COL1A2 by IFN-γ was strengthened further by overexpression of YB-1. On the other hand, overexpression of the mutant YB-1 abrogated the COL1A2 repression by IFN-γ while keeping the basal transcription levels unchanged (Fig. 3E). These results clearly demonstrated that YB-1 not only regulates the COL1A2 transcription but also mediates IFN-γ elicited repression of COL1A2 transcription.

**Y-box Consensus Sequence Confers IFN-γ Responsiveness to a Minimal Promoter**—To determine whether IFN-γ exerts its inhibitory effect solely through the YB-1 binding site or circumvents any additional non-YB-1-specific cis-elements, we constructed (YB-1)-luciferase reporter plasmid in which four tandem repeats of the consensus Y-box sequence were linked to a minimal promoter containing only a TATA box. As shown in Fig. 4A, expression of YB-1 decreased (YB-1)_4-luciferase activity in a dose-dependent manner. Interestingly, a dose-dependent diminution of (YB-1)_4-luciferase activity was also observed by changing the concentration of IFN-γ in culture medium (Fig. 4B). These data suggest that IFN-γ exerts its inhibitory action specifically through the Y-box sequence.

**Nuclear Translocation of YB-1 by IFN-γ**—The results described above led us to investigate the IFN-γ-induced alterations in the relative amount and binding ability of YB-1 to the IgRE. We first evaluated alterations in the relative amount of YB-1 in response to IFN-γ using Western blot analysis. Twenty micrograms of nuclear proteins or 40 μg of cytoplasmic proteins prepared from cells either untreated or treated with IFN-γ for various lengths of time were coelectrophoresed with recombinant YB-1. The amount of YB-1 present in nuclear extracts was significantly increased by IFN-γ treatment for more than 4 h (Fig. 5A, upper panel). Inversely, the amount of YB-1 present in cytoplasmic extracts was obviously decreased (Fig. 5A, lower panel). Then we analyzed IFN-γ-induced alterations in the binding ability of YB-1 to the IgRE using EMSA. As shown in Fig. 5B, the intensity of the YB-1-IgRE complex was increased remarkably by IFN-γ treatment. In contrast, the intensities of both the Sp1-IgRE and Sp3-IgRE complexes remained unchanged (data not shown). Furthermore, Southwestern blot analysis using IgRE as a probe showed that the intensity of the band estimated at ~50 kDa, which is almost identical to the size of YB-1, was significantly increased by IFN-γ treatment for more than 4 h (data not shown). These results clearly demonstrated that IFN-γ promotes the nuclear translocation of YB-1 followed by its binding to the IgRE. To demonstrate directly the nuclear translocation of YB-1 by IFN-γ, human dermal fibroblasts were transfected with 1 μg of YB-1-GFP expression plasmid and treated with 100 units/ml IFN-γ. IFN-γ-GFP fusion protein was located mainly in the cytoplasm of untreated cells, whereas nuclear translocation of YB-1-GFP was observed as early as 4 h after exposure to IFN-γ (Fig. 6).

**DISCUSSION**

We have demonstrated previously that a proximal region of the human COL1A2 promoter is essential for mediating the inhibitory effect of IFN-γ and designated this region the IFN-γ response element, IgRE (13). In the current study, by screening a human fibroblast cDNA expression library, we identified YB-1 as a transcription factor that interacts with the IgRE. Both bacterially expressed recombinant protein and endogenous YB-1 present in fibroblast nuclear extracts exhibited high affinity for the IgRE. Transient transfection assays demonstrated that expression of YB-1 decreased COL1A2 promoter activity in a dose-dependent manner and that IFN-γ suppressed gene transcription through YB-1 binding to the IgRE. Experiments using the carboxyl domain-deleted YB-1 confirmed the role of YB-1 in COL1A2 repression by IFN-γ. Furthermore, a combination of Western blot analysis, EMSA of nuclear proteins, and GFP fluorescence study demonstrated that IFN-γ treatment translocates YB-1 into the nucleus and increases the binding of YB-1 to the IgRE sequence.

