Hepatic stellate cells (HSCs) become activated into myofibroblast-like cells during the early stages of hepatic injury associated with fibrogenesis. The subsequent dysregulation of α(I) collagen gene expression is a central pathogenic step during the development of cirrhosis. Our recent study in rat HSCs (Davis, B. H., Chen, A., and Beno, D. (1996) *J. Biol. Chem.* 271, 11039–11042) found that ERK1,2 activation might be required for maximal α(I) collagen gene expression. However, the role of the parallel JNK cascade in regulating α(I) collagen gene expression was unknown. In this study, we initially found that UV irradiation of HSCs activated JNK but not ERK1,2. Furthermore, UV irradiation increased endogenous α(I) collagen mRNA abundance and stimulated α(I) collagen gene transcription in HSCs. The effect of the activation of JNK and Jun on α(I) collagen gene expression was further evaluated via transfection of chloramphenicol acetyltransferase reporter plasmids with various sizes of truncated 5′ upstream promoter sequence (UPS) of the α(I) collagen gene. This revealed that dominant negative transcription factor JUN suppressed collagen gene transcription in HSCs maintained in media with 20% serum and constitutively activated JUN increased α(I) collagen gene transcription in HSCs cultured in media with 0.4% serum. UV activated JNK utilized a distal GC box in the 5′-UPS of the collagen gene to regulate gene transcription. This observation was confirmed by site-directed mutagenesis. In co-transfection experiments, the col-chloramphenicol acetyltransferase reporter with a mutagenized GC box was not suppressed by dn-JUN and was not stimulated by activated JUN or by UV irradiation. Southwestern blotting analyses and gel shift assays with basic transcription element-binding protein anti-serum suggested that the GC box was bound by basic transcription element-binding protein, a recently described DNA-binding protein. In conclusion, the current study combined with our previous report suggests that ERK1,2 and JNK cascades regulate α(I) collagen expression in HSCs through different regions of the 5′-UPS of the gene. The distal GC box in the 5′-UPS of the α(I) collagen gene may play a central role in receiving extracellular signals through the JNK pathway.

Eukaryotic cells have developed specific signal transduction pathways to respond to and integrate extracellular stimuli. Three of these pathways that have been elucidated in eukaryotic cells can be simplified as follows: 1) raf → MEK1,2 → ERK1,2; 2) MEKK1 → SEK1 → JNK/SAPK; and 3) MEK1 → SEK1 → p38 (1). In each of these kinase cascade pathways, the upstream kinase phosphorylates and activates its immediate downstream substrate kinase. Extracellular signals are thereby transduced through these cytoplasmic kinase cascades to reach their nuclear targets and regulate gene expression. Recent studies indicate that theraf → ERK1,2 pathway has significant effects on α(I) collagen gene expression in rat-derived hepatic sinusoidal stellate cells (HSCs), the major effector cells during the overproduction of collagen which typifies hepatic fibrogenesis and cirrhosis (2–5). The response elements for the cascade involved a NF-1 site in the proximal promoter of the collagen gene, as well as a region within −1620 to −1630 in the distal promoter of the α(I) collagen gene (2). The importance of the NF-1 site in regulating collagen gene expression is in agreement with other studies using non-HSCs (6–9). The effects of JNK activation on α(I) collagen gene expression had not been evaluated in HSCs. Activated JNK can phosphorylate and activate its target protein, JUN, and transduce a signal from cytoplasm to nucleus (10–13). JNKs are activated by a variety of stimuli, such as UV irradiation, heat shock, and osmotic imbalance (14, 15). HSCs have been shown to contain the JNK cascade, which can be activated by fibronectin, interleukins, and tumor necrosis factor (16). These are classic compounds associated with tissue injury and may also be present in serum (16). The current report studied the effects of UV irradiation of HSCs on activation of JNK and on α(I) collagen gene expression. It was found that exposure of HSCs to UV light activated JNK but not ERK1,2, and the activation of JNK by UV irradiation increased α(I) collagen mRNA abundance. Further studies indicated that activated JNK regulated α(I) collagen gene expression through a distal GC box located in the 5′-UPS of the α(I) collagen gene. This response element was distinct from that utilized by the ERK1,2 cascade. A 32-kDa protein, designated basic transcription element-binding protein (BTEB), was found to bind to the GC box. BTEB DNA binding activity was up-regulated in activated stellate cells. The potential mechanism(s) utilized by UV-induced activation of JNK to stimulate α(I) collagen gene transcription is further discussed.

