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Research Article

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Two Different *cis*-acting Regulatory Regions Direct Cell-specific Transcription of the Collagen α_1(I) Gene in Hepatic Stellate Cells and in Skin and Tendon Fibroblasts

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Abstract

The expression of the collagen α_1(I) gene in activated stellate cells plays an important role during liver fibrogenesis. To identify the critical *cis*-elements of the collagen α_1(I) gene in stellate cells, we used transgenic animals bearing various collagen α_1(I) regulatory regions directing the expression of either a human growth hormone minigene or the bacterial β-galactosidase gene. We found that collagen α_1(I)-human growth hormone transgene expression was constitutively high in tendon and skin, provided the transgene contained the −2.3 to −0.44 kb collagen regulatory region. However in the liver, expression was stimulated several-fold, as was the endogeneous gene, by the fibrogenic hepatotoxin carbon tetrachloride. This stimulation occurred whether the collagen 5′ regulatory region extended −2.3, −1.6 or −0.44 kb, and in the presence or absence of much of the first intron (+292 to +1607 bp). In addition, the −0.44 kb 5′ region was sufficient for high-level transgene expression in stellate cells, following their activation by culture on plastic. In contrast, in skin and tendon, high-level transcription of the collagen α_1(I) gene required the −2.3 to −0.44 kb 5′ flanking region. Thus, two different *cis*-regulatory regions direct cell-specific transcription of the collagen α_1(I) gene in stellate cells and in skin and tendon. (*J. Clin. Invest.* 1995. 96:2269–2276.) Key words: gene transcription • stellate cell gene expression • liver fibrosis • oxidative stress • lipid peroxidation

Introduction

Collagen type I, which forms large collagen fibers, is a major component of the excessive extracellular matrix protein in cirrhosis (1, 2). In hepatic fibrogenesis, and following the acquisition of an activated phenotype (3), stellate cells display a high level of collagen type I expression (4). Therefore, the regulation of the type I collagen gene in hepatic stellate cells is crucial in understanding the pathogenesis of fibrosis in chronic liver disease (5, 6).

Although the molecular mechanisms responsible are unknown, the increased production of collagen α_1(I) in liver injury is mainly a result of an enhanced collagen gene transcription (7, 8). The factors known to stimulate collagen gene transcription include acetaldehyde (9), lipid peroxidation and its products (10, 11), and transforming growth factor-β1 (TGFβ1) (12). Several lines of investigation have provided evidence indicating that aldehyde-protein adducts, including products of lipid peroxidation, and TGFβ1, modulate collagen gene expression (9–12) and may be a link between tissue injury and fibrosis (13–16).

At least in part, this cell-specific gene transcription is controlled by diffusible positive transcription factors that are, themselves, enriched in particular cell types (17). By binding specifically to their DNA recognition sites within promoter and/or enhancer regions, such proteins can augment the frequency of transcription initiation of their target genes (18). Positive and negative regulatory elements have been identified in the promoter and first intron of the collagen α_1(I) gene (19, 20). In addition, we identified an enhancer element (Col E-1 [−370 to −344 bp]) that stimulates transcription in both the forward and reverse orientations, when placed before either a heterologous or homologous promoter (21). However, these *cis*-acting elements cannot explain the high expression of the collagen α_1(I) gene in different cell types.

In studies of transgenic animals the critical *cis*-acting element of the collagen α_1(I) gene has been located for skin and tail in the −2300 to −440-bp region (22, 23), and for bone between −2296 and −1672 bp (24). Therefore, in these cell types with high levels of expression of the collagen α_1(I) gene, a key regulatory region excludes the −0.44-kb 5′ region. In the liver, the element responsive to CCl_4, a fibrogenic hepatotoxin (4, 8) that activates stellate cells (3), is present in a human transgene that contains the structural gene, 1.6 kb of promoter and 20 kb of 3′ untranslated region (25). Thus, the *cis*-element(s) and *trans*-acting factor(s) necessary for the high level expression of the collagen α_1(I) gene in activated stellate cells are not known.

To identify the critical *cis*-acting regions of the collagen α_1(I) gene in activated stellate cells, we used transgenic animals bearing various collagen α_1(I) regulatory regions directing the expression of either a human growth hormone minigene (hGH) or the bacterial β-galactosidase gene. In this study, we found that two different *cis*-regulatory regions direct cell-specific transcription of the collagen α_1(I) gene in activated stellate cells and in skin and tendon fibroblasts. The −440-bp 5′ flanking region is sufficient for high level expression of the transgene in stellate cells upon activation of these cells in the liver following treatment with CCl_4 or when cultured on plastic. In contrast, in skin and tendon fibroblasts, collagen α_1(I) gene transcription requires the −2300 to −440-bp region for maximal expression. The first intron is dispensable for collagen α_1(I) expression in both hepatic stellate cells and in tendon fibroblasts, but it is required in skin fibroblasts. These findings should facilitate the

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identification of the gene(s) encoding factors that transactivate the collagen α_1(I) gene in activated stellate cells.

Methods

Cell cultures. Primary stellate cells from transgenic mice treated with mineral oil (control), or CCl_4 were isolated 48 h after injection, essentially as described previously (26). Briefly, after the livers were excised and washed in Hank's balanced salt solution (HBSS) they were minced and incubated at 37°C for 30 min with constant shaking in 0.5% pronase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) 0.05% collagenase B (Boehringer-Mannheim) and 10 μg/ml DNase (United States Biochemical Corp., Cleveland, OH) in HBSS without Ca^{2+}. This digest was filtered through gauze and pelleted at 450 *g* for 10 min and subsequently rinsed four times with HBSS containing 10 μg/ml DNase. Stellate cells were purified by a single-step density Nycodenz gradient (Accurate Chemical & Scientific Corp., Westbury, NY), as described previously (15, 27). Cells were plated on plastic and used either immediately or after 10 d of culture. For determining the morphological characteristics of freshly isolated stellate cells, they were cultured on a collagen type I matrix for 48 h to facilitate attachment and spreading of the cells. Cells were cultured under an atmosphere of 5% CO_2, 95% air in DME medium containing 10% fetal calf serum. Stellate cells were identified by their typical autofluorescence at 328 nm (excitation wavelength), staining of lipid droplets by oil red, and immunohistochemistry with a monoclonal antibody against desmin (15).

