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Research Article

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LAP (NF-IL6) Transactivates the Collagen $\alpha_1(I)$ Gene from a 5' Regulatory Region

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Abstract

Although collagen is known to enhance hepatocyte differentiation and hepatocytes produce collagen *in vivo*, the transcriptional factors responsible for collagen type I gene expression in hepatic cells are not known. LAP (Liver Activator Protein) is a member of the C/EBP family, which in differentiated hepatocytes contributes to the high levels of liver-specific gene expression. In this study we show that LAP binds to the collagen $\alpha_1(I)$ promoter at both reverse CCAAT motifs and activates transcription. Furthermore, an upstream element, collagen element I (-370/-344), which shares homology with the LAP binding *cis*-element of the albumin promoter (9 of 13 bp) is described. This collagen element I stimulates transcription in both orientations and when placed in front of either a homologous or a heterologous chimeric report construct. These experiments suggest that LAP may be important in the expression of collagen in differentiated hepatocytes through both the promoter and a newly described upstream element. (*J. Clin. Invest.* 1994. 94:808-814.) Key words: hepatocyte differentiation • extracellular matrix • liver-specific gene expression • b-ZIP proteins • IL-6

Introduction

Collagen type I, a major component of the hepatic extracellular matrix, is important for normal structure and function of the liver (1). Several studies have documented that hepatocytes contribute to hepatic collagen production *in vivo* (2-4). Hepatocytes produce collagen type I as demonstrated by immunohistochemistry with electronmicroscopy and *in vivo* labeling (3, 4). Furthermore, when fresh hepatic cell types are isolated from normal liver, a sizeable proportion of hepatic collagen $\alpha_1(I)$ mRNA is hepatocyte in origin (5).

Collagen in the extracellular matrix is able to confer a differentiated phenotype to cultured hepatocytes (6-8). Therefore, independent of its quantitative contribution towards the hepatic extracellular matrix, collagen synthesized by hepatocytes may serve as an autocrine or paracrine feedback loop to enhance hepatocyte differentiation.

Primary cultured hepatocytes synthesize collagen only when expressing a differentiated phenotype as indicated by albumin

expression (9). When cultured hepatocytes are stimulated to dedifferentiate, e.g., by the administration of vasopressin, both albumin and collagen $\alpha_1(I)$ gene expression are inhibited (9). The mechanism(s) responsible for this observation is not known but it may be mediated by the release of intracellular calcium or the phosphatidylinositol pathway (10).

CCAAT binding proteins are thought to be important in the regulation of the collagen $\alpha_1(I)$ gene because in human, rat, and mouse two highly conserved CCAAT binding sequences are present in the collagen $\alpha_1(I)$ promoter sequence (11). CBF is a CCAAT binding factor isolated from liver nuclear extracts which binds to both CCAAT sites (12). Furthermore, NF-1 binds to both CCAAT box sites, and it seems to play a role in collagen gene expression in fibroblasts (13). LAP (Liver Activator Protein) (NF-IL6, C/EBP β or IL6-DBP) is a major albumin promoter binding protein in rat liver nuclear extracts (14) and is a member of the C/EBP family of binding proteins (14-17). LAP appears to be a major contributor towards a differentiated phenotype in hepatic cells, including the activation of liver-specific genes and the inhibition of hepatic cell proliferation (14, 17, 18). Because of the reasons outlined above, we studied the role of LAP in the expression of collagen $\alpha_1(I)$ gene in hepatic cells. We found that LAP, but not C/EBP α , stimulates transcription of the collagen $\alpha_1(I)$ gene from the promoter and a novel upstream element.

Methods

Overexpression and purification of recombinant proteins. The plasmid containing the LAP cDNA in the vector pET8c have been previously described (14). LAP_{SMA} contains both the basic domain and leucine zipper as LIP (Liver Inhibitor Protein) but with an additional NcoI-SmaI deletion (19). Recombinant protein was overexpressed from the bacterial strain BL 21/DE-3/plys S transformed with the appropriate plasmid essentially as described by Studier et al. (20). Briefly, bacterial extracts were prepared from bacteria grown to an OD (A_{600}) of 0.5 and subsequently for 4-5 h in the presence of 0.5 M IPTG (14). Lysates were sonicated and fractionated on heparin-agarose columns (14).