Ihn and co-workers (14, 23) have demonstrated that ubiquitous transcription factors Sp1 and Sp3 bind to a TCCCCC motif.
FIG. 3. Both YB-1 and IFN-γ suppress COLIA2 promoter activity via the YB-1 binding site. A, human dermal fibroblasts were transfected with the indicated amounts of YB-1 expression vector. Six hours after transfection, the cells were placed in medium supplemented with 10% FCS and incubated for another 48 h. Total RNA (50 ng) was extracted and quantitatively analyzed for COLIA2 or YB-1 gene expression using real-time RT-PCR. In all cases, pRc/RSV control vector was used to ensure an equal amount of DNA in each sample. The relative levels of COLIA2 or YB-1 gene expression were normalized against the GAPDH levels measured in the same total RNA preparation (mean ± S.D., n = 6). B, the -161WT reporter plasmid was subjected to a substitution mutation at either -160G→159C (-161M1) or -157G→154C (-161M3) within the IgRE in the same way as designed for the EMSA shown in Fig. 1B. Human dermal fibroblasts were transfected with 4 μg of reporter plasmid together with 100 ng of either pRc/RSV (from open bar to closed bar) or YB-1/RSV (from closed bar to open bar) expression vector. Six hours after transfection, the cells were placed in medium supplemented with 10% FCS and incubated for another 40 h. The luciferase activities, normalized by protein concentrations, were expressed in relative luminescence units (mean ± S.D., n = 6). C, human dermal fibroblasts were incubated with different concentrations of IFN-γ (0–100 units/ml) for 48 h. Total RNA (50 ng) was extracted and quantitatively analyzed for COLIA2 or YB-1 gene expression using real-time RT-PCR. The relative levels of COLIA2 or YB-1 gene expression were normalized against the GAPDH levels measured in the same total RNA preparation (mean ± S.D., n = 6). D, human dermal fibroblasts were transfected with 4 μg of wild-type or mutated reporter plasmid. Six hours after transfection, the cells were incubated with different concentrations of IFN-γ (0–100 units/ml) for another 40 h. The luciferase activities, normalized by protein concentrations, were expressed in relative luminescence units (mean ± S.D., n = 6). E, human dermal fibroblasts were transfected with 4 μg of -161WT reporter plasmid together with 100 ng of pcDNA3.1(+)/vector as control or with expression plasmids encoding either full-length YB-1 or deletion mutant YB-1 (N→CSD, corresponding to amino acids 1–129), which lacks the carboxyl-terminal domain. Six hours after transfection, the cells were placed in medium supplemented with 10% FCS. One hour later, they were left untreated (from open bar to closed bar) or treated (from closed bar to open bar) with IFN-γ (1 unit/ml) and incubated for another 40 h. The luciferase activities, normalized by protein concentrations, were expressed in relative luminescence units (mean ± S.D., n = 6). An asterisk signifies that the values are significantly different compared with control. N.S., not significant.

FIG. 4. Y-box consensus sequence confers IFN-γ responsiveness to a minimal promoter. A, human dermal fibroblasts were transfected with 4 μg of (YB-1)4-luciferase reporter gene plasmid together with the different amounts of YB-1/RSV expression vector (0–50 ng). The pRc/RSV vector DNA was used to maintain the equivalent amount of transfected DNA in each dish. Six hours after transfection, the cells were placed in medium supplemented with 10% FCS. One hour later, IFN-γ was added to the medium at different concentrations (0–1 unit/ml) and incubated for another 40 h. Luciferase activities, normalized by protein concentrations, were expressed in relative luminescence units (mean ± S.D., n = 6). An asterisk signifies that the values are significantly different compared with control. N.S., not significant.

