JunD Regulates Transcription of the Tissue Inhibitor of Metalloproteinases-1 and Interleukin-6 Genes in Activated Hepatic Stellate Cells

David E. Smart‡, Karen J. Vincent‡, Michael J. P. Arthur‡, Oliver Eickelberg‡, Marc Castellazzi¶, Jelena Mann§, and Derek A. Mann¶

From the ‡Liver Group, Division of Infection, Inflammation, and Repair, University of Southampton, Southampton General Hospital, Southampton SO16 6YD, United Kingdom, the ¶Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520-8023, and the §Unité de Virologie Humaine, INSERM U412, Ecole Normale Supérieure, 69364 Lyon Cedex 07, France

Activation of hepatic stellate cells (HSCs) to a myofibroblast-like phenotype is the pivotal event in hepatic wound healing and fibrosis. Rat HSCs activated in vitro express JunD, Fra2, and FosB as the predominant AP-1 DNA-binding proteins, and all three associate with an AP-1 sequence that is essential for activity of the tissue inhibitor of metalloproteinases-1 (TIMP-1) promoter. In this study, we used expression vectors for wild-type, dominant-negative, and forced homodimeric (Jun/eb1 chimeric factors) forms of JunD and other Fos and Jun proteins to determine the requirement for JunD in the transcriptional regulation of the TIMP-1 and interleukin-6 (IL-6) genes. JunD activity was required for TIMP-1 gene promoter activity, whereas overexpression of Fra2 or FosB caused a repression of promoter activity. The ability of homodimeric JunD/eb1 to elevate TIMP-1 promoter activity supports a role for JunD homodimers as the major AP-1-dependent transactivators of the TIMP-1 gene. IL-6 promoter activity was induced upon activation of HSCs and also required JunD activity; however, expression of JunD/eb1 homodimers resulted in transcriptional repression. Mutagenesis of the IL-6 promoter showed that an AP-1 DNA-binding site previously reported to be an activator of transcription in fibroblasts functions as a suppressor of promoter activity in HSCs. We conclude that JunD activates IL-6 gene transcription as a heterodimer and operates at an alternative DNA-binding site in the promoter. The relevance of these findings to events occurring in the injured liver was addressed by showing that AP-1 DNA-binding complexes are induced during HSC activation and contain JunD as the predominant Jun family protein. JunD is therefore an important transcriptional regulator of genes responsive to Jun homo- and heterodimers in activated HSCs.

Hepatic stellate cells (HSCs) represent up to 15% of the resident cells of the liver and play a pivotal role in the cellular pathology underlying hepatic fibrosis (1). In response to liver injury of any etiology, the normally quiescent HSC undergoes a progressive process of differentiation into a proliferating myofibroblast-like activated HSC (1). Through increased secretion of extracellular matrix proteins and the tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2, activated HSCs are responsible for deposition and accumulation of the majority of the excess extracellular matrix in the fibrotic liver (2). Furthermore, activated HSCs can contribute to the fibrogenic process through their ability to secrete and respond to a wide range of cytokines and growth factors (3).

Details of the molecular events that regulate HSC activation are beginning to be unraveled, as is the potential for specific members of the AP-1, NF-κB, and Kruppel-like transcription factor families to control key profibrogenic features of the activated HSCs (1, 4–6). Putative AP-1 and NF-κB sites are found in the promoters of many genes that are induced upon HSC activation and contribute to the fibrogenic process, including TIMP-1 (AP-1), IL-6 (AP-1 and NF-κB), and ICAM-1 (NF-κB) (4, 5, 7). Since in vivo activation of HSCs can be closely mimicked by culturing HSCs isolated from normal rat liver on plastic and in the presence of serum, it has been possible to investigate the transcriptional potential of profibrogenic genes during HSC activation (1). Investigators including ourselves have previously demonstrated that basal and cytokine/growth factor-inducible transcription of these genes is dependent on interaction of specific AP-1 and NF-κB (Rel) protein dimers with their putative promoter-binding sites (4–6). These observations indicate that these inducible transcription factors are likely to play a key role in the activation and/or persistence of myofibroblast-like HSCs. Recent studies have identified target genes of NF-κB (IL-6 and ICAM-1) and have also indicated that NF-κB may protect activated HSCs against apoptosis (5, 6, 8). Less attention has been directed at studying the role played by AP-1 in HSC activation. Although in vitro studies have shown that activated HSCs express inducible AP-1 DNA-binding activity (4, 9, 10), there is little direct evidence that AP-1 plays a key role in the transcriptional regulation of the activated HSC phenotype. Chen and Davis (11, 12) recently re-
ported that acetaldehyde- and UV-induced transcription of the α(I) collagen gene is mediated via AP-1-dependent activation of BTEB, a GC box-binding transcription factor that regulates α(I) collagen gene transcription. We have previously shown that an AP-1-binding site in the human TIMP-1 gene promoter is required for high level transcription in activated HSCs (4). In this study, we have addressed the role of the AP-1 transcription factor JunD in the control of TIMP-1 and IL-6 gene transcription in activated HSCs.