RNase protection assay. RNA was isolated from liver, skin, freshly isolated hepatic stellate cells, or cultured stellate cells, and quantitated by a sensitive RNase protection assay as previously described by us (28, 29), using the riboprobe for exon 5 of the human growth hormone gene (22). This and other collagen riboprobes have been used successfully for *in situ* hybridization or RNase protection assays of the collagen α_1(I) gene (15, 16, 27).

Immunohistochemistry. Antibodies directed against α-smooth muscle actin, desmin, or β-galactosidase, were obtained from Sigma Chemical Co. (St. Louis, MO), Cappel Laboratories (Malvern, PA), and Dako Corp. (Carpinteria, CA). Antisera raised against malondialdelyde-protein adducts have been previously described (30). This antiserum is specific for malondialdehyde-lysine adducts as described (10, 15, 16, 30). These antisera were used with fluorochrome-conjugated secondary antibodies (Sigma Chemical Co. and Vector Laboratories, Inc., Burlingame, CA) to visualize the protein of interest. Liver and skin samples were embedded in OCT (Miles Laboratories, Elkhart, IN), frozen in liquid nitrogen, and fixed with 4% fresh paraformaldehyde. Tissues from animals were immunostained, as described previously (31).

Microscopy. Fluorescent labels were visualized using a dual filter Zeiss microscope as described previously (31). A phase-contrast microscope was utilized to visualize antigens with hematoxylin/eosin or alkaline phosphatase secondary antibodies (Vector Laboratories). Cytochromes utilized were alkaline phosphatase and fluorescein, with Evans blue as counterstain (Sigma Chemical Co.).

Animals. Transgenic mouse lines expressing hGH transgenes were as described previously (22). We used transgenic lines containing portions of the human α_1(I) collagen gene, −2300 COL (bases −2300 to +1607), −2300 COL-ΔI (−2300COL without the first intron from bases +292 to +1440) and −440 COL (bases −440 to +1607)]. A plasmid containing −1626/+116 bp of the murine collagen α_1(I) gene ligated to an *E. coli* β-galactosidase reporter gene constructed by Dr. R. Rippe (North Carolina, Chapel Hill, N.C.) (32) was microinjected into fertilized ova by Dr. M. Oldstone and coworkers (Scripps Clinic and Research Foundation, La Jolla, CA) (33). The exogenously introduced transgenes were identified by hybridization of denatured mouse tail DNA with a ^{32}P-radiolabeled probe (33). Hybridization was performed either as dot blots or as Southern blots. Screening mice for expression of β-galactosidase protein was performed by immunohistochemistry of cryostat sections of tail (30, 31). The transgenic founder mice were then mated to normal F_1 hybrids to establish transgenic mouse lines.

Transgenic animals were studied in an animal model of lipid peroxidation and fibrogenesis, CCl_4 intoxication, as reported previously (8, 15, 34). Transgenic animals (25–40 grams) received a single dose of CCl_4 in mineral oil 1:1 (vol/vol) at a dose of 3 ml/kg or mineral oil, and processed as described previously (15). After 48 h, animals were euthanized, and the liver was utilized for RNA isolation, immunohistochemistry and stellate cell isolation as described previously (15, 29–31).

Gel retardation experiments. Gel retardation assays were performed as discussed previously (21, 29). Double-stranded oligonucleotides were end-labeled with [^{32}P] γ-ATP and T4 polynucleotide kinase by the forward reaction (21). Oligonucleotides were used as follows (sense orientation shown): footprint 1 (5′ CTAGCTGATTGGCTGGGGGCCGGGCT 3′) (21); NF1 (5′ ATTTTGGCTTGAAGCCAATATG 3′) (Stratagene); SP1 (5′ GATCGATCGGGGCGGGGCGATC 3′) (Stratagene); and albumin D site (5′ TGGTATGATTTTGTAATGGGG 3′) (21).

Statistical analysis. Results are expressed as mean (±SEM). Either the Student *t* or the Fisher's exact test (two-tailed) was used to evaluate the differences of the means between groups; a P value of < 0.05 was considered significant.

Results

We first analyzed the expression of the collagen α_1(I) gene in the liver following the induction of hepatic injury and lipid peroxidation by the hepatotoxin, CCl_4. Transgenic mice containing various regulatory regions of the human collagen α_1(I) gene were treated with CCl_4 to induce hepatic lipid peroxidation and fibrogenesis as described previously (15). The degree of hepatocellular necrosis in these animals was similar to that described previously for rats (15), judging by liver staining with hematoxylin/eosin (not shown), and by the release of liver enzymes into the blood (serum alanine transaminase: 2004±254 vs 34±18 IU/ml; $P < 0.05$). Similarly, the degree of lipid peroxidation was comparable to that reported by us in rats treated with CCl_4 or overloaded with iron (15, 16). Protein adducts with malondialdehyde, a product of lipid peroxidation, were detected using specific antibodies against the malondialdehyde-lysine epitopes, as reported previously (15, 16, 30). In contrast to the livers of control animals (Fig. 1 *A*), enhanced lipid peroxidation occurred in mice treated with CCl_4 at 48 h in zones 2 and 3 of the hepatic acinus (Fig. 1 *B*), as reported for rats (15).