*This work was supported by National Institutes of Health Grants DK02022, DK42023, DK42086, DK07074-18, and DK47995-01A2 and by the Liver Research Fund, University of Chicago. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HSC, hepatic stellate cell; UPS, upstream promoter sequence; RPA, RNase protection assay; PCR, polymerase chain reaction; BTEB, basic transcription element-binding protein; mut, mutant; EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco's modified Eagle's medium; gal, galactosidase; ERK, extracellular regulated kinase; JUN, transcription factor; colCAT, 3′ promoter region of collagen gene linked to CAT reporter; dn-JUN, dominant negative JUN; v-JUN, constitutively active form of JUN; CAT, chloramphenicol acetyltransferase.

UV Irradiation Activates JNK and Increases αI(I) Collagen Gene Expression in Rat Hepatic Stellate Cells*

(Received for publication, July 28, 1998, and in revised form, September 15, 1998)

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UV and JNK Regulate αd(I) Collagen Gene Expression

MATERIALS AND METHODS

Cell Culture and Transfection—Hepatic stellate cells (HSCs) were isolated from Sprague-Dawley male rats and subcultured in DMEM supplemented with 10% fetal bovine serum and 10% newborn calf serum (10/10 DMEM) by previously described methods (17, 18). Experimental manipulations were performed with cells at passages 2–6. Sixty to eighty percent confluent cells in 6-well cell culture plates were transfected with the LipofectAMINE method following the protocol provided by the manufacturer (Life Technologies, Inc.). Equimolar col CAT reporters (1–1.3 μg of DNA) and equal amounts of dominant negative JUN or empty control plasmid pMNC (2 μg) were used in each well. For each transfection, 0.2 μg of β-galactosidase expression plasmid pSVβ-gal (Promega) was co-transfected for evaluation of transfection efficacy. Each DNA transaction experiment was repeated at least three times.

Chemicals and UV Irradiation—Unless specifically noted, all chemicals were purchased from Fisher or Sigma. Seventy to eighty percent confluent passaged HSCs were incubated in DMEM with 0.4% fetal bovine serum for 48 h before exposure to a UV lamp (254 nm, 38 W, 76-cm distance between uncovered plates and the UV lamp) in a tissue culture hood for 0.2 or 0.4 min. The UV dose was approximately 10 J/m2 (19). Control cells were exposed to room light in a tissue culture hood for 0.4 min. Cells were then maintained in the media with 0.4% serum in a 37 °C incubator for an additional 1 h before total RNA isolations or protein extractions.

RNA Isolation and RNS Protection Assay (RPA)—Total RNA was isolated by the TRI-Reagent (Sigma) following the protocol provided by the manufacturer. Single-stranded RNA probe complimentary to αd(I) collagen gene was synthesized from a primer (5’-AAACCTAGACGGAGTGTTCTTCAC3’) (the 5’-end of the primer). The PCR product was then subcloned into BamHI and SmaI sites in the plasmid pGEM-3Z (Promega). The PCR primer was used to obtain an antisense RNA probe. The template for in vitro transcription was obtained by Ambion and yields a 100-base pair protected fragment. The antisense RNA probes were synthesized and labeled by MaxiScript in vitro transcription kits (Ambion). The synthesized probes were gel purified, RPA was carried out with RPA II (Ambion) following the protocol provided by the manufacturer. The dried gel was exposed to a phosphor imaging system (Phosphor Image SI, Molecular Dynamics, Sunnyvale, CA). The radioactivity in each band was measured by computer-aided densitometry of the phosphorimage using IPLab Gel (Signal Analytics Corp.) as described previously (20).