The jun family proto-oncogenes (c-jun, junB, and JunD) are critical components of the AP-1 transcription factor (13, 14). The Jun proteins are bZip transcription factors that can form either AP-1 homodimers (Jun/Jun) or AP-1 heterodimers. Jun heterodimers are created through interaction of Jun proteins with members of the related bZip protein family, notably those of the fos proto-oncogene family (c-fos, fosB, fra1, and fra2) or the ATF family (ATF2, ATF3, and ATF4) (13–15). An evolutionarily conserved non-canonical AP-1 site (TGAGTAA) in the human TIMP-1 promoter is required for induction of transcription during culture activation of primary rat HSCs and binds Jun/Jun and Jun/Fos dimers (4). Western blot and electrophoretic mobility shift assay (EMSA) studies revealed that JunD is the predominant Jun family protein expressed in culture-activated rat HSCs, with little or no detectable expression of c-Jun and JunB after the first 48 h of culture. This observation indicated a role for JunD not only in the transcriptional activation of TIMP-1, but also in other AP-1-dependent regulatory processes of activated HSCs.

In this study, we demonstrate that JunD is required for high level activity of both the TIMP-1 and IL-6 promoters in activated HSCs. We also show that expression of different combinations of AP-1 proteins leads to differential effects on transcription and that the repressive or stimulatory effects induced by Jun/Jun and Jun/Fos dimers are dependent on the target promoter.

MATERIALS AND METHODS

Cell Isolation and Carbon Tetrachloride (CCl4)-induced Liver Damage—Heparinase were isolated from the livers of male Sprague-Dawley rats (400 ± 50 g) by sequential perfusion with Pronase and collagenase as previously described (16). Induction of acute liver damage in rats was achieved by intraperitoneal injection of a 1:1 ratio of CCl4 (0.2 ml/100 g of body weight) and olive oil as previously described (16). Control rats were administered an intraperitoneal injection of olive oil alone. HSCs were separated from the cell suspension over an 11.5% Optiprep gradient (Nycoderm Pharma AS, Oslo, Sweden), followed by elution. HSCs were seeded onto plastic, cultured in Dulbecco’s minimal essential medium (DMEM), and 16% fetal calf serum (Life Technologies, Inc.), and maintained at 37 °C in an atmosphere of 5% CO2.

Plasmid DNA—All plasmid DNA was prepared using a commercial DNA extraction and isolation kit (Maxiprep, QIAGEN). A chlorophenol acetyltransferase (CAT) reporter plasmid (pTIMP1) containing a 162-bp minimal human TIMP-1 promoter cloned into the HindIII and PstI sites of pBLCAT3 was used to determine TIMP-1 promoter function (4, 17). IL-6 promoter function was studied using the luciferase reporter vector pIL6-Luc651 containing nucleotides −651 to +1 of the human IL-6 gene (7). Construction of pIL6-Luc651 and derivatives carrying site-directed mutations in the AP-1 (20). All plasmid DNA was prepared using a commercial DNA extraction and isolation kit (Maxiprep, QIAGEN). A chlorophenol acetyltransferase (CAT) reporter plasmid (pTIMP1) containing a 162-bp minimal human TIMP-1 promoter cloned into the HindIII and PstI sites of pBLCAT3 was used to determine TIMP-1 promoter function (4, 17). IL-6 promoter function was studied using the luciferase reporter vector pIL6-Luc651 containing nucleotides −651 to +1 of the human IL-6 gene (7). Construction of pIL6-Luc651 and derivatives carrying site-directed mutations in the AP-1 (20).

