E-box-binding Repressor Is Down-regulated in Hepatic Stellate Cells during Up-regulation of Mannose 6-Phosphate/Insulin-like Growth Factor-II Receptor Expression in Early Hepatic Fibrogenesis*

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Hepatic stellate cells become activated during the early stages of hepatic injury associated with fibrogenesis. The mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGFIIr) plays an important role in early fibrogenesis by participating in the activation of latent transforming growth factor-β, a potent inducer of the matrix proteins in activated stellate cells that define the fibrotic phenotype. In this study we examined hepatic stellate cell regulation of M6P/IGFIIr expression and found that M6P/IGFIIr mRNA transcript levels increased in stellate cells from rats exposed to carbon tetrachloride (CCL4), a potent fibrogenic stimulant. Two E-boxes residing in the proximal promoter of M6P/IGFIIr were found to each bind a novel 75-kDa transcription factor (P75) in quiescent stellate cells of normal livers. This E-box binding was down-regulated as an early response in stellate cells exposed to CCL4, coinciding with increased M6P/IGFIIr transcript levels. Mutagenized E-boxes in M6P/IGFIIr promoter-chloramphenicol acetyltransferase (CAT) reporter constructs produced a substantial increase in reporter expression when compared with the corresponding native promoter-CAT construct when transfected in culture-activated stellate cells, suggesting P75’s role as a repressor. The results indicate P75’s participation in the regulation of M6P/IGFIIr transcription in hepatic stellate cells during fibrogenesis.

During hepatic fibrogenesis the hepatic stellate cell becomes activated, transforming from a fat-storing quiescent cell into a myofibroblast-like cell, devoid of fat stores and overproducing type I collagen as well as other matrix proteins that contribute to the fibrotic phenotype (1). This fibrogenic process is not limited to the liver as parallel pathways exist in most solid organs (e.g. kidney, lung, and heart) during pathologic fibrotic motion of TGF-β. Fibrosis of the liver is participating in pathologic states, including hepatocytes in rats exposed to phenobarbital (16), hepatocellular carcinoma (17), and mammary tumors (18).

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many cell types (5). TGF-β stimulates hepatic stellate cells under pathologic conditions and is the most potent inducer of collagen α1(I) expression, a major contributor to fibrosis (1). TGF-β is secreted in a latent, biologically inactive form, and must be activated in order to bind to its own receptor (6). The activation of TGF-β is substantially enhanced by the binding of latent TGF-β to the mannose 6-phosphate/insulin-like growth factor-II receptor (M6P/IGFIIr) (7, 8). M6P/IGFIIr is a dual specificity receptor that binds both insulin-like growth factor-II as well as a variety of proteins bearing mannose 6-phosphate residues (9, 10). Administration of mannose 6-phosphate as well as anti-M6P/IGFIIr antibodies to bovine aortic endothelial/bovine smooth muscle cocultures expressing M6P/IGFIIr surface protein blocks the binding of TGF-β to M6P/IGFIIr and specifically inhibits activation of TGF-β (7). The latency peptides associated with the TGF-β dimer bear two N-linked mannose 6-phosphate residues, which bind latent TGF-β to membrane bound M6P/IGFIIr. This binding, which concentrates latent TGF-β at the cell surface, allows other molecules essential for TGF-β activation to function, which are as yet poorly defined in vivo (7, 11, 12).

M6P/IGFIIr expression is up-regulated early in the process of stellate cell activation. The M6P/IGFIIr protein was found in rat stellate cells in vivo by 48 h after administration of carbon tetrachloride (CCL4) (13), a potent inducer of hepatic fibrogenesis. The M6P/IGFIIr transcript was previously found to be undetectable via Northern analysis in normal (quiescent) stellate cells (13). In whole liver RNA, the M6P/IGFIIr transcript level peaked at 48 h after CCL4 treatment (13). Latent TGF-β secretion by stellate cells increases dramatically during stellate cell activation (14). The parallel induction of M6P/IGFIIr may serve to promote hepatic fibrogenesis by facilitating the TGF-β autocrine loop in the stellate cell through promotion of TGF-β activation. The co-regulation of M6P/IGFIIr and TGF-β occurs not only in hepatic stellate cells in early fibrogenesis (8, 14, 15), but occurs in other cell types as well in which TGF-β is participating in pathologic states, including hepatocytes in rats exposed to phenobarbital (16), hepatocellular carcinoma (17), and mammary tumors (18).

