Identification of a Novel NF-κB-binding Site with Regulation of the Murine α2(I) Collagen Promoter

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HEPATIC FIBROSIS IS DUE TO THE INCREASED SYNTHESIS AND DEPOSITION OF TYPE I COLLAGEN. ACETALDEHYDE ACTIVATES TYPE I COLLAGEN PROMOTERS. NUCLEAR FACTOR κB (NF-κB) WAS PREVIOUSLY SHOWN TO INHIBIT EXPRESSION OF MURINE \( \alpha_1(I) \) AND HUMAN \( \alpha_1(I) \) COLLAGEN PROMOTERS. THE PRESENT STUDY IDENTIFIES BINDING OF NF-κB, PRESENT IN NUCLEAR EXTRACTS OF STELLATE CELLS, TO A REGION BETWEEN -553 AND -537 OF THE MURINE \( \alpha_2(I) \) COLLAGEN PROMOTER. THE NF-κB (p65) EXPRESSION VECTOR INHIBITED PROMOTER ACTIVITY. MUTATION OF THE PROMOTER AT THE NF-κB-BINDING SITE INCREASED BASAL PROMOTER ACTIVITY AND ABROGATED THE ACTIVATING AND INHIBITORY EFFECTS OF TRANSFORMING GROWTH FACTOR \( \beta \) AND TUMOR NECROSIS FACTOR \( \alpha \), RESPECTIVELY, ON PROMOTER ACTIVITY. ACETALDEHYDE INCREASED IκB-\( \alpha \) KINASE ACTIVITY AND PHOSPHORYLATED IκB-\( \alpha \), NF-κB NUCLEAR PROTEIN, AND ITS BINDING TO THE PROMOTER. HOWEVER, THE ACTivating EFFECT OF ACETALDEHYDE WAS NOT AFFECTED BY THE MUTATION OF THE PROMOTER. IN CONCLUSION, ALTHOUGH ACETALDEHYDE INCREASES THE BINDING OF NF-κB TO THE MURINE \( \alpha_2(I) \) COLLAGEN PROMOTER, THIS BINDING DOES NOT MEDIATE THE ACTivating EFFECT OF ACETALDEHYDE ON PROMOTER ACTIVITY. THE EFFECTS OF ACETALDEHYDE IN INCREASING THE TRANSLOCATION OF NF-κB TO THE NUCLEUS WITH INCREASED DNA BINDING ACTIVITY MAY BE IMPORTANT IN MEDIATING THE EFFECTS OF ACETALDEHYDE ON OTHER GENES.

Acetaldehyde, the product of alcohol oxidation, increases \( \alpha_1(I) \) and \( \alpha_2(I) \) collagen gene transcription and messages as well as type I collagen production by cultured stellate cells (10–13). In previous studies, we showed that acetaldehyde enhances the activity of the murine \( \alpha_1(I) \) collagen promoter in stellate cells and that this effect is mediated by increased binding of nuclear proteins including nuclear factor I to region -315 to -295 of the promoter (12). Although Sp1 is important in maintaining basal activity of the \( \alpha_2(I) \) collagen promoter, it plays no role in mediating the effects of acetaldehyde (14).

Acetaldehyde was shown to enhance binding of NF-κB of nuclear extract from HepG2 cells to the NF-κB consensus sequence in one study (15), whereas in another study, acetaldehyde inhibited lipopolysaccharide-stimulated DNA binding of NF-κB by nuclear extracts from Kupffer cells (16). Upon analysis of the nucleotide sequence of the murine \( \alpha_2(I) \) collagen promoter, we found the presence of a NF-κB-binding motif (\( 5’-ACTGGGAAAATTAGGGG-3’ \)) at -553 to -537 from the start of transcription. The purpose of this study was to determine the regulatory role of NF-κB on the \( \alpha_2(I) \) collagen promoter and the effect of acetaldehyde on the regulation.

