Activation of Ito cells involves regulation of AP-1 binding proteins and induction of type I collagen gene expression

Juan ARMENDARIZ-BORUNDA,* Carl P. SIMKEVICH, Nakshatra ROY, Rajendra RAGHOW, Andrew H. KANG and Jerome M. SEYER
VA Medical Center, Research Service (151), 1030 Jefferson Avenue, Memphis TN 28104, U.S.A.

Activation of liver Ito cells is characterized by increased proliferation, fibrogenesis, loss of cellular retinoid and change of cell-shape. Here, we have described fundamental differences between freshly isolated Ito cells (FIC) and long-term cultured Ito cells (LTIC). This process of activation correlates with the absence of expression of Pro α 1(I) gene in FIC. LTIC expressed abundant transcripts of Pro α 1(I) gene. Nuclear run-off experiments showed the inability of FIC to support Pro α 1(I) RNA transcription while LTIC transcribed it greater than 5-fold as compared with FIC. Transforming growth factor β (TGF β)-treated LTIC had a preferential increase in the rate of Pro α 1(I) gene transcription as compared with control LTIC. A human collagen type I promoter–enhancer construct (pCOL-KT) [Thompson, Simkevich, Holness, Kang and Raghow (1991) J. Biol. Chem. **266**, 2549–2556] was readily expressed in LTIC but failed to be expressed in FIC. Furthermore, TGF β treatment of LTIC resulted in an increased expression of pCOL-KT. The

deletion of an activator protein-1 (AP-1) binding site (+598 to +604) in the 360 bp enhancer region of pCOL-KT (S360) caused decreased expression of the CAT reporter gene, suggesting that this bonafide AP-1 site can, at least in part, mediate the transactivation effect of TGF β . Using DNAase I protection, we demonstrate a single foot-print located at +590 to +625 in the S360 fragment; nuclear extracts prepared from TGF β -treated LTIC exhibited greater activity of these AP-1 binding proteins. Gel mobility assays corroborated and extended the footprinting observation. No AP-1-binding activity was found in the nuclear extracts of FIC. Double-stranded oligonucleotides containing the consensus AP-1 motif were able to compete out the binding; consensus NF-1 motif oligonucleotides failed to do so. The preincubation of nuclear extracts from control and TGF β -treated LTIC with antibodies against *c-jun* and *c-fos* rendered a reduced binding of AP-1 proteins to the target S360 fragment.

INTRODUCTION

Increased synthesis and deposition of type I collagen is a bold characteristic of liver fibrosis. Therefore, understanding the molecular mechanisms of type I collagen gene regulation is central to elucidating the pathophysiology of hepatic fibrosis. Ito cells or lipocytes are sinusoidal cells localized within the space of Disse of the liver; these cells express desmin and possess an intrinsic vitamin A autofluorescence (Blomhoff and Wake, 1991). It has been shown that Ito cells play an important role in the development of liver fibrosis (Mak and Lieber, 1988; Bissell et al., 1990). Freshly isolated lipocytes from healthy livers do not express type I collagen mRNA (Maher and McGuire, 1990), but their counterparts obtained from cirrhotic rat livers produce type I collagen and express a 30-fold increase in the corresponding transcripts. Studies both *in vivo* and *in vitro* indicate that Ito cells can undergo activation, a process characterized by increased proliferation, fibrogenesis, loss of cellular retinoid and new expression of smooth muscle-specific α -actin (Rockey et al., 1992). The activation process of Ito cells in culture has been shown to reproduce faithfully virtually all features of activation *in vivo* (Friedman et al., 1989). Although the mechanisms by which Ito cells are activated in the cirrhotic liver or *in vitro* have yet to be elucidated, it is believed that cytokines elaborated *in situ* are probably crucial in such transformation. Among the various cytokines known to be elevated at the sites of hepatic necrosis and inflammation, transforming growth factor β (TGF β) is a premier candidate capable of modulating cellular phenotype and

enhanced extracellular matrix biosynthesis. We have initiated a systematic investigation of the mechanism by which TGF β influences the extracellular matrix phenotype of the Ito cells. Recently, we demonstrated that type I collagen gene expression in Ito cells is regulated at the transcriptional level by TGF β (Armendariz-Borunda et al., 1992). A TGF β -responsive element approximately 1600 bp upstream of the transcription start site in the rat α 1(I) collagen gene was recently identified, this element called TE, was shown to mediate the transcriptional upregulation of the collagen promoter by means of binding nuclear protein complexes independent of nuclear factor-1 (NF-1) or AP-2 (Ritzenhale et al., 1991). Previous evidence showed that TGF β stimulated transcription of the murine α 2(I) collagen promoter through a *cis*-acting element located 300 bases upstream of the transcription start site (Rossi et al., 1988). This DNA sequence [TCG(N)₅GCCAAAG] is similar, but not identical, to a consensus NF-1 site [TGGC(N)₅₋₆GCCAA]. TGF β was also shown to regulate the transcriptional activation of other genes by modifying nuclear factors that bind to the activator protein 1 (AP-1) consensus sequence (Angel and Karin, 1991). Moreover, TGF β -mediated transcriptional activation of its own promoter occurred through an AP-1 binding site (Kim et al., 1989). The transcription element AP-1 was first described as a DNA-binding sequence in the enhancer of the simian virus 40 and the human metallothionein IIa gene (Lee et al., 1987a). The canonical AP-1 sequence TGAC/gTCA (Angel and Karin, 1991; Smeal et al., 1989; Rauscher et al., 1988) was subsequently found in a number of eukaryotic genes and shown to be activated through the in-

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; AP-1, activator protein-1; TGF β , transforming growth factor β ; PAI-1, plasminogen activator inhibitor-1; CAT, chloramphenicol acetyl transferase; NF-1, nuclear factor-1; FIC, freshly isolated Ito cells; LTIC, long-term cultured Ito cells; PKC, protein kinase C; FCS, fetal calf serum; DTT, dithiothreitol; 1 $\times$ SCC, 150 mM NaCl/15 mM sodium citrate.