The proximal region of the M6P/IGFIIr 5′-flanking region was studied herein for changes in protein-DNA binding during hepatic injury. This region was selected for study because it contains two E-boxes, which are often involved in transcriptional regulation (19, 20). E-boxes are transcription factor binding elements that conform to a CANNTG motif and bind proteins that carry a basic helix-loop-helix structural domain, of which more than 200 have been found, categorized into five classes according to the basic helix-loop-helix domain amino acid sequence (19). The specificity of E-box binding relies not only on the central two nucleotides of the CANNTG sequence, but also in the one or two nucleotides immediately flanking...
E-box-binding Protein Regulates M6P/IGFIIR Expression

CANNTG (21). The proximal M6P/IGFIIR E-boxes both contain the sequence GTCACTGTA (E-box underlined). The CACTGTG E-box in particular binds heterodimeric combinations of Myc, Mad, Max, and Mxi (22), as well as upstream stimulatory factor (23), and transcription factor E3 (24). The proximal promoter elements of M6P/IGFIIR were initially investigated using C3H 10T1/2 cells by Liu et al. (20), showing that Myc bound the 3' E-box and that both E-boxes participated in promoting transcription.

In hepatic stellate cells, the M6P/IGFIIR mRNA transcript was up-regulated both in rats exposed to CCl4 and in culture-activated stellate cells. In examining the M6P/IGFIIR proximal promoter, two E-boxes were found to bind a 75-kDa protein (P75). Activation of stellate cells markedly reduces binding of P75, concomitant with the up-regulation of M6P/IGFIIR transcription within 48 h after exposure to CCl4. Mutations of the E-boxes in CAT reporter constructs result in significantly higher expression in transfected stellate cells activated in culture. The putative repressor P75 appears to be a novel transcription factor as its molecular mass of 75 kDa is considerably higher than those of other known CACTGTG-binding factors.

EXPERIMENTAL PROCEDURES

Stellate Cell Isolation and Culture—Hepatic stellate cells were isolated from Sprague-Dawley male rats by previously described methods (25, 26). The cells were either used directly for RNA isolation or nuclear extract preparation, or for culture on tissue culture flasks precoated with type I collagen and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 10% newborn calf serum (10/10 media) (27). Cultures were passaged after reaching confluence; experiments were carried out with cells between passage 2 and 6. Cells quiescent in culture were prepared from serum-activated cells for gel shift analysis. Cells were used in serum-deprived medium for 60 h before harvesting. Cells used in CCl4 studies were harvested either 6, 16, 24, 48, or 72 h after a single administration by gavage of 2 μl of a 1:1 CCl4-mineral oil preparation.

RNA Isolation and Ribonuclease Protection Assay—RNA was isolated using trireagent (Sigma). 1 ml trireagent per rat was used for freshly harvested stellate cells or 4 ml per 75 cm2 confluent cells. Cells were homogenized in trireagent by vortexing, phase separation achieved with addition of 0.1 volume of bromochloropropane (Molecular Co’s modified Eagle’s medium without serum. Cells were incubated in 5% CO2/95% air, according to the manufacturer’s instructions. 18,000 cpm of M6P/IGFIIR transcript preparation, or for culture on tissue culture flasks precoated with type I collagen and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 10% newborn calf serum (10/10 media) (27). Cultures were passaged after reaching confluence; experiments were carried out with cells between passage 2 and 6. Cells quiescent in culture were prepared from serum-activated cells for gel shift analysis. Cells were used in serum-deprived medium for 60 h before harvesting. Cells used in CCl4 studies were harvested either 6, 16, 24, 48, or 72 h after a single administration by gavage of 2 μl of a 1:1 CCl4-mineral oil preparation.