EXPERIMENTAL PROCEDURES

Animals and Materials—Adult male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). All of the animals received humane care in compliance with the guidelines of the Animal Care and Use Committee of the Johns Hopkins University. Animal Care and Use Committee of the Johns Hopkins University. The luciferase construct of the \( \alpha_2(I) \) collagen promoter was provided by Dr. Benoit de Crombrugghe from the M. D. Anderson Cancer Center (Houston, TX). The luciferase construct of the \( \alpha_2(I) \) collagen promoter (pGL3-1009) was made by inserting the 2.0-kb promoter into the HindIII site of the pGL3 enhancer vector as described previously (17). The NF-κB expression vectors RSV-NF-κB (p50) and RSV-Rel (p65) and the empty vector k1 were obtained from Prof. Guidalberto Manfoletti (University of Trieste, Trieste, Italy).

Site-directed Mutagenesis—Oligonucleotides containing 2-bp substitutions were designed for site-directed mutagenesis. The method employed for the mutagenesis of the pGL3-1009 promoter was based on the strategy of overlap extension using PCR as described by Ho et al. (17). The DNA sequencing facility of our Department of Biological Chemistry confirmed successful mutations.

Isolation and Culture of Hepatic Stellate Cells—Rats weighing ~400 g were used in the procedure of hepatic stellate cell isolation, as described previously (13). The procedure follows the method of Friedman and Roll (18) except for the use of a Nycodenz gradient. In brief, the stellate cells were isolated in situ by the perfusion of the portal vein under sterile conditions sequentially with 0.2% Pronase E and 0.015% collagenase in DMEM. The liver cell suspension obtained was centrifuged at 1400 \( \times g \) in a two-step discontinuous Nycodenz gradient. The isolated cells were suspended in DMEM and seeded in 25-cm\(^2\) tissue culture flasks maintained in DMEM containing 10% fetal bovine serum, fungizone (2.5 \( \mu \)g/ml), penicillin (100 units/ml), and streptomycin (100

The most abundant form of collagen deposited in hepatic fibrosis and cirrhosis is type I collagen, which is composed of two \( \alpha_1(I) \) and one \( \alpha_2(I) \) polypeptide chains (1, 2). Type I collagen accumulation during the development of liver fibrosis is initiated by increased expression of the type I collagen genes (2). Hepatic stellate cells are the principal source of type I collagen and other extracellular matrix proteins during liver fibrogenesis (3, 4).

A few common transcription factors such as nuclear factor I and Sp1 are known to bind and activate both the mouse \( \alpha_1(I) \) (5) and human \( \alpha_2(I) \) collagen promoters. The present study identifies binding of nuclear extracts of stellate cells, to a region between -553 and -537 of the murine \( \alpha_2(I) \) collagen promoter. The NF-κB (p65) expression vector inhibited promoter activity. Mutation of the promoter at the NF-κB-binding site increased basal promoter activity and abrogated the activating and inhibitory effects of transforming growth factor \( \beta \) and tumor necrosis factor \( \alpha \), respectively, on promoter activity. Acetaldehyde increased IκB-\( \alpha \) kinase activity and phosphorylated IκB-\( \alpha \), NF-κB nuclear protein, and its binding to the promoter. However, the activating effect of acetaldehyde was not affected by the mutation of the promoter. In conclusion, although acetaldehyde increases the binding of NF-κB to the murine \( \alpha_2(I) \) collagen promoter, this binding does not mediate the activating effect of acetaldehyde on promoter activity. The effects of acetaldehyde in increasing the translocation of NF-κB to the nucleus with increased DNA binding activity may be important in mediating the effects of acetaldehyde on other genes.