* To whom correspondence should be addressed.

teraction with the members of the *jun* and *fos* families of transcription factors. AP-1 was shown to transduce signals generated by TPA activation of protein kinase C (PKC) to TPA-responsive gene transcription (Lee et al., 1987b). PKC can dephosphorylate the DNA-binding domain of *c-jun* protein, augmenting AP-1 activity. Moreover, enhancement of *c-jun* transactivation potential by mitogen-activated protein-serine/threonine (MAP) kinases is also a well documented means for regulating *c-jun* transcriptional activity (Angel and Karin, 1991). In this paper we provide evidence that enhanced expression of AP-1 binding proteins in Ito cells correlates with cell activation, loss of original phenotype (depletion of cellular retinoid) and overexpression of collagen type I. Furthermore, our data suggest that TGF β induces changes in the *trans*-acting factor(s) which recognize AP-1 motif and simultaneously activates transcription of the human Pro α 1(I) collagen gene.

EXPERIMENTAL PROCEDURES

Isolation and culture of Ito cells

Freshly isolated Ito cells were obtained according to the method of Friedman (Friedman et al., 1989). Briefly, the livers of 500 g Wistar strain rats were perfused with 0.2% Pronase for 10 min and then Hanks' balanced salt solution containing 0.03% collagenase and 0.05% Pronase at 37 °C for 30 min. After removal, the liver was minced in 0.03% Pronase and incubated in a shaking 37 °C water-bath for 30 min. The resultant suspension was filtered through gauze, washed three times in Dulbecco's modified Eagle's medium (DMEM) containing DNAase, and then centrifuged through a discontinuous gradient of 6, 8, 12 and 15% arabinogalactan (Stractan, Larex International Corp., Tacoma WA, U.S.A.). Pure Ito cells were recovered from the interphase between 6% Stractan and medium, washed and subsequently used for nuclei extraction. Purity of Ito cell population was approximately 95%, as assessed by the intrinsic autofluorescence at 300 nm u.v. light given by the cytoplasmic-retinoid content. Aliquots of cells were plated in DMEM supplemented with 20% fetal calf serum (FCS) and 2% glutamine. Confluent cultures were trypsinized and split at a ratio of 1:2. After the third subpassage, some of the cells were kept as stock at -70 °C and others were continued in culture and used routinely for the experiments described below.

Nuclear run-off transcription

Preparation of nuclei was performed as previously described (Greenberg and Ziff, 1984). Nuclei from freshly isolated, cultured and TGF β -treated Ito cells were incubated as individual reactions consisting of 2×10^7 nuclei in 200 μ l of reaction buffer. The nuclear suspension was incubated at 26 °C for 10 min, and the radiolabelled RNA (5×10^6 c.p.m.) was hybridized to nitrocellulose filters containing immobilized plasmid cDNAs (10 μ g). For determining non-specific background, 10 μ g of pUC19 DNA were hybridized with radiolabelled run-off transcripts. Hybridization was carried out for 36 h at 65 °C in 10 mM Tris (pH 7.4)/10 mM EDTA/0.2% SDS/0.3 M NaCl. After hybridization, filters were washed twice in an excess of $2 \times$ SSC ($1 \times$ SSC = 150 mM NaCl/15 mM sodium citrate) for 1 h at 65 °C, treated with 80 μ g of RNAase A for 30 min at 37 °C and washed with $2 \times$ SSC for 1 h at 37 °C. Filters were dried and exposed for several different times to Kodak films and the autoradiograms were scanned using an LKB densitometer and signals processed on a Hewlett-Packard 3390A integrator. The scanning of the autoradiograms was performed at the time of exposure which produced a linear response.

Plasmids construction

Construction of the plasmid pCOL-KT, and its deletions using several restriction endonucleases, have been described in detail previously (Thompson et al., 1991). The method of constructing pCOL-KT lacking the AP-1 site has also been previously described (Katai et al., 1992). For DNAase I-footprinting studies, a 360 bp *Sau*3A fragment (+494 to +854) spanning the entire enhancer-like sequence was cloned into the *Bam*HI restriction site of pUC 19 (pUC 19-E) (Katai et al., 1992).

Cell transfection and transient expression assays

Cultured and freshly isolated Ito cells were transfected with pCOL-KT and its deletions by the calcium phosphate precipitation method (Graham and van der Eb, 1973). Cultured Ito cells were plated in a 60 mm diameter dish in DMEM containing 10% FCS in a 37 °C humidified atmosphere of 95%–5% O₂–CO₂ respectively. The next day, 5 μ g of plasmid DNA were transfected into the cultured cells by the calcium phosphate precipitation method followed by a glycerol shock (Parker and Stark, 1979) and the cells were incubated in fresh medium for up to 48 h. For some experiments, the media was replaced by DMEM without serum and TGF β (5–10 ng, R & D, Minneapolis, MN, U.S.A.) were added to the experimental dishes. Transfected cells were harvested and the cell extracts were assayed for chloramphenicol acetyl transferase (CAT) for varied lengths of time by the method of Gorman et al. (1982) using 0.1 μ Ci of [¹⁴C]chloramphenicol per reaction. Efficiency of uptake of DNA was monitored by cotransfection with a plasmid containing the β -galactosidase gene driven by the Rous sarcoma virus long terminal repeat (pRSV *lacZ*). Cell lysates were assayed for β -galactosidase activity by fluorescence using *o*-nitrophenyl β -D-galactopyranoside (Sigma) as substrate (Herbomel et al., 1984). Freshly isolated Ito cells were plated in a 60 mm diameter dish in culture medium containing 20% FCS and after 2 days, transfected with 5 μ g of calcium phosphate-precipitated DNA, and incubated under these conditions for 24 h. After this period of time, the cells were carefully washed with PBS and incubated for additional 24 h in fresh DMEM. The CAT and β -galactosidase assays were essentially performed as described above.

