

Zf9, a Kruppel-like transcription factor up-regulated *in vivo* during early hepatic fibrosis

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ABSTRACT Wound repair in the liver induces altered gene expression in stellate cells (resident mesenchymal cells) in a process known as “activation.” A zinc finger transcription factor cDNA, *zf9*, was cloned from rat stellate cells activated *in vivo*. Zf9 expression and biosynthesis are increased markedly in activated cells *in vivo* compared with cells from normal rats (“quiescent” cells). The factor is localized to the nucleus and the perinuclear zone in activated but not quiescent cells. Zf9 mRNA also is expressed widely in nonhepatic adult rat tissues and the fetal liver. The *zf9* nucleotide sequence predicts a member of the Kruppel-like family with a unique N-terminal domain rich in serine–proline clusters and leucines. The human *zf9* gene maps to chromosome 10P near the telomere. Zf9 binds specifically to a DNA oligonucleotide containing a GC box motif. The N-terminal domain of Zf9 (amino acids 1–201) is transactivating in the chimeric GAL4 hybrid system. In *Drosophila schneider* cells, full length Zf9 transactivates a reporter construct driven by the SV40 promoter/enhancer, which contains several GC boxes. A physiologic role for Zf9 is suggested by its transactivation of a collagen $\alpha 1(I)$ promoter reporter. Transactivation of collagen $\alpha 1(I)$ by Zf9 is context-dependent, occurring strongly in stellate cells, modestly in Hep G2 cells, and not at all in *D. schneider* cells. Our results suggest that Zf9 may be an important signal in hepatic stellate cell activation after liver injury.

Wound repair in parenchymal tissues requires coordinate changes in resident mesenchymal cells that ultimately lead to fibrosis. In the liver, this response involves hepatic stellate cells, which are a nonparenchymal cell type located in the subendothelial space of the sinusoid. Stellate cells express the myogenic intermediate filament desmin and, in normal liver, are the primary storage site for retinoids, which are found in cytoplasmic droplets as retinyl esters (1). Analogous cells in other organs include mesangial cells in kidney (2) and pulmonary fibroblasts in lung (3).

The response of stellate cells to injury is termed “activation” and represents a cellular program with a distinct temporal sequence involving both up- and down-regulation of gene expression (see ref. 4 for review). Its salient features include markedly increased production of extracellular matrix, cellular proliferation caused by induction of mitogens and their receptors (5, 6), increased expression of key cytokines such as transforming growth factor $\beta 1$ (7), contractility (8), increased matrix degrading activity (9), decreased expression of some antioxidant genes (10), and loss of cellular retinoid (11).

The development of methods for isolating and culturing stellate cells has provided an important tool for exploring molecular mechanisms of stellate cell activation. Analysis of gene expression in freshly isolated cells from a normal or injured liver provides an

accurate profile of their behavior *in vivo* (12). Stellate cell activation also occurs when normal cells are grown in primary culture on either plastic or type I collagen, providing a culture model of this response (13). These two approaches—*in vivo* and primary culture—are complementary for identifying regulatory mechanisms in stellate cell activation.

Given the pleomorphic nature of stellate cell activation, attention has been directed toward transcription factors that may regulate induced genes. To date, however, these efforts have focused only on a few known transcription factors whose targets might include inflammation- and fibrosis-related genes. Such factors include c-myc (14), NF- κ B (14), and c-jun/AP1 (15).

To broaden the search for relevant transcription factors, we recently have used subtraction hybridization (16) to identify transcripts induced early in rat stellate cells *in vivo* after a single dose of CCl₄, a known precipitant of hepatic fibrosis. Cloning of induced cDNAs was performed at an early time point after injury to favor the recovery of regulatory rather than structural genes. By using this approach, a partial cDNA was isolated with homology to zinc finger proteins (16). In this report, we have isolated the full length rat and human cDNAs, which we term *zf9*, and characterized production of Zf9 protein. Importantly, we have established Zf9 as a nuclear DNA-binding protein in activated cells that, in a context-dependent manner, transactivates a key promoter driving stellate cell fibrogenesis.

MATERIALS AND METHODS

Rat Hepatic Stellate cDNA Library Construction and Cloning of Zf9. Rat hepatic stellate cells were isolated as described by *in situ* portal vein perfusion and density gradient separation (17) from animals treated 1–3 hr previously with intragastric CCl₄ (18) to induce acute hepatic injury. The rat full length cDNA was cloned by first screening a cDNA library constructed from *in vivo*-activated stellate cells by using the original 589-bp subtracted cDNA as a probe (16), followed by rapid amplification of cDNA ends–PCR (Marathon-Race, CLONTECH) using activated stellate cell cDNA as template.

Cloning of Human Zf9 cDNA. A combination of conventional, degenerate, and rapid amplification of cDNA ends–PCR was used to clone the human Zf9 cDNA homologue. After these experiments were completed, a nearly identical sequence was entered into the GenBank database by Koritschoner (19) describing a protein termed CPBP derived from human placenta.