CAT Assay and Transfection Efficacy Normalization—Cells were harvested and assayed for CAT activity as described previously (21). Briefly, cells were lysed by the freeze–thaw method of five cycles of freezing in liquid nitrogen and centrifugation for 15 min at high speed at 4 °C, protein concentrations were determined by QuantiGold (Diversified Biotech). Protein extracts were heated for 5 min at 65 °C to inactivate any endogenous acetylases. The protein extracts reacted with 50 μl of CAT assay mixture at 37 °C for 90 min. The mixture contains 350 μl of PBS, 110 μl of 10 mM acetyl-CoA, 1.5 ml of chloramphenicol, and 100 μl of 0.5 mM [3H]acetyl-CoA. The reaction mixture was mixed with 1 ml of binding buffer with 4% glycerol, 1 ml of 0.5 M MgCl2, 10 ml of 3 M KCl, and incubated for 10 min at 4 °C. Cells were re-collected by centrifugation for 5 min at 2000 rpm. The cell pellet was resuspended in Solution A, containing 10 mM Hepes, pH 7.9, 1.5 mM MgCl2, and 10 mM EDTA, and incubated for 10 min at 4 °C. The downstream primer (5’-CCCTAATTGCGCCTAGGTC-CTTGATC) was used to generate single strand antisense RNA probe. The template for in vitro transcription was obtained by Ambion and yields a 100-base pair protected fragment. The antisense RNA probes were synthesized and labeled by MaxiScript in vitro transcription kits (Ambion). The synthesized probes were gel purified, RPA was carried out with RPA II (Ambion) following the protocol provided by the manufacturer. The dried gel was exposed to a phosphor imaging system (Phosphor Image SI, Molecular Dynamics, Sunnyvale, CA). The radioactivity in each band was measured by computer-aided densitometry of the phosphorimage using IPLab Gel (Signal Analytics Corp.) as described previously (20).

Plasmid Constructions—Dominant negative JUN (dn-JUN) was originally from Dr. M. J. Birrer (10) The constitutively active form of JUN (v-JUN) was a gift from Dr. N. Hay (University of Chicago). Both dn-JUN and v-JUN were tested; the expected results were obtained when an AP-1 reporter plasmid 3x-TRE-CAT was used, and there was no effect on pBL-CAT. Plasmid 3x-TRE-CAT contains three AP-1 sites to regulate CAT gene expression. The control empty parental vector pBL-CAT has no AP-1 sites. Both plasmids were kindly provided by Drs. B. J. Aneskievich and E. Fuchs (21). The col CAT reporter plasmid p7.1/6 contains 1.7 kilobases of the 5‘-UPS of the rat αd(I) collagen gene and 1.6 kilobases of the first exon and part of the first intron linked to the col CAT reporter gene (22, 23). Plasmids p7.1/6 and p6/4.1 col CAT reporters were derived by treating plasmid p7.1/6 with digestion with restriction enzyme NheI/ThIII I and NheI/MfclI restriction endonucleases, respectively. Plasmids p3.6/1.6 and pdel1.3–0.4 were as described previously (21). pdel 1.4–0.4 was described as follows. pdel 1.4–0.4 was sequenced to ensure that no changes occurred during the PCR.

PCR, DNA Sequencing, and Site-directed Mutagenesis—PCR was performed using Ultra DNA Polymerase (Perkin-Elmer). The sequencing procedures followed the protocol of Sequenase Version 2.0 DNA Sequencing Kit from Amersham Pharmacia Biotech. Plasmid p7.1GC box mut/1.6 was created by the overlap expansion method of site-directed mutagenesis (24). The site-directed mutants were sequenced to confirm the changes. The sites (–1494 to –1468) were mutated from 5’-GGTTGGAGAGGGGGGAGTCTCTGAG3’ to 5’-GGTTGGAGAGGGGGGAGTCTCTGACG3’. This site contains the GC box of interest (i.e. –1484 to –1475).