DNAase protection assays

Nuclear proteins from freshly isolated (FIC) and long-term cultured Ito cells (LTIC) and rat skin fibroblast were extracted by the method of Dignam et al. (1983). For the preparation of the DNA probes, we used the coding strand of the intronic fragment (+492 to +854) which was 5'-end radiolabelled with T4 kinase at the overhanging end created by linearization with *Xba*I. The labelled fragment was obtained by digestion with *Kpn*I and purification in a 1.2% NuSieve agarose gel. For competition assays, complementary oligonucleotides were synthesized by a DNA synthesizer (ABI); oligonucleotides were annealed according to the method of Harrington et al. (1988). DNAase I footprinting was essentially performed as outlined by Briggs et al. (1986). Every reaction mixture contained 1–5 ng of end-labelled DNA (10000–20000 c.p.m.) in a 50 μ l volume of buffer containing 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 2.5 mM MgCl₂, 5% glycerol, 1.0 μ g poly[d(I·C)], 2% poly(vinyl alcohol) and different amounts of the nuclear proteins (or an equal amount of BSC). After 30 min of incubation on ice, the samples were left 5 min at room temperature and 50 μ l of a solution of 5 mM CaCl₂ and 10 mM MgCl₂ were added, followed by addition of DNAase I. Digestion reactions were incubated at room temperature for 1 min and

terminated by the addition of an equal volume (100 μ l) of stop mixture (200 mM NaCl/20 mM EDTA/1 % SDS/1 mg/ml glycogen). After phenol-chloroform-isoamyl alcohol (25:24:1, by volume) extraction and subsequent precipitation, the DNA was suspended in formamide dye and the fragments were separated in a standard 8 % sequencing gel. Maxam-Gilbert G + A sequencing reactions were electrophoresed in parallel lanes. After electrophoresis, the gels were dried and autoradiographed at -70°C with an intensifying screen.

Mobility shift assays

DNA probes used were prepared as described in the section above. Each 10 μ l reaction contained 4.5 μ l of $5\times$ reaction buffer [25 mM Hepes, pH 7.5/10 % glycerol/150 mM KCl/1 mM EDTA/1 mM dithiothreitol (DTT)] 10000 c.p.m.–20000 c.p.m. of DNA probe, 1 μ g of poly[d(I·C)] and 1–3 μ g of nuclear extract proteins. The reaction mixture was incubated for 20 min at room temperature, and then electrophoresed in a 4 % non-denaturing acrylamide gel in $0.25\times$ Tris-borate-EDTA at 10 V/cm. The gel was dried and exposed to X-ray film at -70°C overnight. In some experiments, gel retardation assays were performed by preincubating the nuclear extracts with c-fos antiserum, c-jun antiserum or non-immune serum for 30 min at 37°C . After addition of the probe, the standard gel retardation protocol was performed.

RESULTS

Freshly isolated Ito cells and cells cultivated *in vitro* have distinct phenotypes

Morphological features of freshly isolated Ito cells and their counterparts in culture are shown in Figure 1; freshly isolated Ito cells are slightly rounded cells with a prominent cytoplasm filled with lipid-like droplets (Figure 1b). When the same cells were excited with 300 nm u.v. light, we could detect the characteristic autofluorescence given by the abundant content of retinoids (Figure 1a). We did not detect collagen type I gene expression by

Northern blotting in these freshly isolated Ito cells (results not shown). When we kept a parallel batch of Ito cells in culture for 2–3 weeks, the cells flattened, spread and acquired a fibroblast-like morphology (Figure 1d). The intrinsic autofluorescence given by retinoids when the cells were excited with u.v., shown in Figure 1c, is also dramatically decreased as compared with Figure 1a. Consistent with our previous work (Armendariz-Borunda et al., 1992), collagen type I mRNA transcripts were readily detected in the cultured Ito cells (results not shown).

Transcription of Pro- α 1(I) collagen in freshly isolated Ito cells is repressed and becomes activated when cells are cultivated *in vitro*

In order to determine whether the absence of pro α 1(I) gene expression by freshly isolated Ito cells was due to preferential degradation of the messenger RNA or decreased rate of gene transcription we measured directly the rates of transcription of Pro α 1(I) and β -actin genes (results not shown). As we have shown previously (Armendariz-Borunda et al., 1992), we found a preferential increase in the rate of Pro α 1(I) gene transcription in cultured Ito cells treated with TGF β as compared with the untreated liver cells. More importantly, we found that freshly isolated Ito cells from healthy livers were not able to support Pro α 1(I) RNA transcription. In contrast, Ito cells in long-term culture transcribed Pro α 1(I) gene readily. This data strongly suggest that when these cells undergo activation in culture, they acquire the necessary transcriptional machinery to express Pro α 1(I) collagen gene.