EMSA—AP-1 DNA binding was determined by EMSA as previously described (4) using a 32P end-labeled double-stranded oligonucleotide probe containing a consensus AP-1 site: sense oligonucleotide, 5′-TATAGGATTCGATGCTACCTCT-3′, and antisense oligonucleotide, 5′-AGATGTCGACCTCTGCTATT-3′. Nuclear extracts were prepared from HSCs as described by Dignam et al. (21). Harvested cells were washed twice in ice-cold phosphate-buffered saline (PBS) prior to lysis in Buffer A (21) supplemented with 0.2% Nonidet P-40, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.2 mM EDTA, and 15 μg/ml aprotinin. Lysates were centrifuged for 10 s at 13,000 rpm to collect crude nuclear pellets. Supernatants were washed twice in lysis buffer prior to storage at −80 °C. Addition of proteinase K (1 mg/ml) to the nuclear extract was performed for 1 h at 37 °C prior to lysis in Buffer B (21) supplemented with 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.2 mM EDTA, and 15 μg/ml aprotinin. Aliquots of nuclear extracts were then sonicated in the presence of 2.5 μg/ml aprotinin for 30 s. Cleared nuclear extracts were transferred to fresh Eppendorf tubes, and their protein content was determined using the Bradford DC assay kit (Bio-Rad). EMSA reactions were assembled on ice and consisted of an initial 10-min incubation of 4 μl of Buffer C containing 5 μg of nuclear protein extract and 12 μl of water containing 2 μg of poly(dI-dC). 4 μl of water containing 0.4 ng of radiolaabeled double-stranded AP-1 probe was then added to the reaction and, after incubation, was incubated for a further 20 min. For supershift assays, reactions were incubated for a further 16 h in the presence of 1 μg/ml anti-JunB or anti-JunD polyclonal antibody (Santa Cruz Biotechnology, Inc.). EMSA and supershift reaction mixtures were then resolved by electrophoresis on an 8% non-denaturing polyacrylamide gel (37:5:1).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Whole cell protein extracts were prepared by lysis of PBS-washed cultures in 60 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, and 5% (w/v) 2-mercaptoethanol. Equal quantities (10 μg) of whole cell extract were then fractionated by electrophoresis through a 12.5% SDS-polyacrylamide gel. Gels were run at a 20-mA constant current for 1.5 h prior to transfer onto nitrocellulose as previously described (4, 6). Following blockade of nonspecific protein binding, nitrocellulose blots were incubated for 2 h with primary antibodies (diluted in PBS/Tween 20 (0.05%) containing 5% [w/v] milk) and 5% [w/v] rabbit polyclonal antibody recognizing JunD (Santa Cruz Biotechnology, Inc.) was used at a 1:100 dilution. Blots were then washed twice in PBS/Tween 20 prior to incubation for 1 h with sheep anti-rabbit horseradish peroxidase antibody (1:2000) and after extensive washing in PBS/Tween 20 before being processed to distilled water for detection of antigen using the ECL system (Amer sham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Real-Time Quantitative Reverse Transcription (RT-)PCR—2 μg of RNA extracted from freshly isolated and 7-day culture-activated rat HSCs was used to generate first-strand cDNA using a random hexamer primer (oligo(dN)6). PCR amplification of rat IL-6 and β-actin cDNAs was carried out using specific oligonucleotide primers selected within the coding regions of the rat genes. IL-6 primers used were 5′-CCACCCACACAGGACCATGAT-3′ (sense) and 5′-TCCAGAAAGACCGAGACCTAC-3′ (antisense). Oligonucleotides designed to anneal between nucleotides 180 and 421 of the rat IL-6 cDNA. Primers used for detection of β-actin were 5′-AGAGGGAAATCTGGCTGTCAGA-3′ (sense) and 5′-ACATCTGCTGGAGAGGTTGCCA-3′ (antisense) and were designed to produce a 350-bp product. PCR products were composed of 1 μl of cDNA template, 100 ng each of sense and antisense oligonucleotide primers, 2.5 μl of optimized Taq PCR buffer (Promega), 0.4 mM dNTP mixture, and 2 units of Taq polymerase in a total reaction volume of 25 μl. Following an initial 5-min incubation at 94 °C, PCRs were performed using a 1-min annealing step (at 51.5 °C for IL-6 and 57.0 °C for β-actin), followed by a 2-min elongation step at 72.0 °C and a 30-s denaturation step at 94 °C. A total number of 28 and 30 PCR cycles were carried out for detection of β-actin and IL-6, respectively, followed by a final elongation reaction for 10 min at 72.0 °C. PCR products were separated by electrophoresis at 80 V for 60 min through a 1% agarose gel and were detected by ethidium bromide staining. Expected sizes of specific PCR products (241 bp for IL-6 and 350 bp for β-actin) were verified by reference to a 1-kilobase DNA ladder, and sequence identity of the IL-6 PCR product was confirmed by DNA sequence analysis.

Transfections and Reporter Gene Assays—HSCs were transduced by the retroviral-JunD polycistronic construct (QIAGEN) according to the manufacturer’s instructions. CAT assays were performed as previously described (4, 6, 17) and normalized for differences in transfection efficiency either by the Hirts assay or by measurement of the activity of a cotransfected Renilla luciferase vector. Luciferase assays were performed using a dual luciferase kit (Promega) according to the manufacturer’s instructions. IL-6 promoter-driven expression of firefly lucifer-
JunD Regulates Transcription of the TIMP-1 and IL-6 Genes