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Single stranded RNA probe complementary to M6P/IGFIIR mRNA nucleotides 836–1247 was synthesized with α-[32P]UTP using the MAXIscript kit (Ambion, Austin TX) according to the manufacturer’s instructions. The template for RNA synthesis was generated by PCR with a T7 promoter linked to the downstream primer. Primers used were 5′-CCAGCTCTCTCAGCACTTATG-3′ and 5′-GGATCCCTTAATACGACTTATAGGGAGGAGCTTCTCCT-3′, the T7 promoter is underlined and the M6P/IGFIIR sequence is in small capitals. The T7 promoter and flanking nucleotides were described in the MAXIscript kit manual. The M6P/IGFIIR rat cDNA used for the PCR template was kindly provided by Richard G. MacDonald (9). PCR DNA was ethanol-precipitated, resuspended in water, and used directly in RNA synthesis without further purification. The template for rat glyceraldehyde phosphate dehydrogenase (GAPDH) was obtained from Ambion and yields a 316-bp protected fragment. The synthesized RNA probes were gel purified in 5% 19:1 acrylamide, 8% urea; RNA probes were eluted from the cut bands overnight in 0.5 M NH4OAc, 0.1% SDS, 1 mM EDTA, at 30 °C; radioactivity of the eluted probes was measured by scintillation counting. To account for possible differences in mRNA abundance the ribonuclease protection assay was performed with equal amounts of RNA from normal and CCl4-exposed rats. The integrity of the extracts was determined by probing them with a double-stranded 32P-labeled Sp1 consensus (5′-ATCCATGCGGGGCGGGGGCGAGC-3′) for a gel shift assay, resulting in distinct Sp1 shifts from all extracts (data not shown).

Transfection Studies—Stellate cells were transfected using the LipofectAMINE reagent (Life Technologies Inc.), and cell extract handling, extraction, quantitation, and CAT measurements were described as described previously (28). CAT data was normalized by making results of −2824 to −1 BC transfections = 1 and adjusting for transfection efficiency determined by co-transfection of a β-galactosidase reporter. The level of β-galactosidase expression was determined by a chemiluminescence assay kit (Tropix, Bedford MA) according to the manufacturer’s instructions. The original M6P/IGFIIR promoter DNA was kindly provided by Peter Rotwein in a luciferase reporter plasmid (30). The M6P/IGFIIR promoter sequence was removed by restriction endonuclease digestion and subcloned into a pUC19-based reporter plasmid containing the CAT gene. The full-length promoter includes sequence to −2824 of the M6P/IGFIIR 5′-flanking region plus 109 bp of the 5′-untranslated region. Deletion mutants of the promoter were constructed utilizing a unique SnaBI site at −2808 double-digested with a variety of downstream sites to remove promoter segments and create truncated promoter-CAT plasmids. BstEII, PshAI, and PstI were used to make the −796del, −1del, and −4del plasmids, respectively. The full-length promoter was mutagenized by a PCR-based site-directed mutagenesis described by Ho et al. (31). The primers used to mutagenize both 5′ and 3′ E-boxes changed the CACTGTG E-box sequence to TTCGGA. The mutated PCR product contained the endogenous PstI/RsaI restriction sites found in the promoter which were used to create a 5′- and 3′-E-box mutant M6P/IGFIIR promoter. The two flanking PCR primers used for mutagenesis were as follows: 5′-GGTTGGTCACGGTTCGTCCG-3′, 5′-GACAAGCTCTAGCTCTGCGG-3′; the central two primers for 5′ E-box mutagenesis: 5′-CCTGCCTTCGGAGATCTCCTGC-3′, 5′-AGGATTCCAGGGGAGCTGCACTCCGT-3′; the central two primers for 3′ E-box mutagenesis: 5′-GGAGCTTGGGCAACCTGAGTTCGGG-3′, 5′-GAACTGTGGCAACCTGAGTTCGGG-3′. The mutagenized plasmids were transfected into quiescent stellate cells and sequenced to ensure there were no unintentional mutations produced by PCR, as well as to ensure the correct introduction of the mutated nucleotides into the promoter sequence. The plasmid containing both mutated E-boxes (5′/3′ mutEbox) was constructed by taking the plasmid containing the 3′-mutated E-box (3′ mutEbox) and carrying out a second mutagenesis to incorporate the 5′-mutated E-box. The inability of the mutant TTCGGA sequence to bind the E-box shifted bands was tested with the same 20 mers shown in Fig. 2A with TTCGGA substituted for CACTGTG. These double-stranded oligomers were used as cold competitors in 5′ and 3′ E-box gel shifts. A 200-fold excess of the mutated E-box oligonucleotides was unable to compete with either the 5′ or 3′ E-boxes for binding.