Vectors and oligonucleotides. The mammalian expression vector pSCT C/EBP α was generously provided by S. McKnight (Carnegie Institute, Baltimore, MD). pColcat 1, pColcat 6, and pColcat 3 were kindly provided by M. Breindl (San Diego State University, San Diego, CA) and contain the 5' regions of the mouse collagen $\alpha_1(I)$ gene from -3,600/+110, -1,627/+110, and -220/+110, respectively. Mutations of footprint 1 (FP1m) and 2 (2Am and 2Bm) of Colcat 3 were kindly provided by D. A. Brenner (University of North Carolina) and have been described (13). The entire 5' region (\approx 3,700 bp) of pColcat 1 was removed by XbaI digestion, gel purified and subsequently digested with Hinfl or Sau3AI and used for gel retardation assays. An oligonucleotide was generated of the Col E-1 with the sense strand 5'TCGAGT-TTGTAGTGTTCCTCCAACTCAGATTTCG and antisense strand 5'TCG-ACGAATCTGAGTTGGGAAACTACAAAC, including the region from -368 to -347 and restriction sites XhoI 5' and Sall 3'. Sense and antisense strands were annealed and phosphorylated with T4 polynucleotide kinase and subsequently underwent three rounds of ligation

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and double restriction with *SalI/XhoI* to generate a 5 mer of Col E-1 with each subunit in a 5' to 3' orientation. This multimerized Col E-1 was ligated into the *XhoI* site, which is 5' to a chimeric plasmid containing the -220/+110 collagen $\alpha_1(I)$ promoter in front of the promoterless bacterial CAT gene in pCATBasic (Promega, Madison, WI). Orientation was determined by restriction analysis with *EcoRI* and *XhoI*, and vectors designated pCOL5xF and pCOL5xR for forward and reverse orientations, respectively. Similarly, the multimerized segment was placed in front of the SV₄₀ promoter in pCATPro (Promega) and designated pCp5X. Site-directed mutagenesis was performed using the Amersham system version 2.1 (Amersham, Arlington Heights, IL). A 1,737 bp *PstI-XbaI* fragment of the collagen $\alpha_1(I)$ gene (-1627 to +110) was inserted into Bluescript KS(-) and the antisense strand was rescued. An oligonucleotide in the sense orientation (ACTGATCCCTTAGTAC-TGTTTCCCAAC) that contained both T to A and G to C point mutations was synthesized. Both point mutations together form a unique restriction site, *ScaI*. The resulting mutant segment was cut with *PstI-BglIII* and inserted back into Colcat 6, maintaining the original promoter segment (-220/+110) forming pCol E-1M. The mutation was confirmed by restriction with *ScaI* and sequence analysis.

DNase protection and gel retardation experiments. One-dimensional gel retardation assays were performed as described previously (14). Oligonucleotides were end labeled with [³²P]γATP and T4 polynucleotide kinase by the forward reaction (21). *Sau3AI* and *HinfI* fragments of the *XbaI-XbaI* collagen $\alpha_1(I)$ gene were end labeled with [³²P]γATP using the reverse reaction (21). Two-dimensional gel retardation assays were done essentially as described by Boffini and Prentki (22). DNase I footprinting was performed using either LAP_{Δ21} or the LAP_{SMA} recombinant proteins on the promoter and Col E-1 segments as previously described (14).

Cell culture and transfection. The hepatoma-derived cell line Hep G2 was grown in minimal essential medium with 10% fetal bovine serum in an atmosphere of 5% CO₂. For each experiment, 6 μg total *CsCl* prepared DNA was added in using standard calcium phosphate precipitation in HBS buffer (pH 7.05) (14). Subsequently, glycerol shock (15% in PBS) for 30 s was performed 4 h after the addition of the DNA-calcium phosphate complex. Cells were harvested after 72 h and CAT content determined by ELISA as described by the manufacturer. (5' prime 3' prime; Molecular Devices Corp., Menlo Park, CA).