Expression of wild-type pCOL-KT and its deletions is modulated by serum and TGF β

The effect of serum (Figure 2b) and TGF β alone (Figure 2c) was compared on the level of CAT gene expression when plasmid pCOL-KT [a plasmid containing the human α 1(I) collagen promoter and the first intron] and its deletions were transfected in Ito cells (Figure 2a). The results of transient expression experiments on both FIC and LTIC are shown. Two critical DNA regions present on the first intron of the human Pro α 1(I) gene are a 360 bp enhancer and a contiguous, overlapping negative element (Thompson et al., 1991; Simkevich et al., 1992). This construct behaves as the endogenous gene and it is expressed in a tissue-specific manner in a variety of mesenchymal cells; CAT RNA initiates at the same site utilized by the chimeric gene as does the endogenous Pro α 1(I) gene (Thompson et al., 1991; Simkevich et al., 1992).

The FIC displayed typical vitamin A-filled cytoplasm and could be successfully transfected as shown in Figure 2b. Although there was substantial conversion of chloramphenicol into its acetylated form (20.8 %) in these cells transfected with pSV2CAT construct, these cells failed to express pCOL-KT. It is also noteworthy that none of the deletions of the wild-type pCOL-KT, either in the promoter region (*EcoRV*) or in the enhancer region (*SsrII*, which removes part of the enhancer and the complete negative element) resulted in appreciable change in expression. These results suggest that the FIC retain their *in vivo* phenotype and do not express type I collagen gene.

This behaviour of FIC differs markedly from LTIC; the cultured Ito cells abundantly express pCOL-KT. In contrast to Simkevich et al. (1992) who observed that the deletion of the intronic sequences (e.g. the *SsrII* +670 to +1440) led to substantial reduction in conversion of chloramphenicol when compared with wild-type pCOL-KT, we found a 9-fold increase in CAT expression in cultured Ito cells. It is worth noticing that

Figure 1 Loss of typical Ito cell-autofluorescence given by the cytoplasmic-vitamin A content

Freshly isolated Ito cells were obtained from healthy livers (a and b) and excited with 300 nm u.v. light. The intense cell-autofluorescence is seen in (a). (b) shows the same field observed under regular light microscopy. In contrast, a dramatic decrease in the intensity of the fluorescence was seen when a parallel batch of Ito cells was kept in long-term culture (c). Note the change in cell shape clearly shown in (d).

Figure 2 Expression of wild-type pCOL-KT and its deletions is modulated by serum and TGF β . All the numbers are the representatives values of at least four different experiments

A, Schematic representation of wild-type pCOL-KT plasmid and boundaries of the deletions created with the corresponding restriction enzymes. +1 Indicates the transcriptional initiation site. The overlapping negative element present in the first intron is also indicated. B, Effect of serum. Ito cells were transfected by using the calcium phosphate precipitation method. Long-term cultured cells were plated in 60 mm dishes in DMEM with 10% FCS. The next day, 5 μ g of plasmid DNA were transfected into the cells followed by glycerol shock and the incubation was continued in fresh medium with 10% FCS for up to 48 h. FIC were obtained according to Friedman et al. (1989), plated at equal density as above in 60 mm dishes in DMEM with 20% FCS and after 2 days, transfected with 5 μ g of calcium phosphate-precipitated DNA and incubated under these conditions for 24 h. After this period of time, the cells were carefully washed with PBS and incubated for an additional 24 h in fresh DMEM. Transfected cells were harvested and the cell extracts were assayed for CAT with 0.1 μ Ci of [14 C]-chloramphenicol per reaction. 'Fold change' shows the ratio of activity, normalized against the wild-type plasmid. Efficiency of uptake of DNA was monitored by cotransfection with a plasmid containing the β -galactosidase gene driven by the Rous sarcoma virus long terminal repeat (pRSV *lacZ*) and the cell lysates were assayed for β -galactosidase activity. C, Effect of TGF β . LTIC were transfected with 5 μ g of DNA, the cells were washed with PBS, and the medium was replaced by DMEM without serum. The cultured Ito cells were then incubated for up to 48 h with or without TGF β (10 ng/ml).

Figure 3 Effect of TGF β on the expression of a reporter-CAT driven by wild-type or deleted AP-1 of Pro α 1(I) collagen gene

Cell extracts from Ito cells transfected with M13col-CAT (lanes 1 and 2), M12col(Δ +598 to +604)-CAT (lanes 3 and 4) were prepared and assayed for CAT activity as described in the Materials and methods section. In lanes 2 and 4 the cells were treated with 10 ng/ml of TGF β in serum-free medium after transfection.

the same deletion plasmid failed to exert similar stimulatory effect in FIC (Figure 2b).

The effect of TGF β on the expression of CAT enzyme in transfected cultured Ito cells is shown numerically in Figure 2c. In these series of experiments the cells were transfected and then the culture medium was replaced with serum-free fresh medium

containing TGF β (see Experimental procedures section) and incubated for up to 48 h. An important observation made was that we could detect an increase in the relative CAT activity when TGF β was added to cultured Ito cells transfected with wild-type pCOL-KT. Although this was a modest stimulatory effect, it was substantially potentiated by TGF β when the *SstII* deletion of pCOL-KT was used as the transfected plasmid. Since it is known that putative AP-1 binding sites can mediate TGF β -transcriptional effects, and to precisely define the role of the AP-1-like site at +598 to +604 (TGATTCA), an AP-1 deletion mutant of the wild-type pCOL-KT was tested in LTIC (Figures 2c and 3). In Figure 3 we show representative CAT assays from extracts of cultured Ito cells transfected with wild-type M13col-CAT and M13col (+598 to +604)-CAT. Deletion of the AP-1-like motif rendered a decreased expression of the reporter construct in these cells. These data recapitulate faithfully previous observations by Katai et al. (1992).