The expression of various human collagen α_1(I)-human growth hormone (hGH) transgenes (22) was studied in the liver (see Fig. 2). Collagen α_1(I) and hGH mRNA were analyzed by an RNase protection assay, and simultaneous measurement of GAPDH was used as an internal standard (35). Expression of GAPDH per microgram total RNA was only slightly increased following CCl_4-treatment ($P > 0.05$; NS). In addition, the confounding variable of a different transgene copy number when various lines were compared (22) was avoided by comparing expression under baseline and activated conditions in the same transgenic line (see Figs. 3 and 4). The steady state pool of hGH mRNA (exons 4 and 5) was consistently stimulated by CCl_4, and in parallel to the endogenous mouse collagen α_1(I) gene, whether the collagen α_1(I) regulatory region contained the −0.44 or −2.3-kb 5′ flanking sequence, and in the presence or absence (ΔI) of the first intron (bases +292 to +1607) (Figs. 3 and 4). Because of these unexpected results, and in order to confirm these findings, another transgenic line of −440 COL-hGH (line A) was studied. These animals also responded to CCl_4 intoxication with an increase in hGH transgene expression in the liver comparable to the stimulation of the endogenous collagen α_1(I) gene (Figs. 3 and 4).

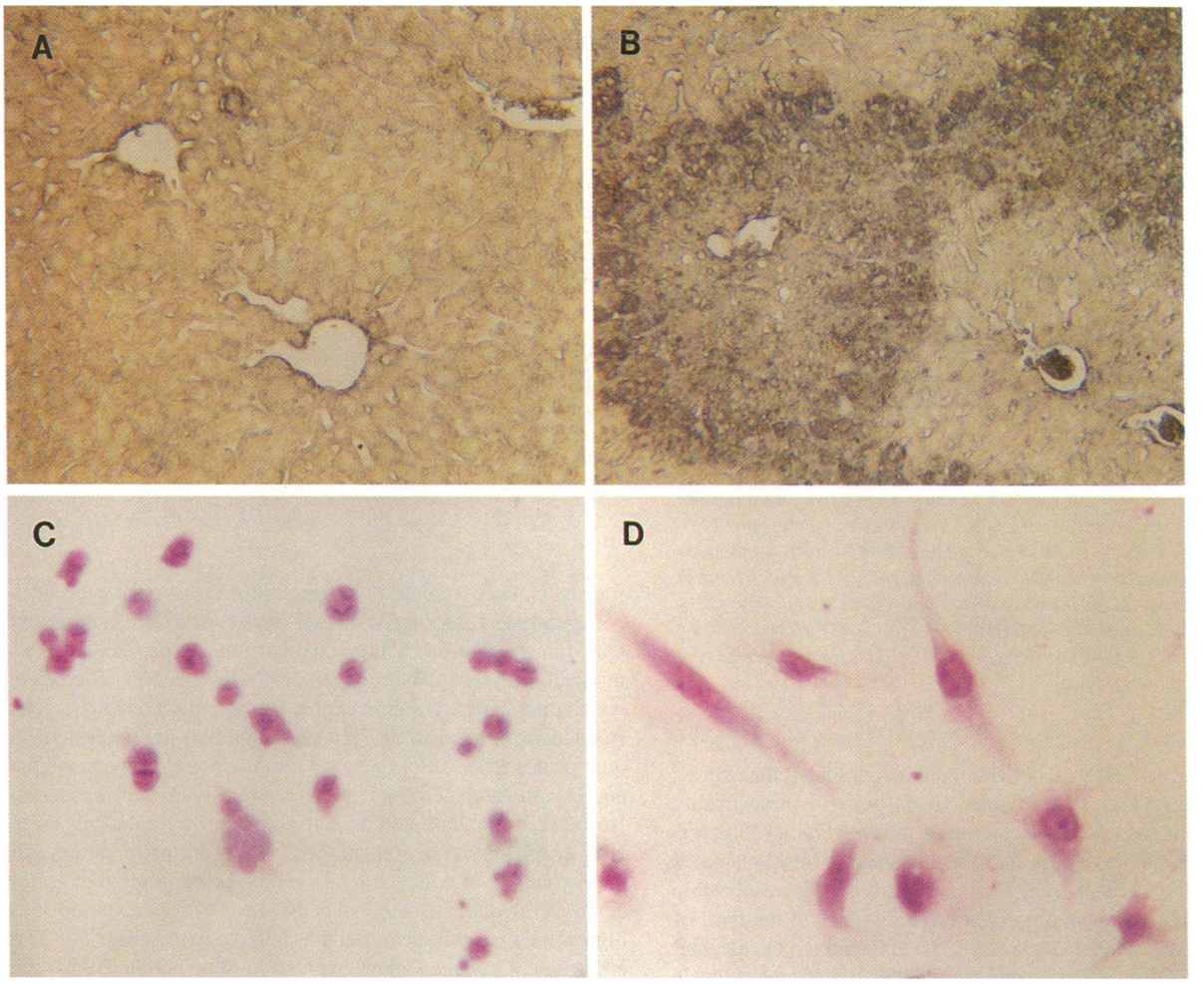

Figure 1. Induction of hepatic peroxidation and stellate cell activation by CCl_4. Animals were studied 48 h after mineral oil (*A* and *C*) or CCl_4 (*B* and *D*) treatment, and liver tissue was processed for immunohistochemistry for malondialdehyde-protein adducts (*A* and *B*), and isolated stellate cells for hematoxylin eosin staining (*C* and *D*) as described in Methods. *(A)* No adducts were detected in mineral oil treated animals. *(B)* Malondialdehyde-protein adducts are detected in the cytosol of hepatocytes in zones 2 and 3 of the acinus. *(C)* Quiescent stellate cells from mineral oil-treated animals. *(D)* Activated stellate cells from CCl_4-treated animals.