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1 The abbreviations used are: NF-κB, nuclear factor κB; NE, nuclear extract; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility shift assay; TNF, tumor necrosis factor; TGF, transforming growth factor; GST, glutathione S-transferase; IκB, inhibitor of NF-κB activity; IKK, inhibitory κB kinase.
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**Fig. 1.** EMSA, with competition and supershift, showing the binding of NE from rat stellate cells to the wild-type NF-κB oligonucleotide (−553 to −537). A, competition (Comp.) was carried out with a 100-fold molar excess of cold oligonucleotide. B, for supershifts, the reaction mixture was incubated with preimmune serum (PI) or antibodies (Ab) to NF-κB (p50) and NF-κB (p65) (1:200 dilution). Arrows indicate the locations of the protein-DNA complexes. Supershifted complexes are indicated by arrowheads. F indicates the free probe.

μg/ml) at 37 °C with a humidified atmosphere of 5% CO₂ and 95% air. The medium was changed every 48 h, while the cells transformed into activated cells after 10–14 days in culture.

**Nuclear Protein Extraction—** Nuclear extracts from cultured stellate cells were prepared as described previously (19). The nuclear protein extracts were aliquoted and stored under nitrogen at −80 °C. The protein content of the nuclear extracts was determined by the method of Lowry et al. (20).

**Electrophoretic Mobility Shift Assays (EMSA)—** The sequence of the wild-type oligonucleotide, corresponding to region −553 to −537 of the α2(I) collagen promoter, used initially for EMSA was 5′-ACTGGGGAATAGGG-3′. Mutated oligonucleotides, which contained 2-bp nucleotide substitutions, are shown in the legend to Fig. 2. Complementary strands of each oligonucleotide were annealed, and the double-stranded oligonucleotides were labeled with [α-32P]dATP and [α-32P]dCTP using Klenow enzyme according to the method of Feinberg and Vogelstein (21). DNA-protein binding reactions were performed following the previously described EMSA procedure (22). Nuclear extracts from stellate cells were incubated with the labeled oligonucleotide probes (2.5–25 fmol) at room temperature for 30 min in 25 μl of reaction buffer containing 25 mM HEPES, pH 7.8, 50 mM KCl, 0.1 mM ZnCl₂, 1 mM dithiothreitol, 2 μg of poly(dI-dC), and 10% glycerol. The competition assays were performed by incubating molar excess of unlabeled oligonucleotides for 30 min with nuclear proteins prior to the addition of labeled oligonucleotide probes. For “supershift” EMSA experiments, rabbit polyclonal antibodies to NF-κB (p65) and to NF-κB (p50) obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were used. These antibodies were added separately to the reaction at the completion of DNA-protein binding and incubated for an additional 30 min at room temperature. The resultant samples were resolved on a 5% nondenaturing polyacrylamide gel and visualized on x-ray film.

**Transient Transfections and Luciferase Assay—** Activated stellate cells, after no more than three passages, cultured in DMEM containing 20% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml) were grown until they were 60% confluent. Each cell culture flask was transfected with 5 μg of pGL3-1009 using calcium phosphate precipitation (23). Where expression vectors were used, the controls were transfected with the empty pκl vector, and the total DNA quantities added to each flask were balanced by the addition of sheared salmon sperm DNA. Transfection efficiency was determined by co-transfection of 0.4 μg of the Renilla luciferase vector pRL-CMV (Promega). Four h after transfecting, the cells were washed twice with DMEM and then shocked with 10% (H₂O)₂SO for 3 min. The cells were then returned to DMEM containing 10% fetal bovine serum. For the experiments with acetaldehyde, the medium was changed after 1 h to serum-free DMEM containing the following six supplemental growth factors: epidermal growth factor (10 μg/ml), transferrin (0.5 μg/ml), selenous acid (5 μg/ml), linoleic acid (0.5 mg/ml), bovine serum albumin (0.5 mg/ml), and fetuin (0.5 mg/ml). The acetaldehyde was added to a final concentration of 200 μM, and the flasks were tightly capped. The cells were spiked with acetaldehyde at 12-h intervals for a total treatment time of 40 h. The cells were harvested 40–44 h after transfection and exposed to one freeze-thaw cycle in reporter lysis buffer (Promega). Firefly luciferase activity was determined and normalized to Renilla luciferase activity using the dual luciferase assay system of Promega.