Cells and Plasmids. Rat and human hepatic stellate cells were isolated and cultured as described above and previously

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Abbreviations: ZAD, Zf9 activation domain; GST, glutathione S-transferase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. U73759 (rat Zf9 cDNA) and AF001461 (human Zf9 cDNA)].

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(17). The following cells and lines were used: Hep 3B and Hep G2 hepatoma cells, rat skin fibroblasts, PAC-1, 293T cells, and S2 *D. schneideri* cells. The following plasmids were used: pGLcontrol, containing the SV40 promoter and enhancer (Promega); pAC-Sp1 (a gift of Robert Tjian, Univ. California, Berkeley); pGL-Col 3 (ref. 20; a gift of Richard Rippe, Univ. North Carolina, Chapel Hill); and GAL4-Sp1 and GAL4 reporter containing the GAL4 upstream activator sequence (a gift of Moshe Oren, Weizmann Institute, Israel) (21).

Plasmids containing regions of *zf9* were amplified by PCR and were cloned into *EcoRI* and *XbaI* sites of either pCIneo (Promega) or a GAL4-DNA binding domain expression plasmid, or they were cloned into *BamHI/XhoI* sites of pAC as follows: rat and human *pCIneo-zf9*, full length rat and human cDNAs; *pAC-zf9*, nucleotides 1–882 of rat *zf9*; *pAC-ZAD* (Zf9 activation domain), nucleotides 1–637 of rat *zf9*; *pAC-zf9 DBD* (DNA binding domain), nucleotides 619–881 of rat *zf9*; *GAL4-Zf9*, the full length rat *zf9* cDNA in the GAL4-DNA binding domain plasmid (21); and *GAL4-ZAD*, nucleotides 1–639 of rat *zf9* in GAL4-DNA binding domain plasmid.

Constructs expressing Zf9 domains as fusion peptides with glutathione *S*-transferase (GST) were cloned into pGEX-2 and were expressed in JM 109 bacteria. These included: full length Zf9 (pGEX2-Zf9) and Zf9 activation domain (pGEX2-ZAD), nucleotides 4–639; and Zf9 DNA binding domain (pGEX2-Zf9 DBD), nucleotides 619–882.

Chromosomal Localization of Zf9. Primers defining a 162-bp region of the human Zf9 cDNA (nucleotides 472–634) were used to select a bacterial artificial chromosome clone from a human bacterial artificial chromosome genomic library

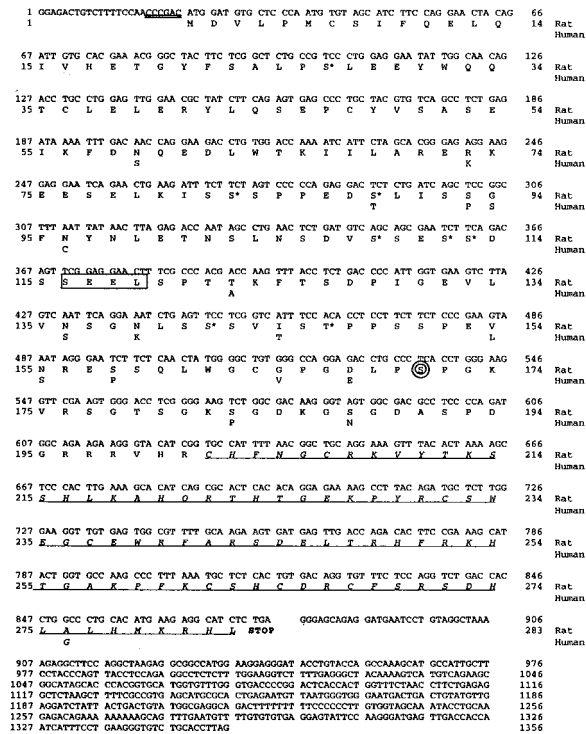


FIG. 1. Rat *zf9* cDNA and deduced amino acid sequences of rat and human Zf9. The rat cDNA sequence is shown, beneath which are the deduced amino acid sequences of rat and human Zf9 (only those amino acids different from rat are shown for the human sequence). The Kozak consensus sequence just preceding the initiator methionine is indicated by a double underline. A potential endoplasmic reticulum targeting signal is boxed (amino acids 116–119). Potential O-linked glycosylation sites are indicated by amino acids with an asterisk. A potential Cdc2 kinase phosphorylation site is indicated by a double circle around serine 171. The C-terminal domain predicting three C2H2 zinc fingers is identified by a single underline.

as described (22). The location of the probes was determined by digital image microscopy after fluorescence *in situ* hybridization and was localized by the fractional length from the p-terminal as described (22, 23). The chromosomal localization subsequently was confirmed by using MACVECTOR software (Oxford Molecular Group) to search all available sequence tag sites with the BLAST algorithm.