Nuclear Extraction—Nuclear proteins were prepared as follows. >95% confluent cells in cell culture flasks were washed twice and harvested in cold PBS. Cells were collected by centrifugation at 2000 rpm for 5 min at 4 °C. The cell pellet was resuspended in 5–10× volume of Solution A, contained 10 mM Hepes, pH 7.9, 1.5 mM MgCl2, and 10 mM EDTA, and incubated for 10 min at 4 °C. Cells were re-collected by centrifugation for 5 min at 2000 rpm. The cell pellet was resuspended in 1.5–2 ml of Solution A with the following inhibitors: 0.5 mM dithiothreitol, 10 μg/ml leupeptin, 2 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml NaF, 10 μg/ml aprotinin, 1 mM NaVO3, and 60 μg/ml β-glycerophosphate. Cells were gently stroked 30 times on ice in a Dounce Type B homogenizer. Nuclei were collected by centrifugation at 2500 rpm for 10 min at 4 °C. The nucleoli pellet was resuspended in Solution A with the above inhibitors and subsequently centrifuged at 15,000 rpm for 20 min at 4 °C. The pellet was resuspended in Solution C, containing 20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 μg/ml leupeptin, 2 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml NaF, 10 μg/ml aprotinin, 1 mM NaVO3, and 60 μg/ml β-glycerophosphate. The lysate was sonicated on an up-and-down rocker for 60–90 min at 4 °C and then centrifuged at 15,000 rpm for 30 min. The clear supernatant was aliquoted in micro centrifuge tubes and stored at –70 °C until use. Nuclear protein concentrations were determined by Bio-Rad reagent.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear protein extracts (5–10 μg) were incubated in 10 μl of binding buffer with 4% glycerol, 5 μM MgCl2, 0.1 mM dithiothreitol, 1 μg/ml Tris-HCl, pH 7.5, and 2.5 μg of poly(dI-dC). In competition EMSAs, the indicated amounts of double-stranded oligonucleotides were added as unlabeled competitor. The mixture was incubated at room temperature for 10 min before adding a radiolabeled probe. In some cases, 2 μl of anti-βTEB or preimmune serum was added to the mixture and incubated at room temperature for 20 min prior to adding 1 ng of a radiolabeled probe. Nucleoprotein complexes were resolved by electrophoresis on 6% nondenaturing polyacrylamide gels in 0.5X Tris borate/EDTA (TBE) buffer. The gels were exposed to autoradiography film (DuPont) overnight at –70 °C. Double-stranded oligonucleotide probes were radiolabeled by T4 polynucleotide kinase (New England Biolabs) and γ[32P] ATP (DuPont). Polyclonal antibody α-βTEB was commercially generated by Research Genetics, Inc., according to the protocol used for BTEB cDNA and its deduced amino acid peptide sequence (28).

Oligonucleotide Probes—The double-stranded oligonucleotides used in the EMSA were as follows. SP-1 consensus oligonucleotides purchased from Promega: 5’-ATTCGATTCGCACCTGGAGAGAGAC-3’; GC box oligonucleotides from –1491 to –1470 of the 5‘-UPS of the rat αd(I) collagen gene were described by Tullius et al. (29). Underlined sequences represent GC box binding domain. All of the oligonucleotide probes were synthesized by Life Technologies, Inc.