Fig. 1. Effects of overexpression of Jun family proteins on TIMP-1 promoter activity in culture-activated rat HSCs. A. 1 μg of the reporter gene pTIMP1, containing nucleotides −102 to +60 of the human TIMP-1 gene located upstream of the CAT gene, was transfected into 7-day culture-activated rat HSCs together with 3 μg of empty vector pCMV2 or pCMV2-derived expression vectors carrying cDNA cassettes for c-Jun, JunB, and JunD. Sister cultures were also cotransfected with 1 μg of the promoterless plasmid pBLCAT3 and 3 μg of pCMV2 as a reference. B. 7-day culture-activated rat HSCs were transfected with 1 μg of pTIMP1 and 3 μg of either empty vector RSVβ (Control) or RSVβ-JunD (dominant-negative JunD). Sister cultures were also cotransfected with 1 μg of the promoterless plasmid pBLCAT3 and 3 μg of RSVβ as a reference. Results are expressed as the mean % CAT conversion with respect to control (pTIMP1 + empty vector RSV) ± S.E. for three independent transfection experiments. Statistical analysis was performed by Student’s t test. * * * * p < 0.05, 0.01, and 0.005, respectively.

RESULTS

JunD Activity Is Required for TIMP-1 Promoter Function in Activated HSCs—TIMP-1 promoter function in activated HSCs is dependent on an intact AP-1 site that binds JunD (4). To determine the influence of JunD on the activity of the TIMP-1 promoter, rat HSCs were culture-activated for a minimum of 7 days prior to cotransfection with a human TIMP-1-CAT reporter (pTIMP1) and expression vectors for c-Jun, JunB, and JunD. Overexpression of JunD in activated rat HSCs resulted in a 2.5-fold enhancement of TIMP-1 promoter activity that was reproducible in replicate experiments (Fig. 1A). In contrast, overexpression of c-Jun or JunB resulted in a 2-fold or greater inhibition of TIMP-1 promoter activity. Overexpression of JunD failed to enhance the activity of a TIMP-1 promoter lacking an AP-1 site and did not alter the low activity of the TIMP-1 promoter in freshly isolated HSCs (data not shown). We next determined if the endogenous JunD activity expressed in activated rat HSCs is required for TIMP-1 gene transcription. Activated HSCs were cotransfected with pTIMP1 and a vector (RSVβ-JunD) producing expression of a mutant JunD protein lacking a functional transactivation domain (20). As shown in Fig. 1B, RSVβ-JunD expression resulted in a profound inhibition of TIMP-1 promoter activity, with levels of transcription that were only marginally higher than those observed with pBLCAT3, which lacks a promoter.