Statistical analysis. The Student paired *t* test was used to evaluate the differences of the means between groups, with a *P* value of < 0.05 as significant.

Results

LAP binds to and activates transcription from the collagen $\alpha_1(I)$ promoter. Two well-characterized reverse CCAAT boxes have been described in collagen $\alpha_1(I)$ promoter of mouse, rat, and human genes, however, the transacting factor(s) that interact with these *cis*-elements have not been clearly identified (13, 23, 24). For the reasons explained above, we used bacterially expressed LAP to determine whether it interacts with the collagen $\alpha_1(I)$ promoter. The LAP_{SMA} has only the DNA binding and dimerization domains of LAP. As it has been shown for LIP (19), LAP_{SMA} has a high affinity for their cognate DNA *cis*-acting elements (18) and it was, therefore, also used in some experiments. Recombinant LAP binds to the collagen $\alpha_1(I)$ promoter in a gel retardation assay (Fig. 1) in a complex pattern, with an increasing number of retarded bands at higher protein concentrations. This suggests that more than one binding site with different affinities exists on the promoter segment or, alternatively, that multimers of the LAP protein form at higher protein concentrations. To confirm that the binding of LAP to the collagen $\alpha_1(I)$ promoter results from specific DNA-protein interaction, DNase I footprints were performed. Binding condi-

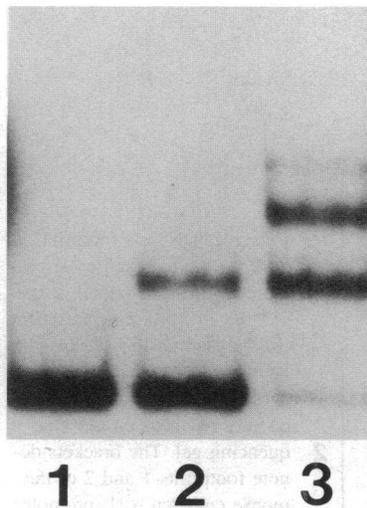


Figure 1. Gel retardation analysis of the interaction of LAP with the collagen $\alpha_1(I)$ promoter. The promoter segment (-220/+110) was isolated and end labeled as described in Methods. The probe (4×10^4 cpm) was incubated without (lane 1) or with LAP_{SMA} recombinant protein at a final dilution of 1:1,000 (lane 2) or 1:200 (lane 3).

tions at room temperature showed no difference between the DNase I pattern with LAP compared with control (data not shown). Similarly, combining DNase I treatment of the DNA-protein complex and gel retardation assay to isolate the 1:1 DNA-protein complex showed again no footprint pattern (data not shown). The results of the latter experiment might suggest nonspecific DNA-protein interaction or short *K_{on}* and *K_{off}* equilibrium constants at the assay temperature conditions. This was resolved by performing the DNase I reaction at 4°C, resulting in two clear footprints (Fig. 2), which are similar to what has been called footprints 1 and 2 or C and D of the mouse collagen $\alpha_1(I)$ promoter (13, 23) and they include both reverse CCAAT boxes. Analysis of the role of LAP on collagen $\alpha_1(I)$ transcription was performed in HepG2 cells, which express negligible levels of LAP-related proteins (18, 19). Transient cotransfection experiments using a chimeric report construct containing the mouse collagen $\alpha_1(I)$ promoter segment (-220/+110) and the bacterial chloramphenicol acetyltransferase gene (Colcat 3) with the LAP expression vector consistently showed a marked increase in reporter activity compared with the chimeric reporter vector alone (*n* = 9; *P* < 0.05) (Fig. 3). These results indicate that LAP interacts with the mouse collagen $\alpha_1(I)$ promoter and can direct a high level of transcription from a chimeric reporter gene in cotransfection experiments. In similar experiments pSCT C/EBP α was cotransfected with Colcat 3 and consistently showed no transactivation (data not shown). In contrast, the expression of AlbuminCAT, containing the albumin promoter, was increased approximately threefold by pSCT C/EBP α as expected (25). Mutations within the CCAAT box of FP1 (FP1m) and FP2 (FP2Am and FP2Bm) decreased the expression of CAT compared with Colcat 3 (Fig. 4). However, only the FP1m significantly inhibited the activation by LAP of Colcat 3 (*n* = 6; *P* < 0.05). These results indicate that FP1 is more important than FP2 in the transactivation of the collagen $\alpha_1(I)$ gene by LAP, which is similar to what was found in NIH 3T3 fibroblasts (reference 13; and our unpublished observations).