Southwestern Blot Analysis—Nuclear extracts (100 μg/lane) were obtained either from HSCs cultured in DMEM with 20% or 0.4% serum for 48 h or directly from normal uncultured rat HSCs or rats pretreated with carbon tetrachloride (CCL4) (0.5 ml + 0.5 ml of mineral oil given intraperitoneally) 16, 24, 48 or 72 h prior to sacrifice. The extracted
proteins and pre-stained protein standards were resolved by 15% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane filter. The filter was washed for 30 min in Buffer A (25 mM NaCl, 25 mM Heps-NaOH, pH 7.9, 5 mM MgCl₂, 0.5 mM dithiothreitol, which was added fresh prior to use). The filter was then blocked by incubation for 3 h at room temperature in Buffer A with 5% nonfat milk. After a short wash with Buffer A containing 0.25% nonfat milk, the filter was probed with 32P-labeled double-strand GC box oligonucleotide (from -1491 to -1470 of the 5'-UPS of the rat α(I) collagen gene) 5'-TTGGAGGAGGCAGCGAGCTCCTTG-3' in 10 ml of Buffer A with 5 μg/ml poly(dI-dC) plus 0.25% nonfat milk on a rocker at 4 °C overnight. The filter was briefly washed twice with Buffer A containing 0.25 mM NaCl and then washed with regular Buffer A twice at room temperature for 15 min each. The filter was wrapped and exposed to autoradiography film (DuPont) overnight at -70 °C.

Statistics—Differences between means were analyzed via Student's t test using the Statworks program for the Macintosh. Statistical significance required differences at the level of p < .05.

RESULTS

JNK Activation by UV Irradiation Increases Endogenous α(I) Collagen Gene Expression—The effects of activation of JNK on α(I) collagen gene expression were studied in passaged HSCs treated with UV irradiation (Fig. 1). HSCs were grown to near confluence and then incubated for 48 h in serum-depleted medium (0.4% serum). The cells were then exposed to ultraviolet light for 0.2 or 0.4 min or to regular light for 0.4 min. The cell extracts were prepared for Western blot analysis (Fig. 1A). It was found that the active forms of JNK 1 and JNK 2 were readily detected in HSCs exposed to UV light by anti-activated JNK polyclonal antibody (Promega), which preferentially recognized the dually phosphorylated active forms of JNK enzymes (Fig. 1A). In contrast, faint bands of active forms of JNK were present in HSCs exposed to regular light (Fig. 1A). UV irradiation did not induce activation of MAPK (ERK1,2) in serum-starved HSCs probed with the anti-activated MAPK polyclonal antibody (Promega) (data not shown). To determine the effects of UV-induced activation of JNK on endogenous α(I) collagen gene expression in HSCs, α(I) collagen mRNA from serum-starved HSCs treated with or without UV irradiation was measured by RPA (Fig. 1B). The radioactivity in each band in the RPA was measured and quantitated by computer-aided densitometry of the phosphorimage using IPLab Gel. As shown in Fig. 1C, it demonstrated that JNK activation by UV irradiation caused an approximate 2.9-fold increase in endogenous α(I) collagen mRNA steady state levels in HSCs.

Activation of JUN Stimulates colCAT Expression in Cultured HSCs—To understand the mechanisms of UV induction of α(I) collagen gene expression, a series of transfection experiments were performed in passaged HSCs (Fig. 2). HSCs were transfected with an AP-1 reporter plasmid 3x-TRE-CAT or an empty parental plasmid pBL-CAT (Fig. 2A). The AP-1 reporter plasmid 3x-TRE-CAT has three AP-1 binding sites to regulate CAT gene expression, whereas the pBL-CAT is the control parental plasmid without AP-1 binding sites. These two plasmids have been used previously to study activation of AP-1 and transcription mediated by AP-1 (27). After transfection, the cells were incubated in media containing 0.4% serum for 36 h before exposure to UV light or regular light. The results indicated that UV irradiation induced a ~4-fold increase in the CAT activity in cells transfected with 3x-TRE-CAT (Fig. 2A). In contrast, UV exposure did not change the CAT activity in HSCs transfected with pBL-CAT (Fig. 2A). These results suggested that UV irradiation increased the ability of the AP-1 complex to regulate gene transcription. To study the significance of the activation of JUN in stimulating α(I) collagen gene expression, HSCs were co-transfected with colCAT plasmids and a v-JUN expression plasmid (Fig. 2B) or a dn-JUN plasmid (Fig. 2C). In Fig. 2B, the results illustrated that v-JUN, but not empty control plasmid pMNC, increased the CAT activity in serum-starved HSCs co-transfected with the p1.7/1.6 colCAT reporter plasmid, which contained 1700 base pairs of the 5'-UPS and 1600 base pairs of the first exon and part of the first intron of the α(I) collagen gene linked to a CAT reporter gene. However, v-JUN did not increase the CAT activity in HSCs co-transfected with the p0.4/1.6 colCAT plasmid (Fig. 2B). Fig. 2C showed that dn-JUN had an opposite effect on p1.7/1.6 in HSCs maintained in medium with 20% serum. dn-JUN significantly
Reduced the CAT activity in HSCs co-transfected with p1.7/1.6 but not in HSCs co-transfected with p0.4/1.6. The lack of the inhibitory effect in HSCs co-transfected with p0.4/1.6 suggests that the necessary response element is missing and that the dn-JUN inhibitory effect is not due to a nonspecific sequestration effect. It should also be emphasized that v-JUN experiments were carried out in 0.4% serum conditions to eliminate serum derived sources of JUN stimulation, whereas the dn-JUN experiments were done in the presence of serum (20%) as a source of JUN stimulation. Taken together, these data indicate that activation of JUN plays an important role in increasing a I(I) collagen gene transcription in vitro and that the response element for activated JUN is located between −1.7 and −0.4 kilobases of the 5′-UPS of the a(I) collagen gene.