Southwestern Blotting—Two gels were run and blotted onto nitrocellulose as in a standard Western assay (32). 100 μg of quiescent and 72-h CCl4 nuclear extracts for each gel were boiled and run in Tris/glycine/SDS-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose in a Tris/glycine/SDS buffer. The blots were incubated for 0.5 h at room temperature in a renaturation buffer of 5 mM dithiothreitol in gel shift binding buffer (10 mM Tris, pH 7.5, 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 50 mM NaCl, 50 mg/ml poly(dI-dC)). The renaturation buffer was then replaced with fresh buffer and non-fat milk added to 5%. This was incubated for 3 h at room temperature. The gel was run again with 0.25% milk in fresh binding buffer. The blots were then incubated another 0.5 h and moved to 4 °C, and 2 μg of labeled 5′ E-box or 3′ E-box probe were added and incubated for approximately 16 h. The DNA was end-labeled with γ32P-ATP, 6000
E-box-binding Protein Regulates M6P/IGFIIR Expression

Ribonuclease Protection—Detection and quantitation of M6P/IGFIIR mRNA in hepatic stellate cells was carried out with a ribonuclease protection assay (Fig. 1). The quantitation of M6P/IGFIIR mRNA was adjusted to GAPDH levels to account for differences in the amount of target RNA used. M6P/IGFIIR mRNA was present in stellate cells from normal liver (Fig. 1A, lane 1). A 2.5-fold increase in the M6P/IGFIIR transcript level in stellate cells occurs by 48 h after exposure of rats to CCl4 (Fig. 1, A, lanes 1–3, and B, bars Q, 6, and 48). This increase was paralleled in vitro, where culture activated stellate cells have a 1.8-fold increase in M6P/IGFIIR transcript when compared with RNA from deprived cells and a 2.8-fold increase compared with stellate cells from normal liver (Fig. 1A, lanes 1, 4, and 5, and B, bars Q, 0%, and CA). The M6P/IGFIIR probe hybridized to stellate cell RNA without GAPDH (Fig. 1A, lane 6) yields a protected band migrating the same distance as the upper bands of lanes 1–5. Denatured single-stranded M6P/IGFIIR and GAPDH probes migrate slightly above their double-stranded counterparts (Fig. 1A, lane 7), and the M6P/IGFIIR probe does not hybridize to any yeast sequence (Fig. 1A, lane 8).

Gel Shift Assays—Protein binding to the two proximal E-boxes was examined in stellate cells using electrophoretic mobility shift assays. Each E-box probe consisted of two 20-mer double-stranded oligonucleotides of native DNA sequence each containing the E-box heptamer plus their respective seven flanking nucleotides found in the M6P/IGFIIR promoter (Fig. 2A). The oligonucleotides were radioactively labeled and used as gel shift probes with nuclear extracts from freshly harvested stellate cells. The 3’ E-box was bound by protein in a single shifted band from quiescent nuclear extract (Fig. 2B, lane 1). The 5’ E-box was bound by protein in two distinct shifted bands from quiescent nuclear extract (Fig. 2B, lane 1). The upper band of the 5’ E-box shift corresponds to the single-shifted band of the 3’ E-box gel shift (Fig. 2E). To assess the relevance of E-box binding to in vivo fibrogenesis, gel shifts were performed using nuclear extracts from freshly isolated stellate cells taken from rats exposed to CCl4 during the previous 6, 16, 24, 48, or 72 h. CCl4 treatment produced a down-regulation, or decrease in E-box binding activity, of both E-box binding proteins (Fig. 2, B, lanes 3–9, and C, lanes 3–11). A computer-aided densitometry scan shows the decrease in protein binding occurring by 24–48 h after CCl4 administration (Fig. 2D). Fig. 2E shows cold 5’ E-box and 3’ E-box competing with labeled 3’ E-box and 5’ E-box probes for binding quiescent nuclear extracts. Cold 5’ E-box competes out the 3’ E-box shifted band. However, cold 3’ E-box can only compete the upper band of the two complexes shifted by 5’ E-box (lane 3). This indicates that each E-box has a distinct binding specificity despite similarities in sequence.