Generation of Anti-Zf9 Polyclonal Antiserum. A 67-kDa GST-fusion peptide containing amino acids 28–199 of the rat Zf9 putative activation domain (pGEX-2-PM) was purified by glutathione affinity chromatography, was suspended in PBS, and was mixed with either complete (initial inoculation) or incomplete (booster inoculations) Freund's adjuvant at a concentration of 20 μ g/ml. New Zealand white rabbits were prescreened for anti-*Escherichia coli* immunoreactivity, and three animals with no reactivity were injected three times, according to standard methods. Antisera was screened and titered by Western blot analysis to 1:100,000.

Zf9 Immunocytochemistry and Western Blot Analysis of Rat Stellate Cells. Normal, freshly isolated rat stellate cells and cells in primary culture for 14 days were placed in two-well glass chambers (Nunc) in the presence of 20% serum. Freshly

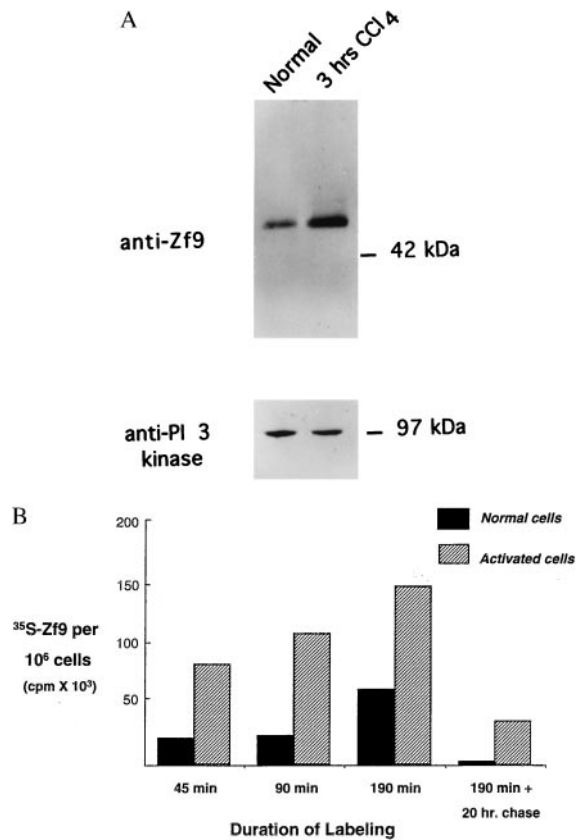


FIG. 2. (A) Induction of Zf9 during stellate cell activation *in vivo*; Western blot analysis of freshly isolated stellate cell lysates from a normal rat and a rat treated 3 hr earlier with CCl₄. A single protein of \approx 46 kDa is identified. The blot was reprobed with anti-phosphatidylinositol 3-kinase. The induction after CCl₄ represents a 3-fold increase. A similar induction was reproducible in three experiments. (B) Increased biosynthesis of Zf9 during stellate cell activation *in vivo*. Pooled stellate cells from either two normal rats or two rats administered CCl₄ were plated in methionine-free medium containing [³⁵S]methionine and harvested at 45, 90, and 190 minutes or after 190 minutes with [³⁵S]methionine followed by a 20-hr chase in unlabeled medium. [³⁵S]Zf9 was purified by immunoaffinity chromatography by using a column containing anti-Zf9. SDS/PAGE autoradiography of eluted protein confirmed specific incorporation of [³⁵S]methionine into Zf9 (not shown). Data is depicted here as specific [³⁵S]methionine incorporation into protein from normal and activated stellate cells, normalized for cell number.

isolated cells were cytospun. Cells were fixed in 2% paraformaldehyde (in PBS containing 0.1% Triton X-100), were blocked with 1% fish gelatin (Sigma), were incubated for 3 hr at 37°C with either anti-Zf9 in PBS (1:10,000 in blocking buffer) or preimmune serum (from the same animal, before immunization), and were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200) (Molecular Probes) for an additional 60 min. After additional washes, cells were mounted (Dako fluorescence mounting medium) and were viewed for epifluorescence.

For Western blot analysis of cultured cells, lysate from 5.0×10^5 cells was loaded per lane. For the Western blot analysis of stellate cells activated *in vivo*, protein concentration was normalized by colorimetric assay (Coomassie Plus protein assay, Pierce), and 40 μ g total protein was loaded per lane. Induction of Zf9 was determined by calculating the relative densitometric intensity of the Zf9 band normalized to phosphatidylinositol 3-kinase detected in the same blot (Upstate Biotechnology, Lake Placid, NY). Blots were incubated in primary antibody (anti-Zf9, diluted 1:100,000) for either 2 hr at room temperature or overnight at 4°C. Secondary antibody [horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham)] was used according to manufacturer's instructions at 1:2,000 dilution, followed by enhanced chemiluminescence protocol (Amersham).