JunD Homodimers Are Strong Transactivators of the TIMP-1 Promoter in Activated HSCs—We have previously reported that culture-activated HSCs express JunD together with Fra2 and FosB (4). To assess the role of Fos family proteins in the transcriptional control of the TIMP-1 gene, we cotransfected activated HSCs with pTIMP1 and expression vectors for c-Fos, FosB, Fra1, and Fra2 (Fig. 2). Overexpression c-Fos or Fra1 caused a moderate reduction of promoter activity that lacked statistical significance; by contrast, overexpression of FosB or Fra2 resulted in a significant 2-fold repression of transcription. As activation of rat HSCs is accompanied by induction of FosB and Fra2 expression (4), it is possible that changes in the activity of these Fos proteins serve to fine-tune TIMP-1 transcription by forming AP-1 dimers that are less active than JunD homodimers. It was therefore of interest to establish if JunD homodimers are able to influence TIMP-1 promoter activity. Activated HSCs were cotransfected with pTIMP1 and a vector (JunD/eb1) that drives expression of a JunD protein in which the JunD dimerization domain is replaced with the dimerization domain from the Epstein-Barr virus EB1 transcription factor (19). This mutant JunD protein is able to form transcriptionally active homodimers, but cannot form dimers with endogenous wild-type JunD, Fra2, or FosB. Expression of JunD/eb1 substantially enhanced TIMP-1 promoter function, generating a 4-fold higher level of CAT activity relative to cells transfected with a control empty expression vector (Fig. 3A). Hence, JunD/eb1 is a powerful positive regulator of TIMP-1 promoter function, and the data suggest that JunD homodimers are stronger AP-1 transactivators than JunD/Fra2 or JunD/FosB heterodimers. As Fra2 can also negatively regulate c-Jun activity (22, 23) and can form heterodimers with JunB that act as transcriptional repressors in keratinocytes (24), it was conceivable that the negative influence of c-Jun and JunB on TIMP-1 promoter function in HSCs may arise from formation of repressive Jun/Fra2 heterodimers. We therefore determined the ability of c-Jun/eb1 and JunB/eb1 dimers to attenuate TIMP-1 promoter activity (Fig. 3B). In contrast to wild-type c-Jun, overexpression of the c-Jun/eb1 homodimer enhanced TIMP-1 promoter activity by 2-fold; however, overexpression of a JunB/eb1 dimer resulted in only a weak and statistically insignificant elevation of transcription.
JunD Acts as a Positive Regulator of IL-6 Promoter Function in Heterodimeric Form—It has previously been established that activated rat and human HSCs express IL-6 and that induction of IL-6 protein expression in response to stimulation of serum-starved HSCs can be suppressed by inhibition of NF-κB (5, 25). To determine if IL-6 mRNA expression is induced during HSC activation, we used reverse transcriptase-PCR to detect IL-6 mRNA in freshly isolated (quiescent) and culture-activated rat HSCs. The presence of IL-6 mRNA was detected in this assay by amplification of a 241-bp cDNA fragment (Fig. 4), which was later verified as a fragment of IL-6 cDNA (nucleotides 180–421) by sequencing. Reverse transcriptase-PCR detection of β-actin mRNA was used as a control for RNA integrity and loading. As shown in Fig. 4, IL-6 mRNA was barely detectable in freshly isolated HSCs, but underwent a dramatic (at least 50-fold) increase upon culturing of HSCs, indicating that induction of IL-6 gene transcription occurs during in vitro activation of rat HSCs. Studies on human fibroblasts have indicated that JunD can act as a regulator of IL-6 transcription via its interaction with an AP-1 site located at nucleotides −283 to −276 in the human promoter (7). To test if JunD is able to exert regulatory control on the IL-6 promoter in HSCs, freshly isolated and activated rat HSCs were transfected with a human IL-6 promoter-luciferase construct (pIL6-Luc651) (7). As previously observed for the TIMP-1 promoter (4), we were unable to detect significant levels of IL-6 promoter activity in freshly isolated HSCs, but could detect high level promoter activity in activated HSCs (Fig. 5A). To control for differences in transfection efficiency between freshly isolated and culture-activated rat HSCs, all transfections were controlled by inclusion of the Renilla luciferase reporter pRL-TK. The activity of cotransfected pRL-TK was routinely only 2-fold higher in activated HSCs compared with freshly isolated HSCs (data not shown); hence, the observed 50-fold elevation of IL-6 promoter activity during HSC activation was due to a specific transcriptional induction. We next tested the ability of JunD to influence IL-6 promoter function by cotransfecting activated HSCs with pIL6-Luc651 together with expression vectors for wild-type JunD, dominant-negative JunD, and JunD/eb1. Overexpression of wild-type JunD modestly (50%) enhanced pIL6-Luc651 activity, whereas expression of dominant-negative JunD reduced IL-6 promoter function to ~50% of control activity, indicating that JunD homodimers are repressors of IL-6 gene transcription in activated HSCs. In an effort to explain this latter observation, activated rat HSCs were transfected with a series of mutant IL-6 promoter-luciferase constructs in which the AP-1, NF-IL6, or NF-κB sites of the promoter were disrupted (Fig. 5B). Unexpectedly, expression of JunD/eb1 inhibited IL-6 promoter function to ~50% of control activity, indicating that JunD homodimers are repressors of IL-6 gene transcription in activated HSCs.

**Fig. 2.** Influence of Fos family proteins on TIMP-1 promoter function in culture-activated rat HSCs. 1 μg of the reporter gene pTIMP1 was transfected into 7-day culture-activated rat HSCs together with 3 μg of empty vector pCMV2 or pCMV2-derived expression vectors carrying cDNA cassettes for c-Fos, FosB, Fra1, and Fra2. Sister cultures were also cotransfected with 1 μg of the promoterless plasmid pBLCAT3 and 3 μg of pCMV2 as a reference. Results are expressed as the mean % CAT conversion with respect to control (pTIMP1 activities were determined 48 h after transfection. Results are expressed as the mean % CAT conversion with respect to control (pTIMP1 + pCMV2) ± S.E. for seven independent transfection experiments. Statistical analysis was performed by Student’s t test, *, **, and *** p < 0.05, 0.01, and 0.005, respectively.