Since we did not find a significant difference in the CAT activity in the cells when transfected with the AP-1-deletion plasmid M13col (+598 to +604)-CAT treated or untreated with TGF β (Figures 2c and 3) we concluded that the bona fide AP-1 binding site in the enhancer region of pCOLKT is mediating, at least in part, the transactivation effect of TGF β .

Activity of AP-1 element at +590 to +615 in the enhancer (S360) of pCOL-KT is modulated by TGF β

Previous work has shown that TGF β -mediated positive regulation of the mouse α 2(I) collagen promoter in mouse NIH 3T3

Figure 4 Analysis of LTICs nuclear proteins interactions with the coding strand of the *Proα1(I)* enhancer (S360)

S360 fragment (+492 to +854) was end-labelled on the 5'-coding strand and incubated with BSA (lane 2) or nuclear proteins from TGFβ-treated (lanes 3 and 4) or control Ito cells (lanes 5 and 6). Radiolabelled S360 (5 ng, 15000 c.p.m.) were incubated with 7.5 μg (lanes 3 and 5) or 15 μg (lanes 4 and 6) of nuclear proteins. The Maxam and Gilbert G+A sequencing reaction of coding strand of S360 is shown in lane 1. The footprinting pattern is indicated by the rectangle and clearly demonstrated only one region of protection corresponding with a putative AP-1 site, which was more easily detected by using nuclear proteins from TGFβ-treated Ito cells.

and rat osteosarcoma cells occurred through an NF-1-like binding site (Rossi et al., 1988). On the other hand, TGFβ regulates the expression of other genes by modifying nuclear transcription factors that recognize the AP-1 consensus sequence (Kerr et al., 1990) and it can also autoregulate its own transcriptional activation through an AP-1 binding site (Kim et al., 1989). More recently, it has been shown that the PAI-1 gene contains two sequences in the promoter region responsive to TGFβ. These two sequences share high homology with AP-1 consensus sequence (Keeton et al., 1991). Thus, different TGFβ-inducible genes may be activated by multiple mechanisms. Since we observed that pCOL-KT and its *SsrII* deletion derivative, which retains an AP-1 binding site (+598 to +604), responded to the action of TGFβ in our system, we reasoned the TGFβ-treated cultured Ito cells over-expressed AP-1 binding proteins. To elucidate this, the enhancer-like S360 fragment (see Figure 2a), was 5'-end-labelled with ³²P on the coding strand and subjected to partial digestion with DNAase I in the absence or presence of nuclear extracts. The DNAase I fragmentation pattern clearly

Figure 5 Gel mobility shift experiments showing the complexes formed between S360 end-radiolabelled probe and nuclear proteins extracted from control of TGFβ-treated Ito cells and rat skin fibroblasts

The gel retardation assays were performed as described in the Materials and methods section. Lanes 1, 5 and 9, 2 μg of nuclear protein; lanes 2, 6 and 10, competition with a 100-fold molar excess of unlabelled S360; lanes 3 and 4, 7 and 8, 11 and 12, competition with a 400-fold and 800-fold molar excess of unlabelled AP-1 or NF-1 synthetic oligonucleotides respectively. The increased amount of S360 binding-proteins in nuclear extracts from TGFβ-treated cultured Ito cells is evident. The free probe is shown in lane 13.

shows only one region of protection (Figure 4). Based on simultaneous analysis of the products of Maxam and Gilbert G+A sequence reaction, the footprinted region corresponds to bases +590 to +615. Nuclear extracts from control cultured Ito cells also showed an identical pattern of DNAase I protection, but three times more protein was required to induce the footprint, suggesting that TGFβ enhanced the activity of AP-1 binding proteins.

In order to identify and quantify this nuclear protein(s) which is interacting with the S360 DNA fragment, we performed gel retardation experiments by using synthetic double-stranded oligonucleotides bearing AP-1 and NF-1 consensus sequences as competitors. Figure 5 shows that nuclear extracts from TGFβ-treated Ito cells contained 3–4-fold more S360 binding proteins as compared with their control counterparts. Competition with cold S360 DNA demonstrated the specificity of the binding assay. The experiments of DNA protection (Figure 4) had suggested that the nuclear proteins are binding through an AP-1-like consensus sequence. The gel shift experiments (Figure 5) corroborated the footprinting observation. A 400-fold molar excess of unlabelled AP-1 oligonucleotide competed out the binding in both control and TGFβ-treated cells, suggesting the presence of similar or closely related AP-1 binding proteins. Competition with NF-1 oligonucleotide, using concentrations as high as 800-fold molar excess, failed to compete out the binding. Nuclear extracts obtained from rat skin fibroblasts were used as a control, showing identical binding complex pattern, correlating with the general notion that most cells constitutively express these DNA-binding proteins.

Freshly isolated Ito cells are deficient in AP-1 activity

In order to elucidate whether freshly isolated Ito cells contained AP-1 binding proteins, we isolated Ito cells from normal animals and used them for nuclear proteins extraction (Dignam et al., 1983). The protein concentration was determined and adjusted to equal concentration for either cell type as described in Figure

Figure 6 Comparison of S360-DNA binding proteins present in nuclear extracts from control and TGF β -treated LTIC and their freshly isolated counterparts, demonstrated substantial differences

The gel mobility shift experiments were carried out as described before. The 5'-end radiolabelled S360 probe was incubated with: lanes 1, 5 and 9, 3 μ g of nuclear extracts of the indicated cell type; lanes 2, 6 and 10, competition with a 100-fold molar excess of cold S360; lanes 3 and 4, 7 and 8, 11 and 12, competition with 200-fold and 400-fold molar excess of unlabelled synthetic oligonucleotides containing AP-1 or NF-1 consensus sequences, respectively. It is important to note that we did not detect functional binding-activity in nuclear extracts from freshly isolated Ito cells. Lane 13 depicts the probe alone.