Immunoaffinity Purification of Newly Synthesized Zf9. Anti-Zf9 IgG was purified from the rabbit anti-Zf9 antiserum by protein G Affi-column (Pharmacia) and was immobilized (2 mg IgG/ml bed volume) on hydrazide-activated beads (Bio-Rad). Stellate cells from paired normal rats or animals treated 20 hr earlier with CCl₄ were isolated, were washed three times in methionine-free medium 199, and were plated in this medium supplemented with 1 mCi of [³⁵S]methionine per 60-mm dish. After 45, 90, or 190 min, the free isotope was removed by washing three times in serum-free medium. After labeling, one set of cells was maintained in unlabeled medium for an additional 20-hr chase period in serum-containing medium, was harvested, and was lysed in 1 \times reporter lysis buffer (Promega) supplemented with protease inhibitors, then

was incubated with immobilized IgG overnight at 4°C with gentle shaking. Unbound material was removed by washing with 40 bed volumes of PBS, and residual buffer was drained by a brief centrifugation (30 seconds at 2,000 \times g). The bound protein was eluted by 6 M urea (in 0.1 M Tris-HCl, pH 7.5) and then was renatured by using a P-6 Biospin column (Bio-Rad) preequilibrated with 20 mM Hepes (pH 7.4), 1 mM MgCl₂, 10 μ M ZnSO₄, 20 mM KCl, and 8% glycerol.

mRNA Analysis. Poly(A)⁺ RNA was extracted from culture-activated stellate cells or whole organs by using a commercial kit (Micro-Fastrack, Invitrogen) and was stored at -70°C in diethylpyrocarbonate-treated water. RNase protection for Zf9 mRNA quantitation was performed exactly as described (12) by using 5×10^5 cpm per probe and 1 μ g mRNA, was incubated overnight, and was digested with T2 RNase.

Gel Shift Assay. Gel shift assay was performed exactly as described (24), with or without anti-Zf9 antiserum. Double-stranded oligonucleotides representing consensus binding sites for Sp1, GATA-1, and Egr-1 were purchased from Santa Cruz Biochemicals, were gel purified in 12% acrylamide, and were end-labeled with [γ -³²P]ATP. For each reaction, 20 ng of recombinant protein was incubated with 2 ng of labeled oligonucleotide in the presence of 1 μ g poly[d(I-C)] and 20 μ l binding buffer (20 mM Hepes, pH 7.4/1 mM MgCl₂/10 μ M ZnSO₄/20 mM KCl/8% glycerol) and was separated on a nondenaturing 5% polyacrylamide in 0.5 \times TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3).

Transactivation Assays. Transient transfection of cultured cell lines (Hep G2 and *D. schneider* S2 cells) was performed by using calcium phosphate precipitation. Primary rat stellate cells were transfected with 5 μ l lipofectamine (2 μ g/ μ l) (BRL) containing 1 μ g total DNA per plate. Transfection efficiency was monitored for each condition by using 1 μ g per plate of a positive control expression plasmid in parallel [either pCMV luciferase or p β gal-Control (CLONTECH)]. Cells were harvested in lysis buffer 48 hr after transfection, and 10 μ g of protein lysate was used for luminescence detection (Analytical Luminescence Laboratory, San Diego).