Identification of an enhancer element in the collagen $\alpha_1(I)$ 5' untranslated region. Transacting factors that bind to the promoter element of a gene frequently bind to other *cis*-elements of the same gene, augmenting their effect on gene transcription (26). To determine if LAP could bind elsewhere in the 5' region of the mouse collagen $\alpha_1(I)$ gene, we used a new technique,

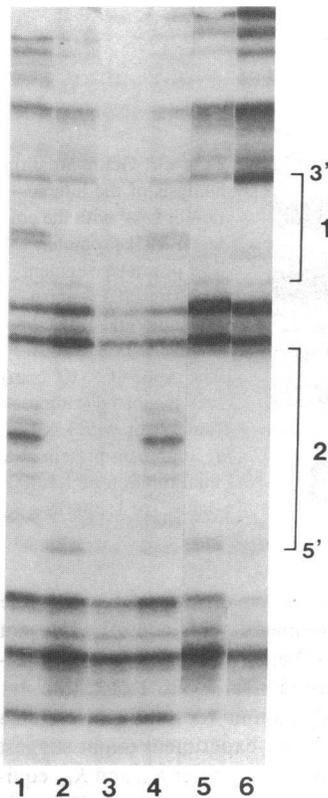


Figure 2. DNase I footprint of LAP on the collagen $\alpha_1(I)$ promoter. The sense strand of the $-220/+110$ promoter fragment was labeled on the 3' end with Klenow. DNase I digestion was performed on ice and subsequently resolved on a 6% sequencing gel. The brackets denote footprints 1 and 2 of the mouse collagen $\alpha_1(I)$ promoter. The same amount of probe was added to all lanes. Lanes 1 and 4 do not contain recombinant LAP. LAP_{SMA} was added to lane 2 (8 footprint units [FPU]) and lane 3 [4 FPU]. LAP was added to lane 5 (8 FPU) and lane 6 (4 FPU). One FPU is defined as the concentration of LAP that shifts 50% of the probe in a standard gel retardation assay.

two-dimensional gel retardation assay (22). Others have shown that the standard technique of transiently transfecting various segments of the collagen $\alpha_1(I)$ gene has not been sufficient to identify *cis*-elements responsible for the high tissue-specific levels of expression observed in transgenic mice containing the same segments of the collagen gene (23, 27). By restricting the 5' region of the mouse collagen $\alpha_1(I)$ gene ($-3,700/+110$) with *HinfI* and *Sau3AI*, multiple bands of different size were obtained and end labeled with ^{32}P . Although retarded bands are observed in a standard one-dimensional gel retardation assay

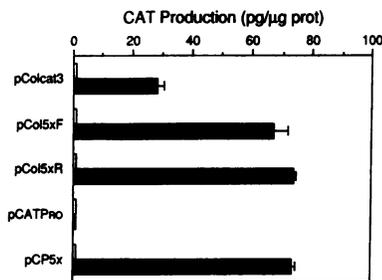


Figure 3. LAP stimulates transcription from the promoter and an upstream element of the collagen $\alpha_1(I)$ gene. HepG2 cells were transfected for 72 h with the respective chimeric reporter genes as described in Methods. pColcat 3 contains the $-220/+110$ fragment of

the mouse collagen type 1 promoter; pCol5xF and pCol5xR contain the fivefold multimerized $-370/-344$ Col E-1 oligonucleotide placed 5' of the $-220/+110$ promoter fragment in either the forward (F) or reverse (R) direction. PCP5x contains the same multimerized segment as above but in front of the SV₄₀ promoter. pCATPro has only the SV₄₀ promoter. The chloramphenicol acetyl transferase (CAT) protein content (pg/μg protein) was determined by ELISA in lysates of cell cultures that were either mock-transfected (white bars) or transfected with a mammalian vector expressing LAP (black bars) as described in Methods. Values are the mean \pm one half the range of duplicate samples.