Nuclear extracts from stellate cells in culture (Fig. 3) contain the same (upper) band as the 3’ and 5’ E-box gel shifts in Fig. 2, B and C, but the lower band seen in the 5’ E-box shift (Fig. 2C) is not present, likely due to differences in in vivo versus in vitro stellate cell proteins. The band shifted by nuclear extract from stellate cells quiesced for 60 h in 0% serum is markedly more intense than the band shifted by extract from culture-activated cells (Fig. 3). Importantly, this mimics the in vivo results of Fig. 2, B and C, which showed that CCl4 (in vivo)-activated stellate cells have reduced binding activity.

Transfection Studies with Native and Mutagenized E-boxes—Since M6P/IGFIIR expression is up-regulated less than 48 h after exposure to CCl4, the coincident loss of E-box binding activity in less than 48 h of CCl4 exposure suggested that the bound proteins are repressors and that induction of M6P/IGFIIR potentially involves de-repression of the gene. To further explore this possibility, 5’ truncations of the M6P/IGFIIR promoter were carried out that selectively eliminated the E-boxes (Fig. 4). The full-length M6P/IGFIIR promoter (−2824 wt) is able to induce CAT expression in activated cells as expected. However, with a large 5’ truncation in the promoter (−716del), which leaves E-box function within the promoter less influenced by distal elements, there is a large (6-fold) decrease in CAT activity compared with the full-length −2824 wild type CAT activity. The −11del construct eliminates the 5’ E-box while maintaining an intact 3’ E-box. This reporter responds with a 4.3-fold increase in CAT expression over the −716del construct containing the intact E-boxes, indicating the presence of a repressor 3’ to −716. The −4del construct cuts the 3’
Fig. 2. E-box gel shift assays. Gel shift assays were performed with the two proximal E-box probes and nuclear extracts from freshly isolated rat hepatic stellate cells. Rats were exposed to CCl₄ for the indicated time points in hours; stellate cells were then immediately harvested and nuclear proteins extracted for use in the gel shifts. Q, quiescent cell nuclear extract (no CCl₄ exposure). Arrows indicate the shifted bands.

A, gel shift probes were derived from the native M6P/IGFIIR sequence. B, gel shift with the 5' E-box probe. C, gel shift with the 3' E-box probe. D, bar graph of densitometry of the shifted bands in B and C. E, quiescent extract incubated with excess of either cold 5' E-box (5') or 3' E-box (3') before adding labeled probe.
E-box in half and eliminates both E-boxes, resulting in a further increase in CAT activity, a 1.4-fold increase over -11del CAT activity. These findings support the gel shift results and indicate the importance of repressor binding to M6P/IGFIIR E-box elements.

Mutagenesis of the E-boxes in the full-length (-2824 to +109) promoter was carried out to characterize E-box function within an intact promoter sequence. Three full-length M6P/IGFIIR reporters were constructed containing mutagenized E-boxes, one construct for each E-box mutagenized individually (5′ mutEbox and 3′ mutEbox) and a third construct containing both E-boxes mutagenized simultaneously (5′/3′ mutEbox). Each mutagenized plasmid was transfected, and the results were compared with the previously transfected plasmids containing only the native sequence (Fig. 4). All three mutagenized constructs resulted in striking increases in CAT activity. When compared with -2824 wild type CAT activity, 5′ mutEbox produced a 2.4-fold increase, 3′ mutEbox produced a 15.3-fold increase, and 5′/3′ mutEbox produced a 7.8-fold increase in CAT activity. The transfection experiments which utilized the mutagenized E-boxes yielded parallel but not identical results (i.e. of dissimilar magnitude) in comparison to E-boxes in truncated promoters and strongly support the evidence of repressor binding to the two proximal E-boxes.

**Southwestern Analysis**—We initiated characterization of the E-box binding protein(s) by determination of molecular weight via Southwestern blotting (Fig. 5). Two gels each containing 100 µg of both quiescent stellate cell and 72-h CCl₄ nuclear extracts were run on SDS-polyacrylamide gel electrophoresis, blotted, renatured, and probed with either labeled 5′ E-box or 3′ E-box probe from Fig. 2A. The 5′ E-box probe bound to a prominent 75-kDa band (P75) from quiescent extract, which is considerably reduced in the 72-h extract, and to a lesser 47-kDa band. The 3′ E-box bound the same 75-kDa band, but not the 47-kDa band, mirroring the gel shift results of Fig. 2, B and C. The identity of the lesser 47-kDa band remains unknown, but it should be noted that it is only the upper band of the in vivo gel shifts that appeared in the in vitro gel shift (Fig. 3), and thus it is likely this upper band contained the protein (P75) that bound the E-boxes in the transfected M6P/IGFIIR plasmids.