The Distal GC Box in the 5′-UPS Is Required for JUN Stimulation—To further analyze the role of JUN in regulating a(I) collagen gene expression in HSCs, and to identify the promoter region responding to activated JUN, a series of colCAT reporter constructs were produced, some of which are shown in Fig. 3A, left. In order to identify the DNA response element, HSCs were co-transfected with dn-JUN and a series of colCAT plasmids with different 5′-UPS truncations of the a(I) collagen gene, and the experiments were performed in DMEM with 20% serum (Fig. 3A, right). dn-JUN resulted in a 2.6-fold reduction in the CAT activity in cells co-transfected with p1.7/1.6 (Fig. 3A and statistical analysis in Table 1). A significant loss of responsiveness to the dn-JUN inhibitory effect was observed in cells co-transfected with p1.3/1.6 or p0.4/1.6 (Fig. 3A and statistical analysis in Table 1). Additional experiments were therefore focused on the general region between −1700 and −1090 base pairs. To prove that this region contained the required response element(s), additional constructs were produced, i.e., pdel 1.3−0.4/1.6 and pdel 1.4−0.4/1.6 (see Fig. 3A for diagram). When transfected with the p0.4/1.6 reporter, there was no response to the co-transfected dn-JUN. In the pdel reporters, the region containing putative responsive element(s) is now adjacent to the unresponsive 0.4/1.6 region. When transfected with these pdel plasmids, the response to the dn-JUN was regained (see Fig. 3A). A computer-aided search revealed a GC box binding site in this region (−1475 to −1484). Because the GC box might be the element responding to dn-JUN, several key nucleotides in the GC box were mutated to evaluate their necessity for the dominant negative JUN inhibitory effect. As shown in Fig. 3A, this mutagenized reporter p1.7 (GC box mut)/1.6 lost the inhibitory response to dn-JUN. This result suggests that the distal GC box plays a key role in the response to the dn-JUN inhibitory effect on a(I) collagen gene transcription. In order to confirm that the GC box was the response element mediating the regulation of a(I) collagen gene expression by activated JNK, HSCs were co-transfected with the v-JUN and colCAT p1.7/1.6 or p1.7(GC box mut)/1.6 plasmid (Fig. 3B). v-JUN had a stimulatory effect on the CAT activity in cells co-transfected with wild-type colCAT p1.7/1.6. The cells co-transfected with p1.7(GC box mut)/1.6 lost the stimulatory effects of v-JUN on CAT expression. Further evidence of GC box function was provided by HSCs transfected with colCAT p1.7/1.6 or p1.7(GC box mut)/1.6 before exposure to UV irradiation (Fig. 3C). The col CAT plasmid with a mutated GC box (p1.7(GC mut)/1.6) lost its response to stimulation by activated JNK. These results collectively suggest that the distal GC box in the 5′-UPS of the a(I) collagen gene is the response element required for UV activated JNK to stimulate a(I) collagen gene expression.