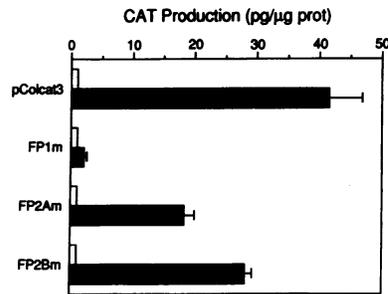


Figure 4. LAP activates transcription from the CCAAT elements of the collagen $\alpha_1(I)$ gene. Experiments were performed as described in Fig. 3. Chimeric reporter genes, FP1m, FP2Am, and FP2Bm are all derived from pColcat 3 and contain point mutations in footprint 1 (FP1) or

footprint 2 (FP2) as described in Methods. HepG2 cell cultures were either mock transfected (white bars) or transfected with a LAP-expressing vector (black bars). Values are the mean \pm one half the range of duplicate samples.

(Fig. 5), the fragments responsible for the retarded bands cannot be clearly identified. Furthermore, the free probe (Fig. 5, lane 1) gives a faint band at the same level as one of the shifted fragments. (Fig. 5, arrow). These issues were clarified by using a two-dimensional gel retardation assay, in which the second dimension is done at high temperatures to dissociate all DNA-protein interactions. Thus, DNA fragments are resolved by their size in the second dimension, and fragments shifted in the first dimension would appear off the diagonal above the fragment of origin. The two-dimensional gel retardation assay shows that one of the retarded fragments correspond to the bands that are ~ 250 bp in length (Fig. 6). As depicted in Fig. 7, it is evident that the promoter and a segment between $-344/-593$ are the only fragments of this size. To further localize the site of DNA-protein interaction, the *HinfI* fragment was isolated and ligated to PCR linkers and uniformly labeled with ^{32}P during PCR amplification. The amplified fragment was further restricted with *Sau3AI* and again subjected to a gel retardation assay (data not shown), which showed that the segment from -344 to -370 contained the site of DNA-protein interaction (Fig. 7). The homology between the identified sequence and the albumin promoter D site, where LAP acts as a potent transcriptional factor

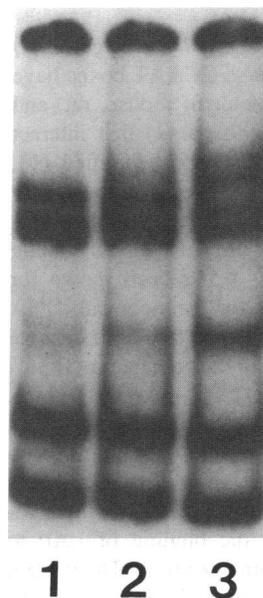


Figure 5. One-dimensional gel retardation assay of LAP with the *HinfI* fragments of the $-3,700/+110$ 5' region of the collagen $\alpha_1(I)$ gene. The 5' region of the collagen $\alpha_1(I)$ gene from $-3,700$ to -110 was digested with *HinfI* to completion and end labeled. Probe without recombinant LAP (lane 1) or with recombinant LAP_{SMA} protein at a final dilution of 1:200 (lane 2) or 1:100 (lane 3) was incubated at room temperature for 20 min before separation on a 5% native PAGE.