Functional assays using four tandem repeats of the YB-1 consensus sequence indicated that IFN-γ exerts its inhibitory action on COLIA2 transcription solely through the YB-1 binding site (Fig. 4). It should be noted, however, that the binding of Sp1/Sp3 was markedly enhanced when using the mutated IgRE sequences (M1 and M5) as EMSA probes (Fig. 2C). YB-1 has been shown to interact with other transcription factors and viral proteins (29–31). In addition, a previous study has suggested that the level of Sp1 activity dictates binding of YB-1 to located between -164 and -159 within the human COLIA2 promoter. Using transient transfection assays, they also showed that this pyrimidine-rich motif is a binding site for a transcriptional repressor (14, 23, 28). However, since the blockade of Sp1/Sp3 binding to the repressor site did not affect the collagen promoter activity (23), the functional role of Sp1/Sp3 interacting with the TCCCCC motif has not been fully understood. Our present study has clearly indicated that the IgRE (-161 to -150), immediately downstream of and partly overlapping the Sp1/Sp3 binding site, is a binding site for a COLIA2 repressor, YB-1. Indeed, Ihn et al. (28) introduced a substitution mutation into -161 GCC –159 within the TCCCCC motif, which was almost comparable with our M1 mutation, and this resulted in a 6-fold increase in the basal promoter activity.
fibroblasts were transiently transfected with 1 µg of pCMV-YB-1-GFP expression plasmid. Six hours after transfection, the cells were placed in medium supplemented with 10% FCS. After incubation for 40 h, the cells were left untreated (A) or treated (B) with 100 units/ml IFN-γ for 4 h. Then the cells were examined under a microscope equipped with a fluorescein isothiocyanate filter set for fluorescence detection.

It has been shown that chk-YB-1b, a chicken homologue of human YB-1, binds to a pyrimidine-rich sequence and activates the rat α1(I) procollagen gene (COL1A1) transcription (33). Amino acid sequences of the nucleic acid binding domain and the carboxyl terminus of chk-YB-1b display a high degree of homology with those of human YB-1. In contrast, the sequence of the amino-terminal domain, which may influence the ability of the protein to interact with other nucleic acid binding proteins, is the most structurally distinct portion (34). Norman et al. (35) have shown that YB-1 overexpression suppresses endogenous COL1A1 expression and collagen protein production in rodent cells. Sequence analysis of the mouse COL1A1 promoter revealed three putative YB-1 binding sites, −83/−72, −103/−92, and −129/−118, all of which are well conserved in human (35). In addition, Yuan et al. (36) have demonstrated transcriptional inhibition of human COL1A1 expression by IFN-γ and located the IFN-γ response element between nucleotides −129 and −109 of the human COL1A1 promoter. In the present study, we have shown that YB-1 suppresses transcription of human COL1A2 gene and that IFN-γ inhibits gene transcription via the YB-1 binding site. Taken together, these results suggest that IFN-γ/YB-1 signaling may coordinate down-regulate both COL1A1 and COL1A2 gene transcription.

Consensus sequence for translocation of proteins to the nucleus does not exist in YB-1. However, nuclear translocation signals are located in its carboxyl terminus, which typically contain a cluster of three to six basic residues in a short peptide of four to nine amino acids (25). In this study, deletion of the carboxyl-terminal domain of YB-1 containing those nuclear translocation signals resulted in an abrogation of COL1A2 repression by IFN-γ. Recently, Stenina et al. (37) have suggested that thrombin induces the release of YB-1 from mRNA by proteolytic cleavage and the truncated YB-1 is translocated into the nucleus and bound to the thrombin response element (CCACCCACC) in endothelial cells. However, Western blot analyses using an amino-terminally Flag-tagged YB-1 expression vector failed to show degradation of YB-1 following IFN-γ treatment of human fibroblasts (data not shown). In contrast, a previous study using human cancer cells showed that the nuclear translocation of YB-1 is induced by UV irradiation or anticancer drugs like cisplatin in a protein kinase C-dependent manner (38). Our results showed that treatment with 100 nM 12-O-tetradecanoylphorbol-13-acetate also initiated the nuclear translocation of YB-1 in human fibroblasts (data not shown). These results may suggest that some of the IFN-γ-induced biological events, such as the induction of YB-1 translocation or the inhibition of type I collagen gene expression, are mediated by the carboxyl-terminal domain of YB-1.
mediated through the protein kinase C pathway (39, 40). The precise mechanism by which IFN-γ initiates the nuclear translocation of YB-1 is currently under investigation.

Altogether, this study represents the definitive identification of the transcription factor responsible for IFN-γ/H9253 -elicited inhibition of COL1A2 expression, namely YB-1. Future studies aimed at the characterization of the molecular mechanism involved in the regulation of type I collagen gene expression by YB-1 may provide a novel insight into the interstitial fibrotic diseases and eventually contribute to the development of useful therapeutic means.

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