**DISCUSSION**

The ribonuclease protection assay shows an increase in M6P/IGFIIR transcript levels in stellate cells activated by exposure to CCl₄. Subsequent experiments define two elements in the proximal promoter of M6P/IGFIIR that are significant to the regulation of M6P/IGFIIR expression by hepatic stellate cells during initial stages of hepatic fibrogenesis. The elements are both CACGTG E-boxes which bind a 75-kDa protein (P75) that is down-regulated in stellate cells as a response to the fibrogenic stimulant CCl₄. This down-regulation occurs concomitantly with the up-regulation of M6P/IGFIIR, suggesting 1) repressor activity for P75 and 2) up-regulation of M6P/IGFIIR expression involves a de-repression of the gene. This is supported by functional removal of the E-boxes by mutagenesis resulting in a marked increase in the ability of the M6P/IGFIIR promoter to drive CAT expression in activated stellate cells.

![Fig. 3. 5′ E-box probed gel shift using extract from stellate cells in culture. 10/10, nuclear extract from activated stellate cells cultured in 10% fetal bovine serum and 10% newborn calf serum; 0%, nuclear extract from cultured stellate cells quiesced in 0% serum for 60 h.](image)

![Fig. 4. Analysis of E-box function. Transfections were carried out with M6P/IGFIIR promoter-CAT constructs containing either wild type (CACGTG) or mutagenized (TCCGAA) E-boxes. The left panel illustrates the transfected plasmids, which contain 109 bp of the 5′-untranslated region (+1 to +109) plus either 2824 bp of the 5′-flanking region with or without mutagenized E-boxes, or with deletions of the 5′-flanking region. These promoter constructs were fused to a CAT gene in a pUC19-based reporter plasmid. Open oval, native E-box; filled oval, mutagenized E-box. Right panel, M6P/IGFIIR-CAT plasmids transfected into primary cultures of activated hepatic stellate cells. The results were normalized by setting -2824 wt = 1. Results are expressed in relative units of CAT activity, mean ± SD, n = 6, adjusted by efficiency of co-transfected β-galactosidase expression.](image)
The ribonuclease protection assay (Fig. 1) shows that the M6P/IGFIIR mRNA level in stellate cells in vivo increases by as much as 2.5-fold during early fibrogenesis. The detection of M6P/IGFIIR transcript in quiescent stellate cells from normal liver is significant because it was previously undetected on a Northern blot (13). More importantly, M6P/IGFIIR is required for lysosomal enzyme sorting (33), and so its low level expression would be expected. Also, the increased mRNA level parallels the increase in M6P/IGFIIR protein, which peaks in stellate cells at 48 h after CCl4 treatment (13).

The gel shift assays (Fig. 2) show a significant decrease in E-box binding as a result of CCl4 exposure. The decrease appears to begin after h 16 and by 24–48 h there is a marked decrease in binding (Fig. 2, B, lanes 7–9, and C, lanes 7–11, and D). This decrease in repressor binding fits the pattern of change in M6P/IGFIIR mRNA transcription abundance, which increases between 6 and 48 h after exposure to CCl4 (Fig. 1, A, lanes 1–3, and B, bars Q, 6, and 48). Thus, the down-regulation of E-box binding would explain, in part, the pattern of transcription for M6P/IGFIIR observed in vivo; as repressor binding decreases, transcription increases. Fig. 2E shows that the single-shifted band from the 3′ E-box gel shift and the upper band of the 5′ E-box gel shift migrate to identical positions in the gel (lanes 1 and 4). The inability of the 3′ E-box to bind protein(s) in the lower band of the 5′ E-box gel shift is further demonstrated by the failure of cold 3′ E-box to compete with 5′ E-box shifting two protein complexes and the 3′ E-box binding the lower band (lane 3) and demonstrates different binding specificities between the two E-boxes.