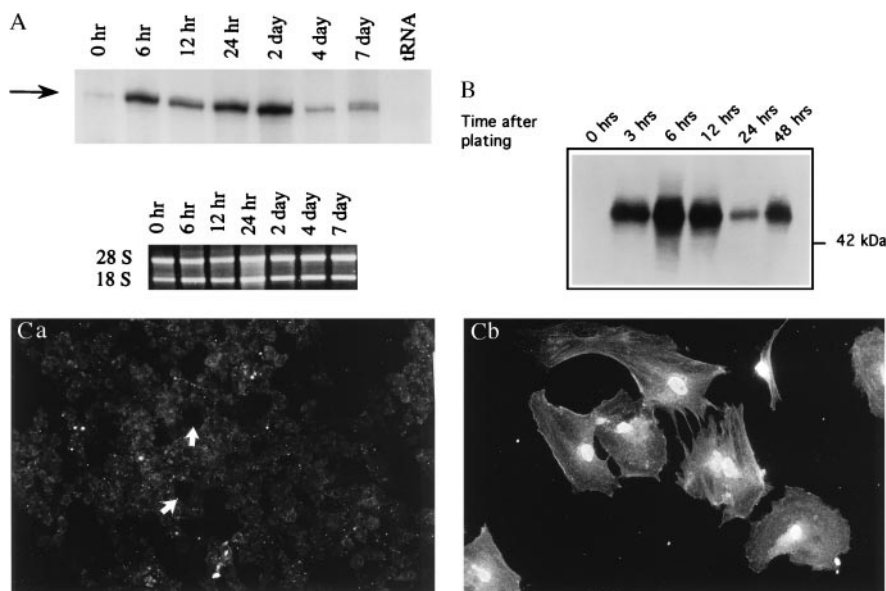


FIG. 3. Induction of Zf9 and nuclear translocation during stellate cell activation in culture. (A) RNase protection in rat hepatic stellate cell isolates at intervals after plating on uncoated plastic. A 624-bp cRNA probe derived from the amino terminus (nucleotides 1–624) was used. The specific band is indicated by an arrow. Time 0 hr indicates mRNA derived from cells of a normal rat before plating. The 28S and 18S ribosomal RNA bands from the identical aliquots of RNA are indicated for each time point. Similar results were obtained in four different experiments. (B) Western blot of cells from the same isolate plated for increasing intervals on plastic identifies a single protein of \approx 46 kDa, which is induced markedly within 3 hr of plating. (C) Immunofluorescence of rat stellate cells with anti-Zf9 antiserum is shown. (a) Freshly isolated cells after cytospin. Faint, patchy fluorescence is apparent surrounding cytoplasmic retinoid droplets, but nuclei are negative (white arrows). Cell borders cannot be appreciated. (\times 250.) (b) Cells maintained in culture on uncoated plastic for 14 days. There is marked nuclear and slight perinuclear fluorescence. Cells from the same isolate incubated with preimmune serum were completely negative (not shown). (\times 250.)

RESULTS

cDNA Cloning, Characterization, and Chromosomal Localization of Zf9. A 589-bp cDNA was cloned previously from activated rat hepatic stellate cells by subtractive hybridization, and early induction of the corresponding mRNA was demonstrated in mechanistically distinct models of liver injury *in vivo* (16). The cDNA was of particular interest because it predicted a translation product with homology to a zinc finger protein, suggesting a potential role as a transcriptional regulator. Full length cDNA cloning of the rat and human *zf9* cDNAs reveals high (95%) interspecies homology at the amino acid level (Fig. 1). The human sequence is 98.5% identical to CPBP, a transcriptional regulator recently cloned from placenta (19). A single ORF of 849 bp is predicted in both rat and humans and is preceded by a short 5' untranslated region. The predicted polypeptide has a calculated molecular weight of 31,821. *In vitro* translation of Zf9 in rabbit reticulocyte lysate confirms a polypeptide of 32 kDa (not shown).

The predicted translation product from *zf9*'s single ORF contains two apparent domains, an N-terminal, 201-aa region rich in serine (25%), leucine, and proline and a highly conserved, 82-aa, C-terminal domain containing three Cys₂-His₂ zinc fingers. Whereas the sequence of the N-terminal 201 amino acid of Zf9 is unique, the carboxy terminal 82 amino acid predicts a typical zinc finger DNA-binding domain of the C2H2 type, with high homology to several "Kruppel-like" factors cloned recently from a variety of tissues. These factors include BTEB2 (25), EKLF (26), BKLF (27), LKLF (28) and GKLF/EZF (29, 30).

The *zf9* gene was mapped by fluorescence *in situ* hybridization to the telomere of chromosome 10P, similar to results recently reported for CPBP (31). This is distinct from the location of related Kruppel-like factors, including EKLF (32) and Sp1 (33). A BLAST search of sequence tag sites localized the human Zf9 sequence to sequence tag sites WI-12084.

Zf9 Protein Induction and Biosynthesis *in Vivo* and Nuclear Localization During Hepatic Stellate Cell Activation. Zf9 protein expression and biosynthesis were examined *in vivo*. Western blot analysis of purified stellate cells documented a 3-fold induction of a single 46-kDa protein 3 hr after administration of CCl₄ (Fig. 2A). This corresponds closely to the time and magnitude of induction *in vivo* of *zf9* mRNA, as reported (16).

To explore the basis for increased expression of Zf9 *in vivo*, we compared the rate of Zf9 biosynthesis between normal and activated cells by using metabolic labeling. This analysis demonstrated a marked increase in Zf9 biosynthesis during stellate cell activation *in vivo* (Fig. 2B). Activated cells had a 3- to 4-fold increased synthesis of Zf9 compared with "normals" ("quiescent" cells from normal animals). Moreover, the amount of labeled Zf9 in activated cells after a 20-hr chase period remained proportionately higher than in normal cells, suggesting that Zf9's rate of degradation may be lower in activated cells (Fig. 2B).

In culture, normal stellate cells activate progressively by plating on uncoated plastic in the presence of serum. Induction of *zf9* mRNA under these conditions of culture activation was documented by RNase protection assay using a 624-bp cRNA probe derived from the unique 5' end sequence of *zf9* (Fig. 3A). There were three separate peaks of induction observed at 6 hr, 2 days, and 7 days, remarkably similar to that seen *in vivo* after CCl₄ (16). The increase in *zf9* mRNA with culture activation was accompanied by an even greater increase in Zf9 protein (Fig. 3B). As reported (16), no transcript for Zf9 was detected in stellate cells by Northern blot analysis, emphasizing the apparent low abundance of this transcript. In the report by Koritschoner (19), a single transcript of 4.5 kilobases for human CPBP was detected in whole tissue extracts from multiple organs.

Induction of protein in culture as detected by Western blot analysis corresponded to the appearance of nuclear Zf9 by immunofluorescence. Whereas freshly isolated cells had only very faint cytoplasmic staining with none in the nucleus (Fig.