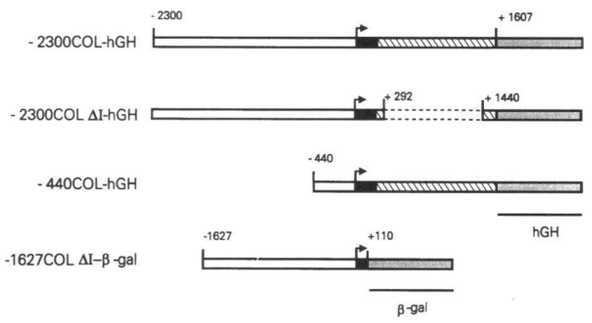

Figure 2. Schematic representation of the human collagen α_1(I)-hGH minigenes and the mouse collagen α_1(I)-β-galactosidase gene. These constructs were used for the generation of transgenic mice. The 5′ flanking regions are shown as open rectangles. The sequences of the first exon and first intron of the collagen α_1(I) genes are indicated in closed and hatched rectangles, respectively. The arrow denotes the start of transcription. The reporter gene sequences of the hGH (exons 4 and 5) and β-galactosidase are indicated in gray rectangles.

The increased transgene expression in the liver following CCl_4 intoxication could reflect expansion of a subpopulation of collagen-producing cells such as stellate cells (6). Because stellate cells are activated (3), and proliferate following CCl_4-induced hepatic injury (Lee et al., unpublished observations), we determined collagen transgene expression per cell. We found that in freshly isolated stellate cells from CCl_4-treated mice, collagen transgene expression was increased per cell, eliminating the confounding variable of stellate cell proliferation. Stellate cells were obtained from control and CCl_4-treated transgenic animals as discussed in Methods. The stellate cell populations were > 95 pure, judging by the autofluorescence at 328 nm or by immunostaining for desmin. Freshly isolated stellate cells from CCl_4-treated animals displayed an activated phenotype (Fig. 1 *D*), and expressed a quantity of α-smooth muscle actin (Lee et al., unpublished observations), similar to that in stellate cells cultured on plastic for 10 d (36, 37). In contrast, stellate cells from control animals had a quiescent phenotype (Fig. 1 *C*), similar to that of stellate cells cultured on plastic for < 3 d (15). The expression of hGH in stellate cells bearing the −440 COL-hGH was markedly increased from control values in mineral oil (vehicle) treated animals (Fig. 3,

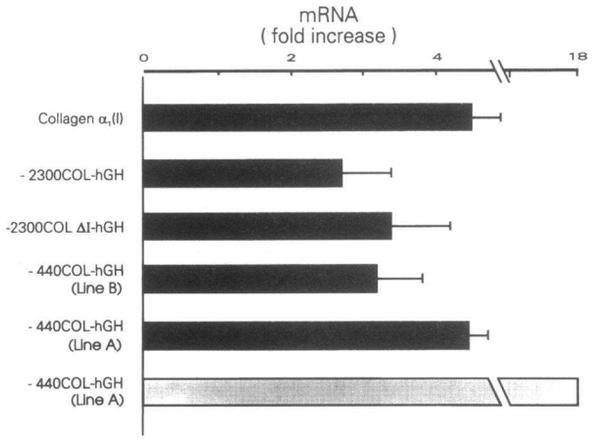

Figure 3. Expression of the human collagen α_1(I)-hGH minigenes in CCl_4-induced hepatic fibrogenesis. Animals were treated with mineral oil or CCl_4 as described in Methods. Liver hGH and collagen α_1(I) mRNA (closed bars), and hepatic stellate cell hGH mRNA *(shaded bar)* were measured by an RNase protection assay 48 h after treatment. Values represent the fold increase from mineral oil (control) to CCl_4-treated animals. In addition, hGH and collagen α_1(I) mRNA values were corrected for GAPDH expression; $P < 0.05$ for all treatments compared to control values.

shaded bar, and Fig. 4), and the increase paralleled the expression of the endogenous collagen α_1(I) gene in these freshly isolated cells, which was greater than sixfold (Fig. 4). Furthermore, the level of expression of the transgene in freshly isolated stellate cells, suggests that these cells are mainly responsible for the increased expression of these transgenes in the liver of CCl_4-treated animals. As expected, the collagen α_1(I) gene and the −440 COL-hGH transgene were expressed at higher levels in freshly isolated hepatic stellate cells than in the entire liver, given that the lower contribution, per cell, of other cell populations toward collagen gene expression (4), would dilute the stellate cell contribution.

The activation of stellate cells in the liver during liver injury and fibrogenesis is characterized by a stellate morphology and by the expression of α-smooth muscle actin (3). These prototypical changes of stellate cell activation are also observed when

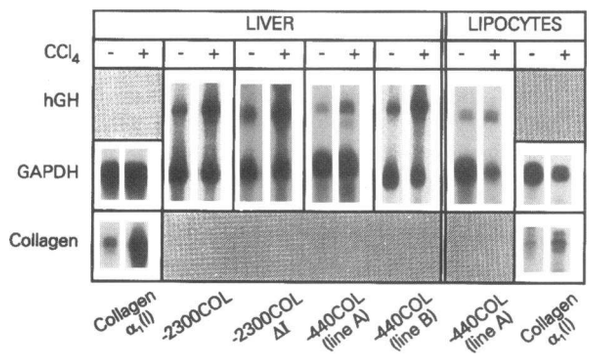

Figure 4. Representative examples of the RNase protection assay comparing the expression of hGH minigenes with the endogeneous collagen α_1(I) gene in CCl_4-induced hepatic fibrogenesis. Samples were obtained as described in Fig. 3. mRNAs for hGH, GADPH and collagen α_1(I) were detected. The specific activity of the probes was identical for each control (−) and experimental (+CCl_4) pair.