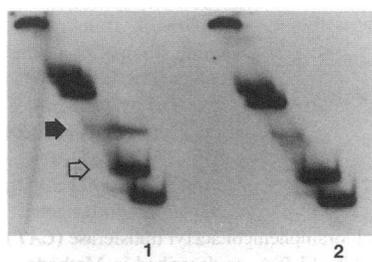


Figure 6. Two-dimensional gel retardation analysis of the *Hinf*I fragments of the -3,700/+110 segment of the collagen $\alpha_1(I)$ and LAP. *Hinf*I fragments were end labeled and incubated with LAP (1:1,000 dilution) alone (lane 1) or with a 20-fold excess of

competitor (lane 2), an oligonucleotide that binds LAP with a high affinity sequence (5'TGGTATGATTTTGTAAATGGGG sense and 5'-TACCACCCATTACAAAATCA antisense). The open arrow identifies the free probe that corresponds to the retarded band (closed arrow).

(14, 17, 19), is striking, with 9 of 13 bases being identical (Fig. 7). To further characterize the DNA-protein interaction, DNase I footprinting was performed and showed a single footprint (Fig. 8), which corresponded to the segment identified by gel retardation. Oligonucleotides identical to the sense and antisense strands of the protected segment identified in Fig. 7 were synthesized. A standard gel retardation assay performed using LAP and the synthesized double-standard oligonucleotide (Col E-1) displayed the expected DNA-protein interaction (Fig. 9). Since the homology with the albumin D site was so striking, a mutation of this site was derived using data from serial point mutations of the D segment, which found that an A \rightarrow C mutation at -100 bp from the start site of transcription was sufficient to inhibit binding (reference 14; M. Chojkier, unpublished observations). Therefore, in the Col E-1 segment, the G at -359 bp was changed to a C. In addition, the T at -363 bp was changed to an A to introduce a convenient restriction site while not altering binding of LAP (reference 14; M. Chojkier, unpublished observations). The double-stranded oligonucleotide did not compete out binding for the Col E-1 oligonucleotide as did cold Col E-1 and the oligonucleotide of the albumin D site (Fig. 9), suggesting that these point mutations were sufficient to prevent the binding of LAP.

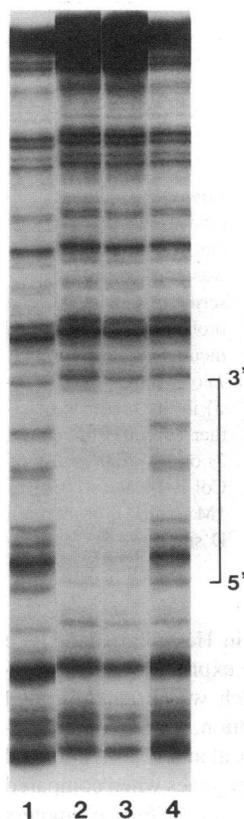


Figure 8. DNase I footprint analysis of DNA-protein interaction of LAP with the Col E-1 element. A segment of the collagen $\alpha_1(I)$ gene containing the Col E-1 element (-370/-344) was isolated and the sense strand labeled on the 3' end. Probe is shown without (lane 1) or with recombinant LAP_{SMA} at a relative concentration of 2 FPU (lane 2), 1 FPU (lane 3), or 0.5 FPU (lane 4). The bracket indicates the extent of the protected region.

Functional analysis of the upstream LAP binding site of the collagen $\alpha_1(I)$ gene. The Col E-1 oligonucleotide described above was multimerized in a head-to-tail fashion and a five mer was selected for further analysis. The 5 mer (5x) was cloned in the forward (F) and reverse (R) orientations in front of either the homologous mouse collagen $\alpha_1(I)$ promoter-CAT (pCol5x) or a SV₄₀ promoter-CAT (pCP5x). These chimeric reporter genes were used in transient cotransfection experiments with