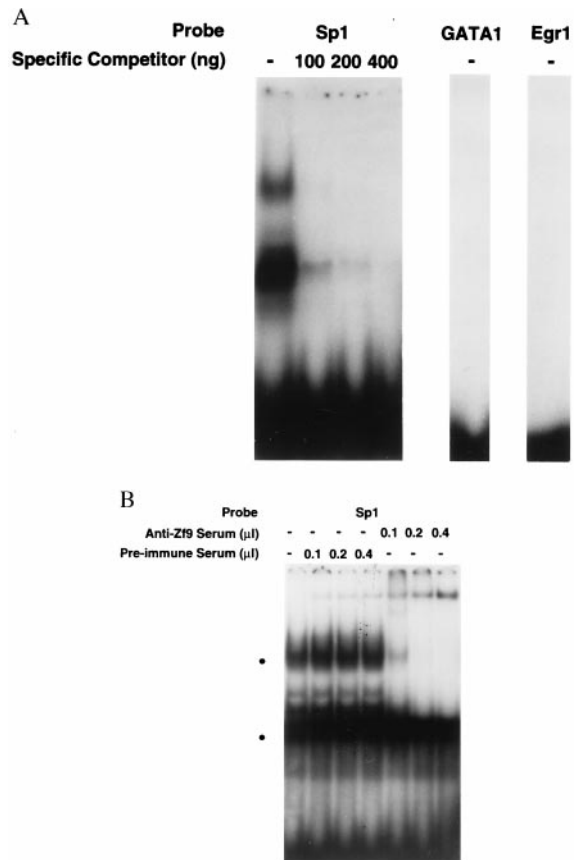


FIG. 4. DNA binding by recombinant Zf9-GST. (A) Gel shift assays performed with recombinant full length Zf9-GST demonstrate strong specific binding to a consensus sequence for Sp1 but none to consensus oligonucleotides for Egr-1 or GATA-1. Nonspecific binding was minimized by inclusion of poly[d(I-C)], as described in *Materials and Methods*. (B) Gel-shift assay using recombinant full length Zf9-GST and an Sp1 consensus probe in which increments of anti-Zf9 antiserum were included led to the disappearance of a higher migrating protein-DNA complex (upper dot, left margin) and a faint intermediate migrating complex but no apparent supershift. A lower migrating complex was unchanged (lower dot, left margin). Similar results were obtained by using stellate cell nuclear extracts (not shown).

3Ca), culture-activated cells demonstrated intense, specific nuclear localization, consistent with Zf9's potential role as a transcription factor (Fig. 3Cb). In addition, eccentric perinuclear staining was clearly visible in culture-activated cells.

RNase protection of adult rat tissues demonstrated *zf9* mRNA in all organs examined (not shown), with highest levels present in lung and intestine, as described for CPBP (19). The transcript also was apparent in fetal liver at gestational days 14–20, primary rat hepatocytes, two human hepatoma cell lines, Hep 3B and Hep G2, primary rat skin fibroblasts, and two mesenchymal cell lines, PAC1 (34) and 293T (not shown). Neither *zf9* mRNA nor protein was detectable in *D. schneider* S2 cells.

DNA Binding of Recombinant Zf9. Based on the high homology of Zf9's DNA binding domain to Kruppel-like transcription factors, we examined its ability to bind to a consensus GC-rich element recognized by other family members and by Sp1. Zf9-GST binds specifically to an Sp1 consensus target sequence but not to either GATA-1 or Egr sequences (Fig. 4A). Addition of EDTA to the binding reaction inhibited Zf9-DNA binding (not shown), confirming that the interaction is zinc dependent. Use of anti-Zf9 antiserum in supershift assays abolished the binding of Zf9-GST to the Sp1 consensus sequence (Fig. 4B). Furthermore, by using truncated Zf9-GST mutants, the C-terminal region was sufficient