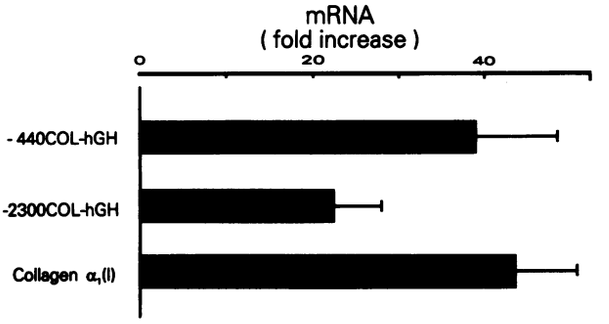

Figure 5. Expression of human collagen α_1(I)-hGH minigenes in hepatic stellate cells. Hepatic stellate cells were isolated from transgenic animals and cultured on plastic for 10 d. Values represent the fold increase from quiescent (day 1) to activated (day 10) stellate cells as measured by an RNase protection assay. $P < 0.05$ for all activated conditions compared to quiescent stellate cells.

freshly isolated stellate cells are cultured on plastic or on a collagen type I matrix for several days (36, 37). Concurrently with the acquisition of the activated phenotype, stellate cells markedly increase their collagen synthesis (36, 37). Therefore, we studied hGH transgene expression in cultured stellate cells isolated from the various transgenic animals. As expected, in quiescent stellate cells there was negligible expression of the endogenous collagen α_1(I) gene as well as of the transgenes. However, following stellate cell activation by culturing these cells on plastic for ten days, there was a dramatic increase in the expression of collagen α_1(I) gene. The expression of collagen α_1(I) in activated stellate cells (day 10) was about 40-fold higher than in quiescent stellate cells (day 1) (Fig. 5). The endogenous collagen gene expression was paralleled by the transcriptional activation of the −440 COL-hGH transgene (Fig. 5). Thus, the transactivation of collagen α_1(I) gene expression in cultured, activated stellate cells requires only −0.44 kb of the 5′ regulatory region, and resembles the regulation of these cells in the intact animal after CCl_4 treatment. As expected, there was also a large stimulation of the expression of the −2300 COL-hGH transgene in activated stellate cells when compared to quiescent stellate cells (Fig. 5). The apparent, reduced expression of this transgene in activated cultured stellate cells, in comparison with the −440 COL-hGH, is due mainly to a higher level of induction in quiescent stellate cells, and suggests a mild but spurious activation of the −2300 COL-hGH under these conditions. Indeed, the hGH mRNA/GAPDH mRNA ratio (expressed in densitometric units) for the −440 COL-hGH and −2300 COL-hGH mRNAs in day 0 stellate cells, was 1.0 and 4.6, respectively. The comparable values for the −440 COL-hGH and −2300 COL-hGH mRNAs in day 10 stellate cells, were 40.3 and 106, respectively. The variable baseline expression of the transgenes is related to copy number and "leakiness" of the construct, and it is therefore less informative than the increase in expression following activation, as described previously (23).

Taken together, these studies indicate that in activated stellate cells, the −0.44 kb region of the collagen α_1 (I) gene contains the responsive element(s) needed to direct transcription of this gene during hepatic fibrogenesis induced by CCl_4. Similarly, the −0.44 kb 5′ flanking sequence is sufficient to stimulate hGH transgenes in stellate cells that are activated by culture on plastic.

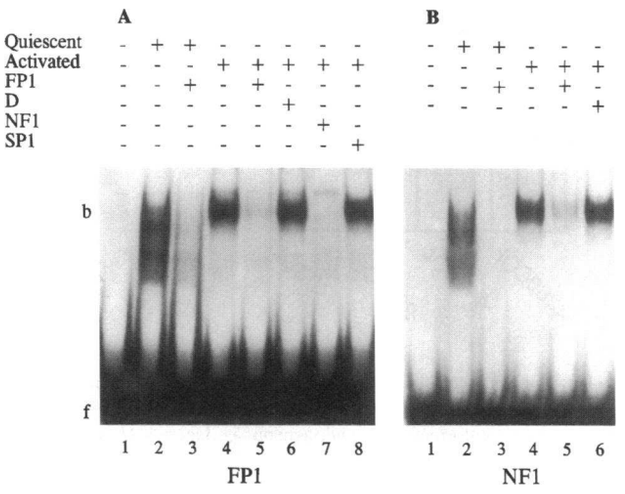

Figure 6. NF1 binding activities are increased in activated stellate cells. *(A)* Mobility shift analysis with nuclear extracts from quiescent (lanes *2* and *3*) and activated (lanes *4–8*) stellate cells, and an oligonucleotide corresponding to footprint 1 (FP1; −103 to −82). Samples were incubated with ^{32}P-labeled FP1 oligonucleotide (1 ng). Nuclear extracts on lanes 3 and 5–8 were preincubated with FP1, NF1, D, or SP1 oligonucleotides (25 ng), as indicated. *(B)* Mobility shift analysis with nuclear extracts from quiescent (lanes *2* and *3*) and activated (lanes *4–6*) stellate cells, and an oligonucleotide corresponding to a consensus sequence for binding of NF1. Samples were inculated with ^{32}P-labeled NF1 oligonucleotide (1 ng). Nuclear extracts on lanes *3* and *5*, and *6* were preinculated with FP1 or D oligonucleotides (25 ng), as indicated. The DNA-protein complexes were resolved by electrophoresis on a 6% nondenaturing polyacrylamide gel. The positions of the free *(f)* and bound *(b)* DNA are shown. Equal amounts of nuclear protein were used in all lanes, except in lanes *1*, which were incubated without nuclear extracts.