GC Box-binding Protein Is Altered During Stellate Cell Activation—The GC box sequence is one of the most common regulatory DNA elements of eukaryotic genes. A GC box is usually bound by a member of the Sp transcription factor family (e.g. Sp-1 transcription factor) or BTEB (26). To analyze the GC box-binding protein in HSCs, nuclear extracts were obtained from culture-activated HSCs and from HSCs isolated from rats injected with CCl4 (72 h prior to sacrifice). Injection of CCl4 results in HSC activation typified by HSC proliferation and ultimately enhanced a(I) collagen gene expression (3–5). The extracts from cells activated in vitro both contained a single GC box-binding protein, which was demonstrated in the gel shift assay as a single intense band with the same
mobility (Fig. 4, lanes 6 and 11, lower arrow). In contrast, a faint band with the same mobility was present in quiescent HSC extracts obtained directly from normal rats (lane 10) and in serum-deprived HSCs in culture (lane 5). Competition with either GC box oligonucleotides or a DNA fragment from base pairs -21400 to -21500 base pairs of the 5' UPS, which contained the GC box, eliminated this binding (lanes 7 and 8). Although Sp-1 binding could be involved in the GC box binding, this did not appear to be likely. As shown in Fig. 4, an Sp-1 consensus oligonucleotide resulted in a gel shift that differed considerably from the gel shift using the GC box probe (see Fig. 4, lanes 1–3 versus lanes 6–11). Its mobility was slower, and the response to serum-containing media was less significant. Also, competition with excess Sp-1 oligonucleotide had a minimal effect on the GC box gel shift (Fig. 4, lane 9). The GC box gel shift appeared to require 72 h following CCl4 injection for maximal activity (see Fig. 5). Extracts from earlier time points were intact as they had the ability to bind to a consensus Sp-1 domain (data not shown). This gradual onset of the GC box-binding protein was consistent with the gradual increase in collagen gene expression in vivo (4). The process might require the recruitment of other cells and cytokine release to adequately stimulate the HSCs (28).

**BTEB Is the GC Box-binding Protein**—The results of the EMSA (Fig. 4) suggested that the DNA-binding protein is unlikely to be an Sp-1 transcription factor because the nucleopro-
The extracts are demonstrated in lanes 1 and the upper arrow.

**Table I**

| CAT (units/mg of protein) | pMNC | dn-JUN | t test |
|---------------------------|------|--------|--------|
| p-1.7/1.6                 | 1.8±0.3 | 0.68±0.2 | <0.05  |
| pdel1.4–0.4/1.6           | 1.9±0.4 | 0.65±0.15 | <0.05  |
| p-0.4/1.6                 | 0.80±0.3 | 0.81±0.2 | Not significant |
| p-1.7(GC-box mut)/1.6     | 1.5±0.25 | 1.4±0.2 | Not significant |

**Fig. 4.** GC box gel shift coincided with stellate cell activation. Nuclear extracts were prepared either from cultured HSCs in medium with 0.4% serum (lanes 1, 3, and 5) or 20% serum (lanes 2 and 6–9) or directly from freshly isolated HSCs without any treatment (lane 10) or with CCl4 injection 72 h prior to isolation (lane 11). The nuclear extracts were incubated with 32P-labeled Sp-1 consensus oligonucleotides (lanes 1–3) or 32P-labeled GC box oligonucleotides (lanes 4–11) (see under “Materials and Methods” for details). In lane 4, there was no protein extract. Lane 4 was used to indicate the position of the free probe. In some assays, competition was performed with excess (100×) of cold GC box oligonucleotides (lane 7), a cold DNA fragment from −1400 to −1500 base pairs (lane 8), or cold Sp-1 consensus oligonucleotides (lane 9). The lower arrow on the left indicates a GC box gel shift band, and the upper arrow indicates a Sp-1 gel shift band. Intact proteins in the extracts are demonstrated in lanes 1 and 2.