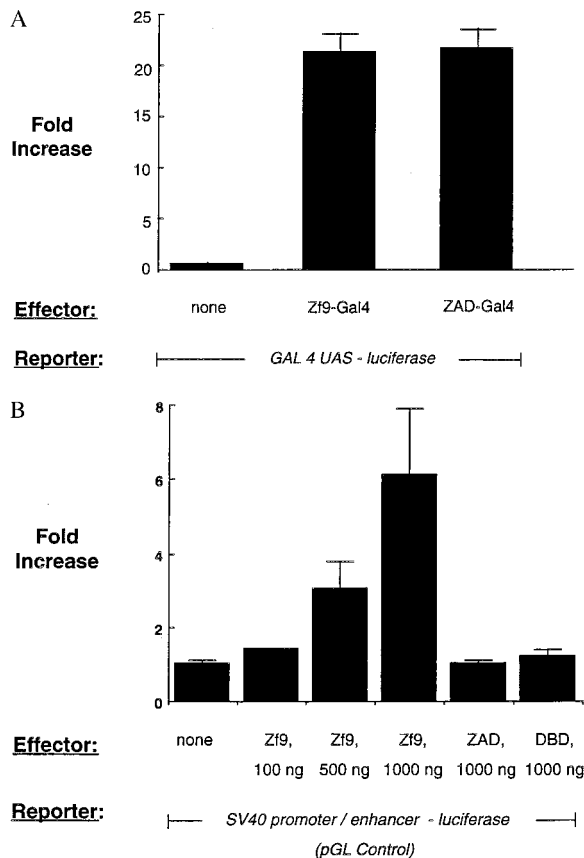


FIG. 5. Transactivation by Zf9 following transient transfection. (A) Cotransfection of 500 ng of either full length Zf9 (Zf9-GAL4) or the 201-aa N-terminal domain (ZAD-GAL4) fused to the GAL4 DNA binding domain and a GAL4 responsive luciferase reporter results in equally strong transactivation in Hep 3B cells. (B) Cotransfection of 100- to 1,000-ng Zf9 (pAC-Zf9) and a 1- μ g luciferase reporter containing the SV40 promoter/enhancer (pGL Control) in *D. schneider* S2 cells demonstrates transactivation by full length Zf9 but not by either the N-terminal 201-aa (ZAD) or the 82-aa zinc finger domain (DNA binding domain). Data represents mean of two experiments normalized to cell number.

for DNA binding whereas the N-terminal domain (amino acids 1–201) alone did not bind DNA (not shown).

Zf9 Is a Modular Transcription Factor. Cotransfection of *zf9* in cultured cells with different reporter systems was used to characterize its transcriptional activity. First, we tested the ability of either the 201-aa N-terminal domain of Zf9 or the full length protein fused with a GAL4 DNA binding domain to transactivate a GAL4 responsive promoter in mammalian cells (Fig. 5A). Both constructs led to robust activation of the reporter, establishing Zf9 as a transcription factor and identifying the 201-aa N-terminal region as a true activation domain. Based on these findings, experiments were extended to *D. schneider* cells, which lack Zf9 mRNA and thus provide an ideal cellular context for testing the transactivating capacity of exogenous Zf9 without interference by endogenous Zf9. Because of Zf9's binding to a GC box motif, we used a reporter construct containing six GC-rich elements within the SV40 promoter/enhancer (pGL Control). As shown in Fig. 5B, when full length Zf9 was expressed under the control of the actin promoter (pAC-*zf9*), concentration-dependent transactivation of the SV40 promoter-luciferase reporter was observed whereas neither the N-terminal domain nor the C-terminal zinc-finger (DNA binding) was transactivating. These results establish Zf9 as a modular transcription factor in which both activation and DNA binding domains are required.

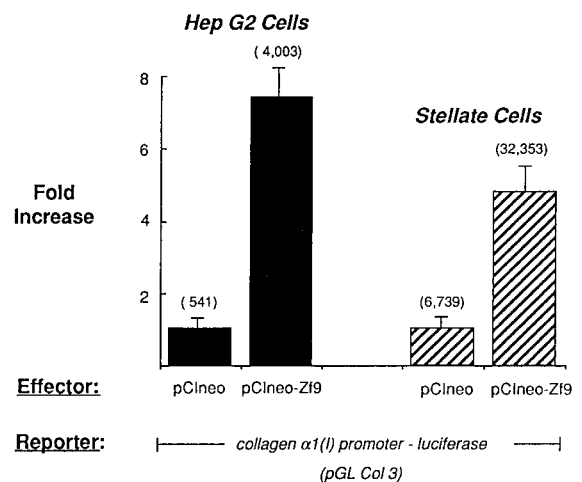


FIG. 6. Transactivation of the collagen $\alpha 1(I)$ promoter by Zf9 in primary rat stellate cells and Hep G2 cells. Cotransfection of 0.5 μ g Zf9 driven by the cytomegalovirus promoter (Zf9-pCIneo) and 1 μ g of a collagen $\alpha 1(I)$ 1-luciferase promoter (pGL Col3) was performed in equal numbers of stellate cells from a normal rat 2 days after plating on plastic, and in Hep G2 cells. Although the fold activation was similar in the two cell types, the basal and Zf9-stimulated relative luciferase activities (shown in parentheses above each bar) were ≈ 12 -fold greater in stellate cells. Data represents mean \pm SD of sextuplicate values normalized for transfection efficiency. Similar results were obtained in four separate experiments.

Transactivation of a Key Gene of Fibrogenesis by Zf9 Is Cell Context-Dependent.

The original strategy of cloning early genes induced during hepatic stellate cell activation was designed to reveal regulatory proteins that might drive the expression of key structural genes associated with hepatic fibrosis. The promoter for collagen $\alpha 1(I)$, the key component of the hepatic scar, contains two GC-rich regions within a ≈ 200 -bp minimal promoter required for collagen transactivation in hepatic stellate cells (20). Furthermore, the temporal expression of the collagen I gene after the early induction of Zf9 during stellate cell activation implied that Zf9 could regulate collagen I gene expression. To address this issue in the appropriate biologic context, we transiently cotransfected Zf9 and a luciferase reporter driven by the minimal promoter of collagen $\alpha 1(I)$ into rat stellate cells in primary culture (Fig. 6). As expected, significant endogenous activity of the reporter was apparent in the absence of exogenous Zf9 (see luciferase values above bar, Fig. 6), with an additional ≈ 5 -fold transactivation when exogenous Zf9 was expressed. Transactivation also was observed in Hep G2 hepatoma cells, but both the basal and Zf9-stimulated luciferase activities in this cell type were 1/12 that of primary stellate cells (see luciferase values above bars, Fig. 6). Thus, the greater "fold induction" in Hep G2 cells than in stellate cells reflected a much lower basal activation in Hep G2 in the absence of exogenous Zf9. Of interest, Zf9 failed to transactivate the collagen promoter in *D. schneider* cells (not shown), implying that transactivation of collagen $\alpha 1(I)$ requires an appropriate cell context.