CCAAT binding proteins are thought to be important in the regulation of the collagen α_1(I) gene because in human, rat, and mouse two highly conserved CCAAT binding sequences are present in the collagen α_1(I) promoter sequence (19). CBF is a CCAAT binding factor isolated from liver nuclear extracts which binds to both CCAAT sites (38). Also, LAP, a bZIP transcription factor, binds to and activates transcription from the proximal CCAAT element (footprint 1;FP1) (21). Furthermore, NF-1 binds to both CCAAT box sites, and might play a role in collagen gene expression in fibroblasts (39). For these reasons, we studied the binding affinity of nuclear proteins from quiescent and activated stellate cells to *cis*-acting elements of the collagen α_1(I) promoter. As shown in Fig. 6 *A* (lane *4*), nuclear extracts from activated stellate cells formed a complex with a [^{32}P]-FP1 cognate DNA element (bp-82 to-103 of the collagen α_1(I) gene). The formation of this DNA-protein complex was efficiently competed by FP1 oligonucleotide, as expected, and by an NF1 oligonucleotide (lane *7*). In contrast, neither LAP (albumin D site) (21) nor SP1 *cis*-acting elements were able to disrupt the DNA-protein complex (lanes *6* and *8*). Moreover, nuclear proteins from activated stellate cells formed a similar complex with a [^{32}P]NF1 oligonucleotide (Fig. 6 *B*, lane *4*), which was competed by a FP1 oligonucleotide (lane *5*) but not a D site oligonucleotide (lane *6*). The different pattern of DNA-protein complexes produced by the binding of nuclear extracts from quiescent stellate cells to both [^{32}P]FP1 and [^{32}P]NF1 was also competed by FP1 oligonucleotides, indicating the binding of different or modified nuclear proteins to these sites. These results suggest that the proximal CCAAT element, and in particular the NF1 cognate sequence, may be important in the increased transcription of the collagen α_1(1) gene in activated stellate cells.

In addition, we analyzed the expression of the transgenes and collagen α_1(I) gene, in skin and tendon from these animals (Fig. 7). As depicted in Fig 7, *A* and *B*, in skin and tendon the −2.3-kb region directs the transcription of the hGH reporter gene at much higher levels than does the −0.44-kb region. The expression of the −440 COL-hGH transgene was only 10–20% that of the −2300 COL-hGH transgene in skin and tendon. Moreover, the −2.3-kb region was required for the level of expression of the transgene to reflect closely the expression of the endogenous collagen α_1(I) (data not shown). Collectively, these data indicate that the −0.44-kb 5′ region of the collagen α_1(I) gene contains the *cis*-element(s) critical for maximal *trans*-activation of this gene in the liver of animals during the induction of fibrogenesis, but not for maximal expression of the gene in skin and tendon.

Complementary information was obtained with another transgenic line of mice containing the β-galactosidase reporter gene driven by the mouse collagen α_1(I) promoter (−1627/+110 bp) (see Fig. 2). The functional expression of the transgene was determined by slot blots of tail DNA probed with a ^{32}P-labeled β-galactosidase gene fragment (not shown). Transgene expression was confirmed by β-galactosidase immunohistochemistry of cryostat sections of the tail (see below). This transgene was also activated in the liver by CCl_4 treatment, resulting in a marked increase in the expression of β-galactosidase, as detected by immunofluorescence, almost exclusively in sinusoidal cells with the phenotype of activated stellate cells (Fig. 8, *B* and *C*). No expression of the chimeric −1627 COL-β-galactosidase reporter gene was seen when the first antibody was omitted (not shown), or when transgenic animals were treated with mineral oil (Fig. 8 *A*). This experiment supports our earlier conclusion that the first intron is not essential for high expression of the collagen α_1(I) gene in activated stellate cells (see Fig. 3).

As expected, the expression of the −1627 COL-β-galactosidase transgene was negligible in skin (Fig. 9 *B*), since collagen gene expression in dermal fibroblasts has been shown to require the first intron (23). However, expression of the −1627 COL-β-galactosidase transgene was readily detected in tendon, indicating that the first intron is not required for collagen α_1(I) gene expression in tendon fibroblasts (Fig. 9 *B*). No expression was observed when the first antibody was omitted (Fig. 9 *A*), or in nontransgenic animals (not shown). These findings suggest that the −1627/+110-bp region of the collagen α_1(I) gene is sufficient to direct the expression of the β-galactosidase transgene in tendon fibroblasts. Collectively, these data indicate that two different *cis*-regulatory regions direct cell-specific transcription of the collagen α_1(I) gene; the −0.44-kb 5′ region is sufficient in activated stellate cells, while the −2.3 to −0.44-kb region is required for maximal expression in skin and tendon fibroblasts.

Discussion

In this study, we have identified different regulatory regions of the collagen α_1(I) gene that are activated in a cell-specific manner in either stellate cells or skin and tendon fibroblasts, and result in high levels of expression of the gene. The −0.44-kb region is sufficient to fully direct the transcription of a hGH

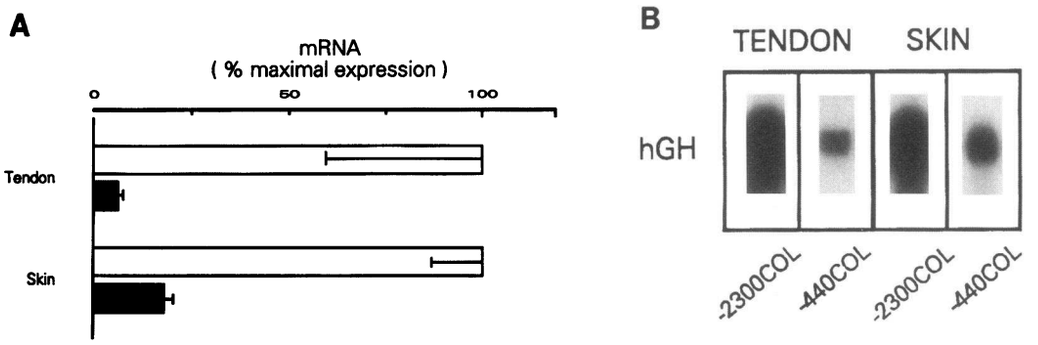

Figure 7. Expression of human collagen α_1(I)-hGH minigenes in tendon and skin. Tissues were obtained from 3-wk-old transgenic animals. hGH mRNA was measured by an RNase protection assay. *(A)* Values are percentages of expression for −2300 COL-hGH *(open bars)* and −440 COL-hGH *(closed bars)* minigenes, with the value for −2300 COL-hGH set at 100. Values were corrected for endogeneous collagen α_1(I) expression. $P < 0.05$ for −2300 COL-hGH for tendon and skin. *(B)* Representative examples of the tendon and skin RNase protection assays comparing the expression of the hGH minigenes (0.4 and 3 μg RNA, respectively).