**Western Blot Analysis—** The cells were lysed in Nonidet P-40 lysis buffer containing 50 mM Tris-HCl, pH 8.0, 400 μM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 1 h at 4 °C and cleared by centrifugation at 12,000 × g for 15 min at 4 °C. The total protein from the cell lysates was separated on mini-SDS gels at 100 V for 1 h. The membranes were washed in phosphate-buffered saline, pH 7.6, containing 0.1% Tween 20 (PBS-T) and subsequently blocked with 5% (w/v) dried nonfat milk in PBS-T for 1 h at room temperature. The membranes were incubated with rabbit polyclonal antibodies to NF-κB (p50), NF-κB (p65), IκB-α, IκB-β, phosphorylated IκB-α, IκK-α, and IκK-β (Santa Cruz Biotechnology) at 4 °C overnight. After repeated washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution; Amersham Biosciences) at room temperature for 1 h. The membranes were then washed again and visualized by enhanced chemiluminescence reaction (ECL Plus; Amersham Biosciences).

**Ultraviolet Cross-linking of Nuclear Proteins to Oligonucleotides and Immunoblot Analysis—** The binding of nuclear proteins to the oligonucleotide probe was performed as for EMSA. Reactions using 8 μg of nuclear protein and 50 fmol of the radioactively labeled oligonucleotide were used. Following the binding reaction, UV cross-linking was performed as described previously (24). The membranes were then incu-

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**Fig. 2.** Mutational analysis of the NF-κB-binding sequence of the α2(I) collagen promoter. EMSA was performed with 1 μg of the labeled wild-type and mutated oligonucleotides and 8 μg of the rat stellate cell nuclear protein extract. The bold letters indicate the mutated sequences in each mutated oligonucleotide. The arrow indicates the location of the protein-DNA complexes. F indicates the free probe. WT, 5′-ACTGGGGAATAGGG-3′; M1, 5′-ACTTTTGGAAATTTAGGG-3′; M2, 5′-ACTGTTTAAATAGGGG-3′; M3, 5′-ACTGGGTTTATTAGGGG-3′; M4, 5′-ACTGTTGGAAATTAGGG-3′; M5, 5′-ACTGGGGAATATGGGG-3′; M6, 5′-ACTGGGGGAATATTTGG-3′; and M7, 5′-ACTGGGGGAATTAGGGTT-3′.
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RESULTS

Nuclear Factor κB Binds to the Murine α5(I) Collagen Promoter, and This Binding Activity Is Increased by TNF-α—Increased nuclear protein binding to the oligonucleotide specifying the wild-type −553 to −537 region of the α5(I) collagen promoter is shown in Fig. 1A. The nuclear protein binding is competed away by a 100-fold molar excess of the cold oligonucleotide. Antibodies to NF-κB (p50) and to NF-κB (p65) resulted in the appearance of supershifted bands (Fig. 1B). Only the antibody of NF-κB (p50) resulted in the complete disappearance of nuclear protein binding.

A mutational analysis of the NF-κB-binding site of the α5(I) collagen promoter was performed by EMSA of protein-DNA complex formed with labeled oligonucleotides that were sequentially mutated by 2 base pairs between −550 and −537.
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Fig. 5. EMSA of the effects of acetaldehyde on the binding of proteins in NE to the wild-type NF-κB oligonucleotide. The cells were not exposed (C) or exposed to acetaldehyde (A) 200 μM for 4 or 24 h. The arrows indicate the protein-DNA complexes formed. F indicates the free probe.

(Fig. 2). This analysis revealed that the 2-bp mutation of the oligonucleotide M2 or of the oligonucleotide M3 eliminates the upper protein-DNA complex. The 2-bp mutation of oligonucleotide M6 results in a decrease in the upper protein-DNA complex. Thereafter the α2(I) collagen promoter (pGL3-1009) was mutated by the 2-bp mutation of the M2 oligonucleotide and named pGL3-1009mut.