DISCUSSION

Zf9/CPBP is a member of the Kruppel-like family of transcription factors that is induced in the well defined, biologically important context of hepatic stellate cell activation. The modular structure of Zf9 has several interesting features. Post-translational modifications of Zf9 *in vivo* are possible based on the 14-kDa difference between the native protein in stellate cells (46 kDa) and the *in vitro* translation product (32 kDa). These modifications may include both phosphorylation and glycosylation because consensus sequences for both serine phosphorylation by Cdc2 kinase and O-linked glycosylation

are present. Additionally, an endoplasmic reticulum targeting signal is evident, indicating that the protein could be membrane-bound and post-translationally modified in the endoplasmic reticulum.

Given *zfp*'s ubiquitous expression and the growing list of transcription factors with nearly identical DNA binding domains, what regulates its specificity in hepatic stellate cells compared with other tissues? In particular, CPBP [recently renamed COPEB (31)], the human homologue of Zf9, has been identified as a placental factor driving expression of pregnancy-specific glycoproteins (19). Although it originally was described as a factor interacting with TATA-less promoters, our data indicate that CPBP/Zf9 also can interact with a promoter containing a TATA box, that of collagen $\alpha 1(I)$. The biologic context in which Zf9 is expressed in stellate cells and subcellular localization are likely to be important. Although Zf9 is expressed widely, we have characterized its activity in a well defined biological context. Stellate cell activation represents a highly regulated process in which the temporal sequences of gene activation and protein expression are well characterized. Within this context, Zf9 may interact with general and/or specific regulatory factors in a cell-specific manner, as dictated by the structure of its activation domain and conformation. The transactivation of the collagen $\alpha 1(I)$ promoter in stellate cells but not in *D. schneider* cells emphasizes the importance of cellular context because collagen gene transactivation was most active in that cell type that expresses matrix genes *in vivo*.

The other determinants of Zf9's specificity could be the relative amount of protein expressed, its rate of degradation, and its subcellular localization. The clear increase in nuclear Zf9 with stellate cell activation may create a critical concentration necessary for target gene activation. The metabolic labeling data suggest that increased Zf9 may arise from both transcriptional and translational up-regulation and possibly increased protein stability. After metabolic labeling, a slower rate of decrease in labeled Zf9 in activated vs. normal (quiescent) cells suggests that Zf9 protein may be degraded more slowly during cellular activation. The presence of an ER targeting signal, cytoplasmic staining in quiescent cells, and perinuclear fluorescence in activated cells indicates that cellular activation may be associated with nuclear translocation of Zf9 in addition to a net increase in protein amount.

An essential component of understanding tissue fibrosis is the characterization of regulatory mechanisms in the mesenchymal cells that produce extracellular matrix. Induction of *zfp* mRNA and increased expression and synthesis of Zf9 protein occur rapidly during stellate cell activation, preceding induction of structural and cytokine genes, including collagens (35), platelet-derived growth factor receptor (36), and transforming growth factor $\beta 1$ and its receptors (37). Induction occurs in mechanistically distinct models of liver injury *in vivo* (16) as well as in culture-induced activation, supporting the notion that Zf9 up-regulation is a general feature of stellate cell activation. Moreover, Zf9 transactivates collagen I, a key component of the fibrotic matrix. In addition to the immediate-early induction within hours, there are also later peaks of *zfp* gene expression, as also observed *in vivo* (16). This pattern of *zfp* mRNA expression similarly was observed in culture and raises the possibility that the factor has functionally distinct activities at different points along the temporal sequence of cellular activation, possibly with distinct transactivation targets and coactivators in early (1–6 hr) vs. late (48–96 hr) intervals after liver injury.

The cloning of a Kruppel-like transcription factor induced during stellate cell activation that transactivates a relevant target gene provides an important entry point in dissecting molecular pathways underlying this key event in liver injury, one about which little is known at the transcriptional level. Nonetheless, a direct role of Zf9 in regulating stellate cell activation *in vivo* must still be established. These studies emphasize the value of a cloning strategy that utilizes a well

characterized, *in vivo* model of a tissue response to identify cDNAs with maximal biologic relevance. Finally, the findings in this study point to multiple mechanisms for increasing gene expression in fibrosis and could provide general clues for how the fibrotic response is regulated in parenchymal tissues.

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