Figure 7 Mobility shift experiments with polyclonal antisera to c-fos (α c-fos) and c-jun (α c-jun) showed that c-jun plays an important role in mediating the binding to the S360 DNA-fragment

Preincubation of nuclear extracts from TGF β -treated Ito cells with two different increasing concentrations of rabbit non-immune serum (lanes 4 and 5), α c-fos (lanes 6 and 7) and α c-jun (lanes 8 and 9). Fixed amounts of nuclear protein (2 μ g) were used in lanes 2–9. The preincubation was carried out for 30 min at 37 $^{\circ}$ C and after addition of the probe the standard gel retardation protocol was performed. Lane 1 shows the free probe, lane 2 shows the control and lane 3 depicts the competition with AP-1 oligonucleotide, using 200-fold molar excess.

6. This series of experiments showed identical patterns of gel retarded DNA–protein complexes as in Figure 5, with the exception that we did not find any DNA-binding activity in the nuclear extracts of freshly isolated cells (even when we increased the amount of nuclear proteins). These data are consistent with the previous observations shown in Figure 2b, where we did not find a substantial expression of CAT activity when these cells

were transfected with pCOL-KT and its corresponding deletions. Thus, these differences in the expression of AP-1 binding proteins could account, at least in part, for the differential control of Pro α 1(I) by FIC and LTIC. Furthermore, it appears that TGF β potentiates the AP-1 DNA-binding activity, and thus could be contributing to an enhanced expression of the gene.

Treatment of Ito cells extracts with polyclonal antisera to c-jun (α c-jun) diminishes its binding to the S360 DNA fragment

It has been shown that different members of the *jun* and *fos* gene families are components of the AP-1 transcriptional complex (Kovary and Bravo, 1991; Castellazi et al., 1991; Vogt and Bos, 1990; Abate et al., 1990). *c-Jun* can form either jun/jun homodimers or jun/fos heterodimers via the leucine repeat present in both proteins. Homo- and hetero-dimers bind to the same consensus sequence, TGACTCA, yet providing varied gene activation. Since our experiments demonstrated that the most prominent shifted band was competed out by a double-stranded AP-1 oligonucleotide, we addressed the question of whether polyclonal antisera against c-fos and c-jun, could compete for the binding to the target DNA. Figure 7 shows representatives of three different experiments when nuclear extracts from TGF β -treated Ito cells were preincubated with antiserum to c-jun and to c-fos, and subjected to the standard gel-shift protocol. In order to analyse quantitatively the nature of this protein–DNA interaction, we used a fixed amount of nuclear proteins and then preincubated them with two different concentrations of the corresponding polyclonal antisera and non-immune serum. We found that the preincubation with non-immune serum, somewhat stabilized the protein–DNA complex, increasing the intensity of the shifted band. However, when nuclear proteins were preincubated with α c-fos and α c-jun, we found that mostly the latter polyclonal antiserum was able to reduce the binding for the target S360 DNA. The preincubation with α c-fos also decreased the intensity of the major shifted band, albeit to a lesser extent. These data confirm that the S360-bound nuclear factors are AP-1-like and that the protein–DNA interaction is possibly being mediated mostly through the DNA-binding domain of a jun-like protein.

Antisera α c-jun abolishes the AP-1 footprint on S360

In order to determine whether α c-jun or α c-fos could abolish the delineated footprint, encompassing from +590 to +615 in S360, we preincubated the nuclear extracts from control and TGF β -treated Ito cells with the different antisera; the DNAase I protection assay was essentially performed as described for Figure 4. The results shown in Figure 8 confirmed and extended our previous observations with gel shift experiments. As shown before in Figure 4, the region of protection defined by DNAase I cleavage, corresponds to a 25 bp region in both control and TGF β -treated nuclear extracts. Nonetheless, the footprint was more marked when using the latter ones (compare lanes 6 and 7 with 12 and 13). The preincubation with α c-jun completely abolished the footprint at the two concentrations of TGF β (lanes 10 and 11) or control nuclear proteins used (lanes 3 and 4). Again, the functional consequence of incubating with α c-fos showed a lesser effect, correlating with the previous data. Therefore, we conclude that the DNAase I footprint in S360 likely results from binding of jun-like transcriptional factors. It is important to notice that this was true for both control or TGF β -treated cells. Nonetheless these results also pointed out a major role for TGF β in increasing the activity of jun proteins, by either the induction of new proteins, or by modifying and activating pre-existing transcriptional factors of the *jun* gene family.

Figure 8 Competition of the footprint in cultured Ito cells on coding strand with antiserum α c-jun

Nuclear extracts from control and TGF β -treated Ito cells (lanes 2, 4, 6, 8, 10 and 12: 7.5 μ g of protein; lanes 3, 5, 7, 9, 11 and 13: 15 μ g of protein) were preincubated with identical amounts of rabbit non-immune serum, α c-fos antiserum and α c-jun antiserum as indicated. After 30 min at 37 $^{\circ}$ C, the different nuclear extracts were further incubated with the 5'-radiolabelled S360 probe and DNAase I digestions were performed as indicated in the Materials and methods section. The region of protection from DNAase I cleavage, indicated by the rectangle, shows that mainly α c-jun antiserum competed for binding to nuclear factor. The Maxam and Gilbert G + A sequence ladder generated from the radiolabelled coding strand of this probe is shown in lane 15.