Exposure of the stellate cells to TNF-α (0.6 nM) for 30 min resulted in an increase in the upper protein-DNA complexes formed by nuclear extracts with the wild-type NF-κB oligonucleotide (Fig. 3, lane D). Antibody to NF-κB (p65) results in a decrease in the upper complex formed in the presence of 7 μg of NE (lane 5) and in the appearance of a supershifted complexes when the NE concentration used in the EMSA was increased to 12 μg (lanes 8 and 9). TNF-α increased NF-κB (p65) but not NF-κB (p50) protein in nuclear extracts of the stellate cells (Fig. 4).

Nuclear Factor κB Inhibits the Activity Murine α2(I) Collagen Promoter—The effects of NF-κB (p65) and NF-κB (p50) expression vectors were determined on the activity of the wild-type (pGL3-1009) and mutated (pGL3-1009mut) α2(I) collagen promoters in co-transfection experiments. The NF-κB (p65) expression vector resulted in the inhibition of the activity of pGL3-1009 (p < 0.05) (Table I), whereas the NF-κB (p50) expression vector had no effect. The combination of the NF-κB (p65) and NF-κB (p50) expression vectors caused inhibition of pGL3-1009 activity (p < 0.05) similar to that observed with NF-κB (p65) expression vector alone. The basal activity of the transfected pGL3-1009mut was greater than the activity of the wild-type pGL3-1009 (p < 0.01). NF-κB (p65) alone or NF-κB (p50) alone had no significant effect on pGL3-1009mut, but the combination of NF-κB (p65) and NF-κB (p50) resulted in a paradoxical increase in the activity of pGL3-1009mut (p < 0.05).

TGF-β (0.5 nM) increased the activity of pGL3-1009 (p < 0.05), whereas TNF-α (1.4 nM) decreased the activity of pGL3-1009 (p < 0.01) (Table II) but had no effect on the activity of pGL3-1009mut.

Acetaldehyde Increases Nuclear Factor κB Binding to the Murine α2(I) Collagen Promoter—Exposure of stellate cells to acetaldehyde (200 μM) for 4 and 24 h increased the binding of NF-κB to the wild-type NF-κB oligonucleotide (Fig. 5). Acetaldehyde increased nuclear NF-κB (p65) protein after 4 h of exposure but had no effect on nuclear NF-κB (p50) protein (Fig. 6). The relative densitometries of cross-linked NF-κB (p65) shown in Fig 6B were 100 ± 9 for control and 139 ± 7 after 4 h of acetaldehyde exposure (p < 0.05).

Acetaldehyde Activates NF-κB by Enhancing IkB Kinase and IkB Phosphorylation—Acetaldehyde resulted in a small decrease in IkB-α kinase activity at 0.5 h, followed by an increase at 4 h. (Fig. 7A). Acetaldehyde exposure for 4 h resulted in a decrease in phosphorylated IkB-α at 0.5 h followed by an increase at 4 h (Fig. 7B). Acetaldehyde increased IkB-α at 0.5 and 4 h and increased IkB-β at 4 h (Fig. 7B). Acetaldehyde increased IKK-α protein but did not affect IKK-β protein (Fig. 7C). The effects of acetaldehyde in enhancing IkB kinase and IkB phosphorylation at 4 h were associated with increased cytosolic NF-κB (p65) protein, but there was no change in cytosolic NF-κB (p50) protein (Fig. 7D).

The Activating Effect of Acetaldehyde on Enhancing the Activity of the Murine α2(I) Collagen Promoter Is Not Mediated by NF-κB Binding—Acetaldehyde (200 μM) increased the activity of both pGL3-1009 and pGL3-1009mut (Table III).

**DISCUSSION**

This study demonstrates that NF-κB binds to a novel site in the murine α2(I) collagen promoter and that binding to this site results in inhibition of promoter activity. Prior studies have shown that NF-κB binds and inhibits the murine α2(I) (8) and human α2(I) collagen promoters (9).