**Fig. 3.** Effects of expression of Jun/eb1 homodimers on TIMP-1 promoter activity in activated rat HSCs. 7-Day culture-activated rat HSCs were cotransfected with 1 μg of pTIMP1 and 3 μg of empty vector pDP7 (Control) or a pDP7-derived vector carrying a junD/eb1 fusion gene (A) or 3 μg of empty vector pDP7 (Control) or a pDP7-derived vector carrying c-junD/eb1 or junD/eb1 fusion genes (B). In both experiments, sister cultures were also cotransfected with 1 μg of the promoterless plasmid pBLCAT3 and 3 μg of pDP7 as a reference. CAT activities were determined 48 h after transfection. Results are expressed as the mean % CAT conversion with respect to control (pTIMP1 + pDP7) ± S.E. for three independent transfection experiments. Statistical analysis was performed by Student’s t test, *, **, and *** p < 0.05, 0.01, and 0.005, respectively.
amplified cDNA fragments, and the sequence identity of the cDNA
rats with CCl4 for 1–3 days results in acute liver injury that is
Fig. 7
CCl4-injured animals. Supershift analysis with anti-Jun anti -
activity was detected in nuclear extracts of HSCs isolated from
of HSCs isolated from normal rats lack significant AP-1 DNA-
tent nuclear extracts. As previously reported, nuclear extracts
sentative of results obtained from analysis of three independ-
stimulatory effect of JunD heterodimers on IL-6 transcription
must operate via an alternative sequence in the promoter.
JunD Is a Major Constituent of AP-1 DNA-binding Com-
plexes Induced during in Vivo Activation of Rat HSCs—Having
shown that JunD is required for TIMP-1 and IL-6 promoter
function, it was of interest to determine if JunD DNA-binding activity is expressed in activated HSCs in vivo. Treatment of
rats with CCl4 for 1–3 days results in acute liver injury that is
accompanied by HSC activation (26, 27). Nuclear extracts were
prepared from HSCs isolated from rats injured for 48 h by
intraperitoneal injection of CCl4 or olive oil carrier as a control.
Fig. 7A shows an EMSA for AP-1 DNA binding that is repre-
sentative of results obtained from analysis of three independ-
ent nuclear extracts. As previously reported, nuclear extracts
of HSCs isolated from normal rats lack significant AP-1 DNA-
binding activity (4). However, substantial AP-1 DNA-binding activity was detected in nuclear extracts of HSCs isolated from
CCl4-injured animals. Supershift analysis with anti-Jun anti-
odies revealed reactivity with both anti-c-Jun and anti-JunD
antisera; by contrast, anti-c-Jun antiserum was without reac-
tivity (Fig. 7B). Although both anti-JunB and anti-JunD anti-
sera generated supershift complexes, we consistently observed
a greater loss of the AP-1 DNA-protein complex in the presence
of anti-JunD antiserum. This loss of reactivity in supershift
EMSA experiments is usually due to formation of large mul-
timeric complexes that are unable to enter the gel or can result
from disruption of the DNA-protein interaction by the anti-
body. The greater loss of AP-1 DNA-protein complex using
anti-JunD antiserum compared with the loss when using anti-
JunB antiserum suggests that JunD is the major Jun family component of the CC1α-induced complex. Immunoblot analysis
of HSC nuclear extracts was performed to monitor any changes
in the expression of JunD protein that occur during the in vivo
activation of rat HSCs. Previous studies have shown that JunD
is detected as two biologically active isoforms with apparent
molecular masses of 43 and 39 kDa (28). HSCs isolated from
control rats expressed a low level of JunD that was predomi-
nantly expressed as the shorter 39-kDa isoform (Fig. 7C). In
three independent experiments, we were able to consistently
demonstrate a strong induction of JunD in HSCs isolated from
CCl4-injured rats. We also observed a shift in isoform expres-
sion, with loss of the 39-kDa form detected in control rats in
favor of expression of the longer 43-kDa JunD protein. These
data confirm our earlier observations with the in vitro model of
HSC activation (4) and indicate the need to further investigate
the function of JunD in fibrogenesis.

DISCUSSION

We have previously reported that JunD is the predominant
Jun family protein expressed in primary culture-activated rat
HSCs grown in the continual presence of serum and in the
absence of stimulation by specific growth factors, cytokines, or
mitogens (4). Under these conditions, rat HSCs undergo a
time-dependent program of activation that, over a period of
7–14 days, results in a myofibroblast-like phenotype that re-
sembles the HSC phenotype in the injured liver (1). In this
study, we provide evidence that JunD functions in culture-
activated HSCs to regulate transcription of at least two genes
of relevance to fibrosis, TIMP-1 and IL-6.