DISCUSSION

We believe that Ito cell activation is a critical requirement for the enhanced deposition of extracellular matrix associated with liver fibrosis. In this paper, we have presented evidence that this process of activation of Ito cells correlates with the expression of AP-1 binding proteins. Furthermore, we have also shown that TGF β increases the activity of nuclear proteins of the AP-1 family in LTIC. The results suggest that TGF β may upregulate the transcriptional activity of human Pro α 1(I) gene, at least in part, through modulating the concentration of AP-1 *trans*-acting factors that bind with high affinity to the enhancer-spanning S360 DNA fragment. Moreover, we have demonstrated, by using specific polyclonal antisera, that the main transcriptional molecule involved in mediating the binding belongs to the steadily growing family of the cellular *jun* genes. It is noteworthy that the

inability to detect the same kind of S360 DNA binding-factors in nuclear extracts obtained from freshly isolated cells, strongly suggest that increased levels of *trans*-acting AP-1-like protein could likely be involved in the activation of the quiescent Ito cells. Evidence presented here and elsewhere, has shown that normal Ito cells change their phenotype after they are isolated and plated in culture (Friedman et al., 1989; Davis et al., 1987; Weiner et al., 1990). Quiescent normal Ito cells do not produce collagen type I. However, after several days in culture they begin to switch their normally collagen type IV- and III-producing phenotype, becoming very active producers of collagen type I. Detailed information concerning this mechanism of transcriptional gene switch and activation is lacking. Ito cells isolated from rats intoxicated with CCl $_4$ or with cholestatic liver injury (i.e. activated *in vivo*) are the predominant cells expressing extracellular matrix genes (Maher and McGuire, 1990). Nevertheless, it is evident that the activation of Ito cells *in vivo* is rather complex and involves a complex interplay between other liver specific cells, local soluble mediators and the extracellular matrix itself. Therefore, it is difficult to analyse the process of activation *in vivo*. One alternative to this experimental caveat is using the Ito cell culture-activation model that recapitulates virtually all characteristics of activation *in vivo*.

Several studies have suggested that the first intron of the collagen α 1(I) gene is intricately involved in the transcription of that gene, but the mechanisms responsible for that regulation remain a matter of speculation (Katai et al., 1992; Liska et al., 1990). Furthermore, the presence of an AP-1 site in the first intron (enhancer-containing DNA sequence) of the Pro α 1(I) collagen gene raises many questions. Katai et al. (1992) have shown, consistent with previous observations (Bornstein and McKay, 1990), that a 360 nucleotide Sau 3A fragment (S360, spanning from +492 to +854) enhanced transcription, and its deletion from pCOL-KT resulted in drastic reduction in CAT expression in different mesenchymal cells. Furthermore, the deletion of an AP-1 consensus motif (from +598 to +604) on the parental plasmid also led to decreased transcription of the Pro α 1(I) gene. The AP-1 sequence is a recognition target for the *jun* family of transacting factors, which include *jun B*, *jun D* and *c-jun* (Angel et al., 1987; Ryder et al., 1988; Hirai et al., 1989). These three *jun* proteins are almost identical to their C-terminal regions, which are involved in dimerization and DNA-binding, whereas their N-terminal parts, which are involved in transcriptional activation, diverge. All three form heterodimers with themselves or with *c-fos* or other members of the *fos* family. The existence of gene families with identical or very similar DNA-binding specificities is not restricted to the *jun* or *fos* families; it is rather frequently observed in several nuclear proteins (Karin, 1990). Thus, the multitude and cell specificity of the *jun* and *fos* factors and their binding affinity sites could allow for the differential regulation of the response to cytokines like TGF β in different cells and under different environmental circumstances. Moreover, the *jun* family of genes is disparately regulated in different tissues and can regulate either transcriptional activation (Angel et al., 1987) or repression (Takimoto et al., 1989). In addition, Pertovaara et al. (1989) and Li et al. (1990) have reported that TGF β differentially activates members of the *jun* protein family. Rossi et al. (1988) reported that mouse α 2(I) collagen gene transcription is stimulated by TGF β through the binding of NF-1 to NF-1-like binding-sites localized at position -350 in the promoter. The S360 DNA fragment (enhancer-like) does not contain an NF-1 binding-site since NF-1 double-stranded oligonucleotides failed to compete out the binding of nuclear factors to S360 fragments (Figures 5 and 6). A plausible explanation of these data is that TGF β induces the transcriptional

activation of collagen type (I) gene expression through the induction of jun proteins. These jun proteins, possibly forming dimers with themselves or with other fos proteins, might be regulating the expression, at least in part, of type I collagen through the binding to the AP-1 binding-site present in the enhancer of the first intron.

The mechanism by which TGF β is mediating the increase of AP-1 transcriptional factors in our experimental system is unknown. Experiments are currently being carried out in our laboratory addressing this question. In this regard, it has been shown that 12-*O*-tetradecanoylphorbol 13-acetate (TPA) can enhance jun/AP-1 activity by two different mechanisms. The first step, which occurs immediately after stimulation with TPA, involves a post-translational event that leads to increased activity of pre-existing jun/AP-1 molecules. The second step involves increased synthesis of jun/AP-1, mediated by the interaction of activated jun/AP-1 with the *jun* promoter, resulting in transcriptional activation (Angel et al., 1988). Undoubtedly, one of the most important post-translational events involved, is the degree of phosphorylation of the different jun and fos molecules (Kovary and Bravo, 1991), which would enable these transcriptional regulators to form a specific homo- or hetero-dimer. Thus, the relative proportion of the different jun proteins (c-jun, junB, junD) and fos (c-fos, fosB, Fra-1 and Fra-2) proteins, will be constantly varying under any given set of circumstances; the different jun/fos heterodimers will also be changing, providing the cell with a very fine tuning mechanism for controlling the expression of particular genes.