transgene in activated stellate cells. High expression of the hGH transgene occurred in the presence or absence of the first intron of the collagen α_1(I) gene. Using another transgenic animal bearing −1.6 kb of the 5′ region of the collagen α_1(I) gene, we confirmed that the critical *cis*-element(s) for hepatic stellate cells were not situated in the first intron. However, the −440-

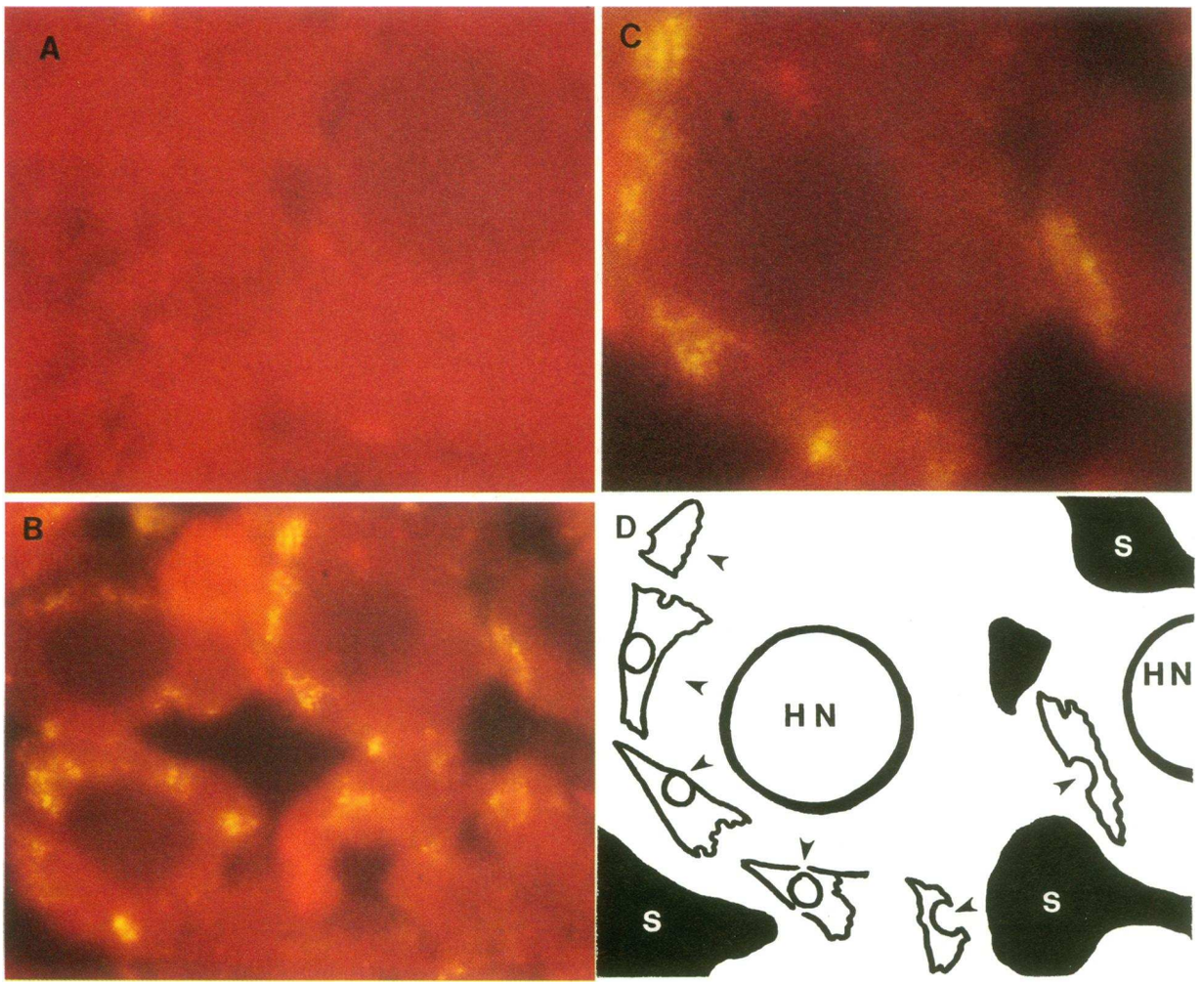

Figure 8. Increased expression of the −1627 COL-β-galactosidase in the liver following CCl_4 treatment. Immunodetection of β-galactosidase *(yellow)* was performed using β-galactosidase antibodies and fluorescein-labeled second antibodies. Evans blue *(red)* was used as counterstain. Liver tissue was obtained from mineral oil (*A*, ×1000) and CCl_4 (*B*, ×400; and *C*, ×1000) treated animals. *D* is a schematic of *C*. The sinusoids *(S)*, hepatocyte nuclei *(HN)*, and hepatic stellate cells *(arrows)* are depicted.