Stellate cells in culture spontaneously transform into the activated phenotype (34). Significant to subsequent transfection experiments, the quiescent versus activated change in E-box binding seen in the in vivo experiments is mimicked in cultured stellate cell extracts. Cultured stellate cells in passage 3 quiesced in 0% serum show a marked increase in E-box binding activity; therefore, E-box binding decreases during activation of stellate cells in culture, recapitulating the in vivo response (Fig. 3). M6P/IGFIIR transcript level changes in vitro also mimic the in vivo response, increasing 2.8-fold compared with quiescent cells from the rat and 1.8-fold compared with cells quiescent in culture. It was previously shown that M6P/IGFIIR transcript is detectable in Northern blots of RNA from cultured cells only after 6 days in culture (13). Freshly isolated quiescent stellate cells become activated at approximately the 6th day of culture (13).

Since the decrease in E-box binding is concomitant with increased M6P/IGFIIR expression, the E-box binding factor appears to be a repressor, and its down-regulation (or deactivation) contributes a de-repression to M6P/IGFIIR gene expression. Fig. 4 shows that both the elimination of the E-boxes in plasmids −11del and −4del as well as their mutagenesis produces an increase in the ability of the promoter to drive CAT expression. The increases in CAT activity from the −11del and −4del plasmids shows not only that the E-box is binding a repressor, but that an inducer is likely to be found downstream of −4 in the 109 bp of the 5′-untranslated region. The functional elimination of the E-boxes in the mutagenized full-length plasmids produced a dramatic increase in CAT activity, a strong demonstration of the repressor function of the E-box binding protein. The large difference in CAT activity between transfected mutagenized plasmids and the deletion plasmids that physically remove the E-boxes is likely due to the remaining full promoter being uncoupled by the E-box repressors, showing the presence of strong transcriptional activators in the 5′-flanking region, which will need to be identified in future studies. Interestingly, the combined effect of the 5′ mutEbox and 3′ mutEbox is not additive, and CAT activity of the 5′/3′ mutEbox is half that of the 3′ mutEbox. However, gel shifts (Fig. 2E) show that the two E-boxes have binding affinities that are not identical, suggesting they may differ functionally in vivo. Protein-protein interactions of the 5′ P75 may differ from 3′ P75 interactions, which could also explain this nonadditive effect.

Of the two bands shifted by the 5′ E-box (Fig. 2C), only the upper band is shifted by the 3′ E-box (Fig. 2B). Cultured stellate cell extracts (Fig. 3) also contain only the upper shifted band seen in Fig. 2, B and C. Since it is in the cultured stellate cells that transfections occur, it is likely the protein in this upper (75 kDa) band whose E-box binding is being prevented in the mutagenized plasmids, resulting in the high level CAT expression, and thus it is this protein, P75, that is acting as the repressor, rather than the lower band seen in Fig. 2C.

Southwestern analysis showed both E-box probes bound to a prominent 75-kDa band (P75) from quiescent nuclear extract (Fig. 5). P75 is considerably reduced in the 72-h extract, which supports the results of the gel shift experiments. The minor 47-kDa band was bound by the 5′ E-box (Fig. 5, left panel), but was not bound by the 3′ E-box. This reflects the E-box gel shifts in Fig. 2, with the 5′ E-box shifting two protein complexes and the 3′ E-box shifting only the upper of those two bands. The 75-kDa repressor is a size that does not correspond to any known CACGTG E-box-binding proteins, which range in size from 21 to 59 kDa (22–24). A literature search did not reveal any E-box-binding protein in the 75-kDa range. Also, incubating the nuclear extracts with anti-Max, anti-Myc, or anti-upstream stimulatory factor did not result in supershifts in the gel shift assays (data not shown). Thus it is likely that P75 is a novel protein.

It is interesting to note that the same promoter elements are used in opposing ways in two different cell types. In C3H 10T1/2 cells, the two M6P/IGFIIR E-boxes promote transcription apparently through Myc binding (20). Hepatic stellate cells, on the other hand, use the same E-boxes to repress (and de-repress) transcription of M6P/IGFIIR through regulation of an E-box binding repressor. CCl4 treatment does not abolish repressor binding to the E-boxes in vivo, rather repressor E-box binding is down-regulated. This suggests a fine-tuned regulation of M6P/IGFIIR transcription by hepatic stellate cells in early fibrogenesis.

The presence of a repressor which participates in stellate cell regulation of M6P/IGFIIR expression may eventually provide a target for therapeutic intervention in liver fibrosis. Without M6P/IGFIIR, activation of TGF-β is greatly reduced. Through high level expression of specific repressors such as P75, the progression of fibrogenesis could be blocked.
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