Induction of high levels of TIMP-1 promoter activity during
culture activation of rat HSCs is dependent on an intact AP-1
site shown to interact with JunD (4). In this study, we show
that overexpression of wild-type JunD and homodimeric JunD/
dominates TIMP-1 promoter activity; by contrast, expres-
sion of a dominant-negative JunD protein in activated HSCs
strongly inhibits TIMP-1 promoter function. We have also
observed that an antisense JunD oligonucleotide can substan-
tially reduce TIMP-1 promoter activity in activated HSCs.2
Taken together, these data indicate that JunD is a vital com-
ponent of the transcriptional machinery that regulates TIMP-1
expression in activated HSCs. However, because overexpres-
sion of JunD did not enhance TIMP-1 promoter activity in
frequently isolated HSCs, other factors must cooperate with JunD
to regulate induction of TIMP-1 transcription. A strong candi-
date factor is the 30-kDa Upstream TIMP-1 element-1 (UTE-
1)-binding protein, which we have shown is induced during
HSC activation and associates with the TIMP-1 promoter in
these cells (17). Overexpression of c-Jun, JunB, Fra2, or FosB
in activated HSCs repressed TIMP-1 promoter activity,
whereas c-Fos and Fra1 had little effect. Both Fra2 and FosB
are induced in activated HSCs and can associate with the AP-1
DNA-binding site in TIMP-1 (4). As JunD/e1 homodimers are
strong stimulators of TIMP-1 gene transcription, the most
likely explanation for the inhibitory effects of exogenously
added Fra2 and FosB is that they form heterodimers with
endogenous JunD that are less active than JunD homodimers.
We therefore propose that the high level of TIMP-1 expression
in activated HSCs is controlled by the balance between JunD
homodimers and heterodimers, with the former promoting high
rates of TIMP-1 mRNA synthesis. Of relevance to this idea,
both in vitro (4) and, as shown in this study, in vivo HSC
activation is accompanied by increased JunD protein expres-
sion. This increase in JunD expression would serve to elevate
TIMP-1 expression by increasing the pool of JunD available for
assembly of transcriptionally active homodimers. The process
by which JunD expression is elevated during HSC activation
and the mechanisms underlying the suppressive effects of Fra2
and FosB on JunD-mediated transcription therefore warrant
further investigation.

Effects of jun and fos proto-oncogenes on TIMP-1 promoter
function have previously only been studied in the context of F9
teratocarcinoma cells, which, in their undifferentiated state,
lack detectable AP-1 activity (29). Overexpression of c-Jun,
JunD, and c-Fos in undifferentiated F9 cells enhances TIMP-1
promoter activity and, in contrast to HSC combinations of
either c-Jun or JunD with c-Fos, generates the highest pro-

2 D. E. Smart and D. A. Mann, unpublished data.
moter activities (29). The negative influence of c-Jun in activated HSCs may be explained by its ability to form repressive AP-1 heterodimers with Fra2, which has been shown to inhibit c-Jun activity in F9 cells (21–22). In support of this idea, we have shown that expression of c-Jun in a homodimeric form (c-Jun/eb1) will enhance TIMP-1 promoter activity in HSCs. However, the absence of c-Jun DNA-binding activity in both in vitro and in vivo activated HSCs argues against the protein.

**FIG. 5.** Induction of IL-6 promoter activity during culture activation of rat HSCs is regulated by JunD. A, freshly isolated (Quiescent) and 7-day cultured (Activated) rat HSCs were cotransfected with 1 μg of pIL6-Luc651 and 50 ng of pRL-TK, and luciferase values were determined 48 h after transfection. B, 7-day culture-activated HSCs were cotransfected with 1 μg of pIL6-Luc651, 50 ng of pRL-TK, and 3 μg of either a control empty vector (pCMV2 or RSVβ gave similar values) or expression vectors for JunD, dominant-negative JunD, and the JunD/eb1 homodimer. Sister cultures were also cotransfected with 1 μg of the promoterless plasmid pGL3-Basic, 3 μg of pCMV2, and 50 ng of pRL-TK as a reference. Normalized (to pRL-TK activity) luciferase activities are expressed as the means ± S.E. of three independent transfection experiments and in B are expressed as a % of the control activity (pIL6-Luc651 + empty vector). Statistical analysis was performed by Student's t test. * and ***, p < 0.05 and 0.005, respectively.

**FIG. 6.** Mutagenesis of AP-1, NF-κB, and NF-IL6 DNA-binding sites in the IL-6 promoter. A, map of the human IL-6 promoter showing approximate locations of AP-1, CRE, NF-IL6, NF-κB, and TATA elements. B, normalized luciferase activities for 7-day culture-activated rat HSCs cotransfected for 48 h with 50 ng of pRL-TK and either wild-type pIL6-Luc651 or mutated pIL6-Luc651 vectors carrying mutations in the AP-1, NF-κB, and NF-IL6 sites. Sister cultures were also cotransfected with 1 μg of the promoterless plasmid pGL3-Basic and 50 ng of pRL-TK as a reference. Results are expressed as the means ± S.E. of three independent transfection experiments. Statistical analysis was performed by Student's t test. * and ***, p < 0.05 and 0.005, respectively.
having any role in the regulation of TIMP-1 expression. The negative influence of c-Fos in HSCs is more difficult to explain when considering that the combination of c-Fos and JunD acts as a strong enhancer of TIMP-1 promoter function in F9 cells (29). One possibility is that there may be cell-specific differences in the interaction of AP-1 dimers with other transcription factors implicated in the control of TIMP-1 promoter function such as UTE-1 or c-Ets (4, 17). Logan et al. (29) showed that AP-1 and c-Ets-1 synergistically activate the TIMP-1 promoter in F9 cells and moreover form a direct interaction that requires the presence of c-Fos in the AP-1 dimer. Our group (4) and other investigators (30) have reported that c-Ets-1 expression is strongly down-regulated during HSC activation to barely detectable levels. In addition, mutation of the Ets/PEA3-binding site in the TIMP-1 promoter has only a minor effect on transcription in activated HSCs (4). If c-Fos does act synergistically with c-Ets-1, then the relatively low levels of c-Ets-1 in activated HSCs may be one explanation for the lack of responsiveness of the TIMP-1 promoter to overexpression of c-Fos in HSCs.