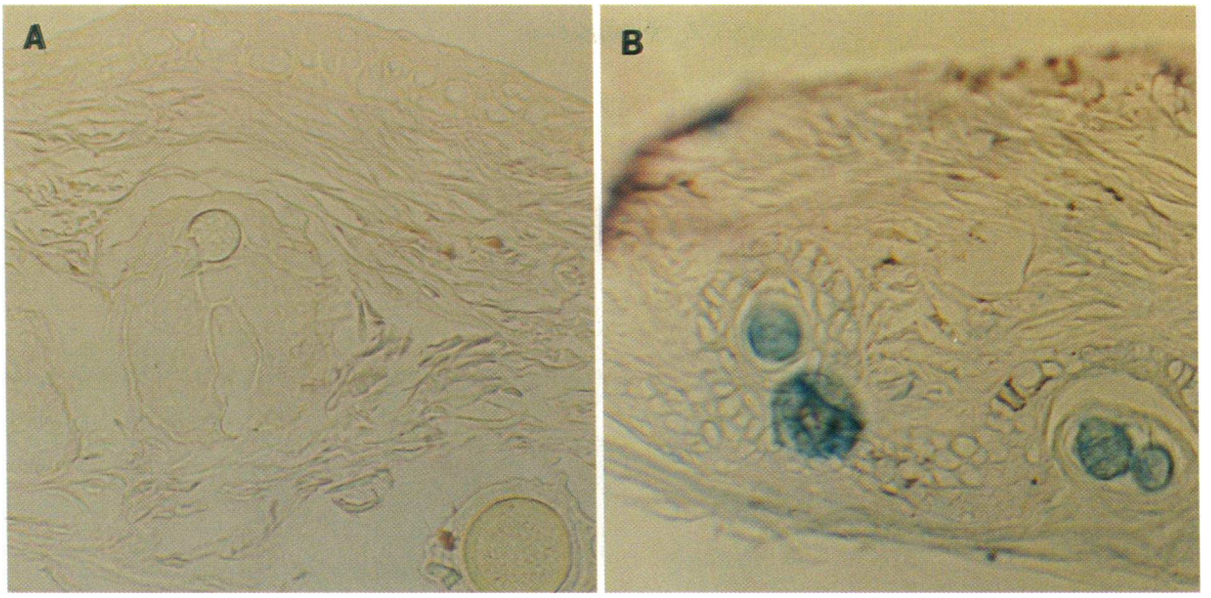

Figure 9. Expression of the −1627 COL-β-galactosidase transgene in tendon. β-galactosidase was detected by immunohistochemistry using β-galactosidase antibodies and avidin-biotin-alkaline phosphate labeled second antibodies *(blue)* (×100). Tail tissue was obtained from nontransgenic *(A)* and transgenic *(B)* animals.

bp 5′ region is unable by itself to direct high level transcription of the collagen α_1(I) gene in skin or tendon fibroblasts.

In tissues with high expression of the collagen α_1(I) gene, the −0.44-kb 5′ flanking sequence has previously been shown to be insufficient to direct expression of the gene. Krebsbach et al. (24), found that in calvarial bone, the region between −2296 and −1672 bp of the collagen α_1(I) gene was indispensable for its expression. In addition, Bornstein and co-workers have reported (22, 23) that the region between −2.3 to −0.44-kb was necessary for collagen α_1(I) expression in skin and tail. Expression of the −440 COL-hGH transgene in the tail was about 10-fold lower than that of the −2300 COL-hGH transgene (22). However in the tail, tendon contributes only a small percentage to the total RNA, and other tissue components of the tail (skin, bone, etc.), which do not express the −440 COL-hGH transgene, could have diluted the contribution of tendon when whole tail is analyzed (22). Our finding that the −0.44-kb 5′ region is insufficient to direct maximal transcription of the hGH transgene in tendon fibroblasts, indicates that full expression of the collagen α_1(I) transgene requires the −2.3 to −0.44-kb region of the collagen gene.

Tissue-specific transcriptional control of the collagen α_1(I) gene has been suspected, since the insertional mutation of the Moloney murine leukemia virus affects collagen expression in fibroblasts but not in odontoblasts (40). This altered transcriptional activity of the collagen α_1(I) gene is probably the result of a change in chromatin conformation induced by the viral insertion (41). Our finding of cell-specific *cis*-acting regulatory regions within the collagen α_1(I) 5′ regulatory region has another precedent. The α-amylase gene contains a double promoter acting selectively in parotid gland cells and hepatocytes, as reported by Schibler and co-workers (42). However, the expression of the α-amylase gene is weak in hepatocytes, whereas the expression of the collagen α_1(I) gene is strong in both activated stellate cells and in skin and tendon fibroblasts.

Stellate cells play a key role in the pathogenesis of hepatic fibrosis (4, 5). Although we (27) and others (36, 37) have reported that quiescent stellate cells produce little collagen type I, activated stellate cells display a high level of collagen α_1(I) gene expression (4, 36, 37). Therefore, stellate cell activation is essential for hepatic fibrogenesis. Relevant to this study, co-culture experiments of hepatocytes and stellate cells treated with CCl_4 indicate that hepatocytes exert a paracrine stimulation of both lipid peroxidation and collagen gene expression in stellate cells (15). These results strongly suggest that lipid peroxidation in the absence of inflammation is capable of inducing, directly or indirectly, the expression of collagen α_1(I) in stellate cells. In agreement with our findings, stellate cell collagen gene expression is stimulated by 4-hydroxynonenal, a product of lipid peroxidation (26).

Analysis of the expression of relevant deleted or mutated collagen α_1(I) chimeric genes in activated stellate cells, should allow us to identify the *cis*-acting element(s) within the −0.44-kb region responsible for activation of the collagen α_1(I) gene in these cells. The finding that the proximal CCAAT box of the promoter may play a role in activated stellate cells, and the recent identification of the *cis*-element Col E-1 (21), within this critical region for activated stellate cells, could facilitate the isolation of the gene(s) encoding the *trans*-acting factors that bind to the collagen α_1(I) gene in these cells. In conclusion, our results provide novel insights into potential mechanisms for the cell-specific regulation of collagen α_1(I) gene expression in hepatic stellate cells and support previous studies of the regulation of this gene in skin and tendon fibroblasts.

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