TNF-α is well known to activate latent NF-κB by degradation of IκB inhibitory cytoplasmic retention proteins, leading to nuclear translocation and gene activation (26). In this study, TNF-α increased NF-κB (p65) protein and binding to the newly described NF-κB-binding site in the murine α2(I) collagen promoter and inhibited the activity of the transfected promoter in stellate cells. Previously, TNF-α had been shown to increase NF-κB binding activity to the murine α2(I) (8) and to the human α2(I) (9) collagen promoters, resulting in depressed collagen gene expression (8, 9).
Fig. 7. A, effect of acetaldehyde on IκB-α kinase activity in rat stellate cells. Cytosol was precipitated with antibody to IKK-α. The kinase assay used GST-IκB-α as a substrate and [γ-32P]ATP. The changes in 32P-phosphorylated GST-IκB-α are shown. B, immunoblot of the effects of acetaldehyde on cytosolic IκB-α, IκB-β, and phosphorylated IκB-α proteins. C, immunoblot of the effects of acetaldehyde on cytosolic IKK-α and IKK-β proteins. D, immunoblot of the effects of acetaldehyde on cytosolic NF-κB (p50) and NF-κB (p65) proteins. The stellate cells in culture were not exposed (C) or exposed to 200 μM acetaldehyde (A) for 0.5 and 4 h. The relative densitometry readings (means ± S.E.) from three samples for each determination are shown. *, p < 0.05 versus respective control. **, p < 0.01 versus respective control.
The data are expressed as the means ± S.E. of six determinations.

| Additions | Luciferase activity expressed as a percentage of the wild-type or mutated promotera |
|-----------|----------------------------------------------------------------------------------|
| Control   | pGL3-1009: 100 ± 6; pGL3-1009mut: 100 ± 8                                       |
| Acetaldehyde (200 µt) | pGL3-1009: 575 ± 26; pGL3-1009mut: 412 ± 10³ |

The mean pGL3-1009 and pGL3-1009mut luciferase activities were 72,918 and 93,552 relative light units/mg protein.

TGF-β1 is a strong stimulator of the production of extracellular matrix proteins such as collagen and fibronectin (27). The activation by TGF-β of the α5(I) collagen promoter in this study required NF-κB binding to the newly described binding site, because activation was abrogated by mutation of this binding site. This finding was surprising because TGF-β was shown to regulate the human α5(I) collagen promoter principally through the cellular Smad signal transduction pathway (28). However, it has become apparent that the receptor-activated Smads regulate transcription in the context of other transcription factors such as Sp1 (29) and that in addition other signal factors such as Sp1 (29) and that in addition other signal pathways including the RAS pathway may play a role as antagonists in mediating the effects of acetaldehyde on other genes.

Although our study demonstrates that acetaldehyde increases the partial blockage of the activating effect of acetaldehyde on the proximal TGF-β-responsive elements. In previous studies, we showed that acetaldehyde enhances the activity of the α5(I) collagen promoter in stellate cells and that this effect is mediated by increased binding of nuclear proteins, including nuclear factor 1 to a region, which is located in the TGF-β-responsive element (12). The binding of Sp1, by contrast, was not affected by acetaldehyde (14). In studies with the mouse α1(I) collagen promoter, it was found that the activating effect of acetaldehyde was mediated by increased binding of CCAAT/enhancer-binding protein β to a region that overlaps with the TGF-β-responsive element (34, 35), but in this case, the activating effect of acetaldehyde was not modified by neutralizing antibody to TGF-β (34).

In conclusion, this study shows that NF-κB binds to a novel site in the murine α5(I) collagen promoter and that binding to this site results in inhibition of promoter activity. Acetaldehyde increases the translation to the nucleus and the binding of NF-κB to the promoter by increasing the phosphorylation-dependent degradation of IκB-α. This increased NF-κB binding, however, plays no role in the activating effect of acetaldehyde on the activity of this collagen promoter. The effects of acetaldehyde in increasing the binding activity of NF-κB to DNA may be important in the regulation of other genes.
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