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REFERENCES

- Abate, C., Luk, D., Gentz, R., Rauscher, III, F. J. and Curran, T. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1032–1036
- Angel, P., Hattori, K., Smeal, T. and Karin, M. (1988) *Cell* **55**, 875–885
- Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. and Karin, M. (1987) *Cell* **49**, 729–739
- Angel, P. and Karin, M. (1991) *Biochim. Biophys. Acta* **1072**, 129–157
- Armendariz-Borunda, J., Katayama, K. and Seyer, J. M. (1992) *J. Biol. Chem.* **267**, 14316–14321
- Bissell, D. M., Friedman, S. L., Maher, J. J. and Roll, F. J. (1990) *Hepatology* **11**, 488–498
- Blomhoff, R. and Wake, K. (1991) *FASEB J.* **5**, 271
- Briggs, M. R., Kadonaga, J. T., Bell, S. P. and Tjian, R. (1986) *Science* **234**, 47–52
- Bornstein, P. and McKay, J. (1990) *J. Biol. Chem.* **265**, 13351–13356
- Castellazzi, M., Spyrou, G., LaVista, N., Dangy, J.-P., Piu, F., Yaniv, M. and Brun, G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8890–8894
- Davis, B. H., Pratt, B. M. and Madri, J. A. (1987) *J. Biol. Chem.* **262**, 10280–10286
- Dignam, J. P., Lebovitz, R. M. and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
- Friedman, S. L., Roll, F. J., Boyles, J., Arenson, D. M. and Bissell, D. M. (1989) *J. Biol. Chem.* **264**, 10756–10762
- Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) *Mol. Cell Biol.* **2**, 1044–1051
- Graham, F. and van der Eb, A. (1973) *Virology* **52**, 456–457
- Greenberg, M. E. and Ziff, E. B. (1984) *Nature (London)* **311**, 433–438
- Harrington, M. A., Jones, P. A., Imagawa, M. and Karin, M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2066–2070
- Herbomel, P., Bourachot, B. and Yaniv, M. (1984) *Cell* **39**, 653–655
- Hirai, S.-I., Rysec, R.-P., Mehta, F., Bravo, R. and Yaniv, M. (1989) *EMBO J.* **8**, 1433–1439
- Karin, M. (1990) *New Biol.* **2**, 126–131
- Katai, H., Stephenson, J. D., Simkevich, C. P., Thompson, J. P. and Raghov, R. (1992) *Mol. Cell. Biochem.* **118**, 119–129
- Keeton, M. K., Corriden, S. A., van Zonneveld, A. J. and Loskutoff, D. J. (1991) *J. Biol. Chem.* **266**, 23048–23052
- Kerr, L. D., Miller, D. B. and Matrisian, L. M. (1990) *Cell* **61**, 267–278
- Kim, S.-J., Denhez, F., Kim, K. Y., Holt, J. T., Sporn, M. B. and Roberts, A. B. (1989) *J. Biol. Chem.* **264**, 19373–19378
- Kovary, K. and Bravo, R. (1991) *Mol. Cell Biol.* **11**, 2451–2459
- Lee, W., Haslinger, A., Karin, M. and Tjian, R. (1987a) *Nature (London)* **325**, 368–372
- Lee, W., Haslinger, A., Karin, M. and Tjian, R. (1987b) *Nature (London)* **325**, 368–372
- Li, L., Hu, J.-S. and Olson, E. N. (1990) *J. Biol. Chem.* **265**, 1556–1562
- Liska, D. J., Slack, J. L. and Bornstein, P. (1990) *Cell Regul.* **1**, 487–498
- Maher, J. J. and McGuire, R. F. (1990) *J. Clin. Invest.* **86**, 1641–1648
- Mak, K. M. and Lieber, C. S. (1988) *Hepatology* **8**, 1027–1033
- Parker, B. and Stark, G. (1979) *J. Virol.* **31**, 360–369
- Pertovaara, L., Sistonen, L., Bos, T. J., Vogt, P. K., Keski-Oja, J. and Alitalo, K. (1989) *Mol. Cell Biol.* **9**, 1255–1262
- Rauscher, F. J., Voulalas, P. J., Franza, B. R. and Curran, T. (1988) *Genes Dev.* **2**, 1687–1699
- Ritzenhelen, J. B., Goldstein, R. H., Fine, A. and Smith, B. D. (1991) *J. Biol. Chem.* **266**, 13625–13631
- Rockey, D. C., Maher, J. J., Jarnagin, W. R., Gabbiani, G. and Friedman, S. L. (1992) *Hepatology* **16**, 776–784
- Rossi, P., Karsenty, G., Roberts, A. B., Roche, N. S., Sporn, M. B. and de Crombrughe, B. (1988) *Cell* **52**, 405–414
- Ryder, K., Lau, L. F. and Nathans, D. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1487–1491
- Simkevich, C. P., Thompson, J. P., Poppleton, H. and Raghov, R. (1992) *Biochem. J.* **286**, 179–185
- Smeal, T., Angel, P., Meek, J. and Karin, M. (1989) *Genes Dev.* **3**, 2091–2100
- Takimoto, M., Quinn, J. P., Farina, A. R., Staudt, L. M. and Levens, D. (1989) *J. Biol. Chem.* **264**, 8992–8999
- Thompson, J. P., Simkevich, C. P., Holness, M. A., Kang, A. H. and Raghov, R. (1991) *J. Biol. Chem.* **266**, 2549–2556
- Vogt, P. K. and Bos, T. J. (1990) *Adv. Cancer Res.* **55**, 1–35
- Weiner, F. R., Giambone, M. A., Czaja, M. J., Shah, A., Annoni, G., Takahashi, S., Eghbali, M. and Zern, M. A. (1990) *Hepatology* **11**, 111–117