**Fig. 5.** GC box gel shift versus time post-CCl4. HSC extracts were prepared at different time points, 6–72 h post-CCl4 injection. The GC box oligonucleotide probe (GC box in the −1.4 to −1.5 kilobase region) was identical to the probe used in Fig. 4. The arrow on the right of the gel indicates a single shifted band.

protein complex of interest moved much faster than the Sp-1 complex in the gel. Further studies were performed to identify the GC box DNA-binding protein in the 5′-UPS of the α(I) collagen gene in HSCs. The Southwestern blot revealed a single 32-kDa protein, which could form a nucleoprotein complex with a GC box oligonucleotide (Fig. 6). The 32-kDa size is incorrect for Sp-1 (29). The band also became prominent at 72 h following CCl4 injection (Fig. 6, lane 6), which was consistent with our previous observation (Fig. 5). As expected from the gel shift experiments, serum stimulation of cultured HSCs also resulted in a GC box-binding protein that had the same molecular mass (Fig. 6, lane 2). Recent studies involving other cell types unrelated to HSCs had identified and cloned a GC box-binding protein that had the same molecular mass referred to as BTEB (26, 30). In another EMSA (Fig. 7), nuclear protein extract was pretreated with polyclonal anti-BTEB. The pretreatment with anti-BTEB significantly diminished the gel shift retarded band of interest (lane 3). In great contrast, pretreatment with preimmune serum had no effect on the abundance of the retarded band (lane 4). These gel shift assays suggest that BTEB is the distal GC box DNA-binding protein.

**Fig. 7.** Anti-BTEB pretreatment diminished the GC box gel shift retarded band. The nuclear extracts were obtained from HSCs maintained in medium containing 0.4% fetal calf serum (lane 1) or 20% serum (lanes 2–4). The extracts either had no pretreatment (lanes 1 and 2) or were preincubated with antiserum to BTEB (lane 3) (αBTEB) or with preimmune serum (lane 4) (preI) followed by incubation with the radiolabeled GC box oligonucleotide probe (see under “Materials and Methods”). Lane 5 had no nuclear extract, indicating the position of the free probe.

**DISCUSSION**

The sinusoidal HSCs represent the major effector cells during hepatic fibrogenesis. During this process, the normal quiescent, vitamin A-storing HSCs transform into actively proliferating, collagen-producing cells. The HSCs excessive collagen matrix production and increased cellular proliferation lead to the collagenization and disruption of the space of Disse and formation of the fibrous septae seen in cirrhosis. Eukaryotic cells have developed specific signal transduction pathways to
UV and JNK Regulate α(I) Collagen Gene Expression

directly on and integrate extracellular stimuli. A recent study of HSCs found that fibronectin and tumor necrosis factor α activated both JNK and ERK1,2 and increased AP-1 DNA binding ability and α(I) collagen mRNA abundance (16). However, that study could not clearly indicate the effects of JNK or ERK1,2 on α(I) collagen gene expression because fibronectin and tumor necrosis factor α activated both pathways. Our previous studies found that activation of ERK1,2 induced by serum was important for the maximal expression of α(I) collagen gene in passaged HSCs (2). The function of activated JNK in regulating α(I) collagen gene expression was studied in the present report. It was found that UV irradiation activated JNKs, but not ERK1,2 (Fig. 1A) and that UV irradiation increased endogenous α(I) collagen mRNA abundance in passaged HSCs (Fig. 1B). Both activated ERK1,2 and activated JNK have the capacity to translocate to the nucleus and phosphorylate transcription factors (31, 32). The current transfection studies indicated that activated JNK induced by UV irradiation increased the ability of AP-1 to induce gene transcription in HSCs (Fig. 2A). Additional studies suggested that the active form of JUN increased AP-1 activity and M. Karin, B. Aneskievich, R. Davis, L. Zon D. Rowe, and D. Breault for plasmids used in transfection.

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