HSCs isolated in an activated form from CCl4-treated rats expressed DNA-binding forms of both JunB and JunD. In vivo activated HSCs therefore differ from culture-activated HSCs, in which JunB is only induced transiently during the early (first 48 h) phase of activation and is not detected in fully activated cells (4). One explanation for this difference is the possibility that the population of HSCs isolated from injured livers includes cells in states of early and late activation and that expression of JunB DNA-binding activity is provided by a subpopulation of cells still undergoing the transition to the myofibroblast phenotype. Alternatively, the difference may reflect the more complex paracrine and autocrine signaling events occurring in the injured liver compared with the purely autocrine events driving HSC activation in vitro. If this latter explanation is true, then TIMP-1 expression would be regulated in vivo by competition between JunB- and JunD-containing AP-1 dimers, as well as by the activity of Fra2 and FosB. Since overexpression of JunB exerted a repressive effect on TIMP-1 promoter activity, whereas expression of JunB/eb1 was without significant effect, we suggest that JunB heterodimers inhibit transcription probably by competing with JunD homodimers for DNA binding. Consistent with this suggestion, previous reports have described the JunB/Fra2 heterodimer as a powerful negative transcriptional regulator (22, 24).

Many genes induced during HSC activation carry functional AP-1 sites in their regulatory DNA sequences, which raises the possibility that JunD may stimulate transcription of several genes associated with the profibrogenic phenotype of activated HSCs. Our demonstration that JunD is a regulator of IL-6 as well as TIMP-1 gene transcription in HSCs supports this idea. IL-6 is involved in the pathogenesis of many diseases, including liver cirrhosis (31–35). Although recent studies with IL-6−/− mice have indicated an overall inhibitory role for the cytokine in the pathology of experimental liver fibrosis (32), other investigators have demonstrated a profibrogenic role for IL-6 as a stimulator of HSC activation (34, 35). Unstimulated activated rat HSCs produce high levels of IL-6 and are responsive to the cytokine, which was shown to stimulate HSC proliferation and collagen synthesis (34, 35). IL-6 is therefore a marker of HSC activation and may be provide an autocrine pathway that helps perpetuate the activated phenotype; the mechanism responsible for induction of the cytokine is therefore of interest. The human and rat IL-6 gene promoters have a very similar structure, with functional AP-1, cAMP-responsive element (CRE), NF-IL6 (CAAT/enhancer-binding protein-β), and NF-xB sites conserved between the genes (36). Mutagenesis studies have shown that all four sites contribute to transcriptional activity of the promoter in macrophages, a murine mesangial cell line, rat osteoblasts, and lung fibroblasts (7, 37–40). In this study, the human IL-6 promoter (−651 to +1) was found to be transcriptionally inert in freshly isolated rat HSCs, but was highly active in culture-activated rat HSCs. Since we were able to show a similar level of induction of endogenous IL-6 mRNA expression during HSC activation, we suggest that regulation of IL-6 expression is mainly controlled at the transcriptional level. These data closely resemble the events described for induction of TIMP-1 mRNA expression and gene promoter activity in HSCs (4, 16) and indicate that transcriptional induction of the IL-6 and TIMP-1 genes is coordinately controlled during HSC activation. HSCs overexpressing wild-type and dominant-negative JunD proteins displayed enhanced and reduced IL-6 promoter activities, respectively. From these data, we conclude that JunD is one factor responsible for coordinating TIMP-1 and IL-6 gene transcription. However, the manner in which JunD regulates transcription from the two promoters differs in that JunD/eb1 homodimers repressed IL-6 promoter activity. This latter result suggests that JunD can function both as an activator and a repressor of IL-6 gene transcription depending on whether it is in the form of a heterodimer or homodimer. Although we have not yet established the precise mechanism underlying this dual function of JunD, we were surprised to find that an AP-1 site (−284 to −276) in the IL-6
JunD Regulates Transcription of the TIMP-1 and IL-6 Genes

JunD is an important transcriptional regulator in activated HSCs, and as such will play a key role in the molecular pathogenesis of liver fibrosis. Our demonstration that JunD regulates both TIMP-1 and IL-6 gene expression in activated HSCs supports this proposal and raises the need for studies aimed at determining the role of JunD in HSC activation and liver fibrosis.

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