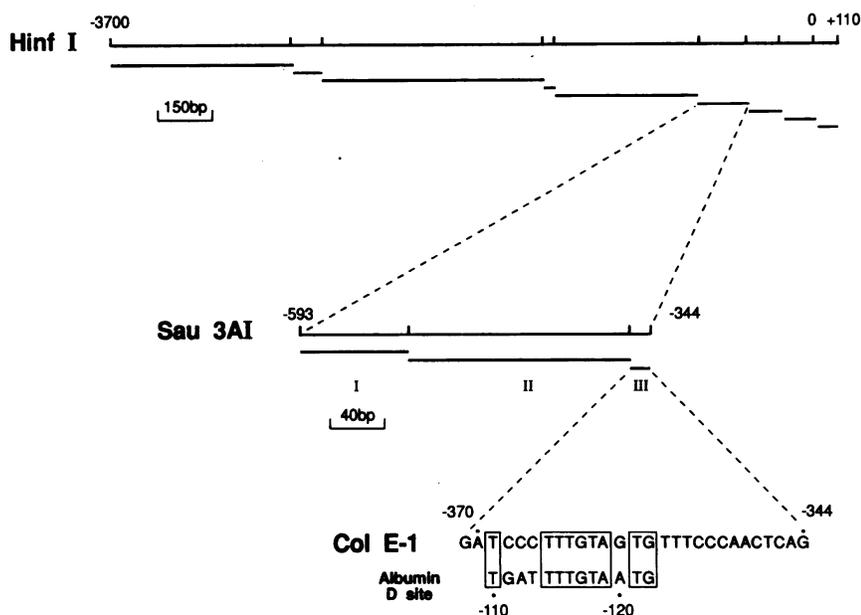


Figure 7. Isolation and sequence identity of the LAP binding element in the 5' region of the collagen $\alpha_1(I)$ gene. *Hinf*I digestion of the -3,700/+110 collagen $\alpha_1(I)$ region resulted in eight upstream fragments as shown in the upper part of the figure. Two-dimensional gel retardation assay identified the -593/-344 segment to contain a LAP binding element. *Sau* 3A1 digestion of the -593/-344 fragment and one-dimensional gel retardation assay further narrowed the LAP binding element to -370/-344. The sequence of the -370/-344 segment, which contains the LAP binding element (Col E-1), is shown. Sequence homology to the albumin D site is indicated by the boxes.

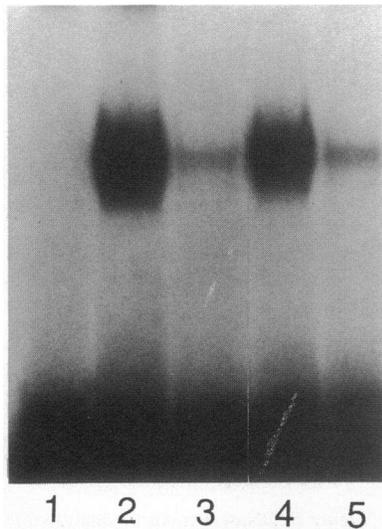


Figure 9. LAP does not bind to the Col E-1 mutant. Col E-1 segment was end labeled as described in Methods. The probe (4×10^4 cpm) was incubated without (lane 1) or with LAP (lanes 2–5) in the presence of either no competitor (lane 2) or 100-fold excess of Col E-1 (lane 3), Col E-1M (lane 4), or albumin D site (lane 5).

the eukaryotic LAP expression vector in Hep G2 cells. There was a marked increase in reporter gene expression in the presence of LAP ($n = 4$; $P < 0.05$), which was orientation and promoter independent (Fig. 3). In addition, the upstream element was effective in enhancing both basal and LAP-stimulated transcription from these chimeric reporter genes when compared with the collagen (pColcat 3) and SV₄₀ (pCATPro) promoters (Fig. 3). These results are consistent with the behavior of other *cis* elements (28) and suggest that the segment identified between -344 and -370 of the mouse collagen $\alpha_1(I)$ gene has the ability to function as a *cis*-acting element for LAP in hepatic cells. We will refer to this element as Col E-1 (for collagen element 1). This element was mutated (Col E-1M) within Colcat 6 (-1,627/+110) to assess whether Col E-1 was functional in the context of a large segment of the collagen $\alpha_1(I)$ gene. In cotransfection experiments, LAP activates transcription from pCol E-1M only ~ 25% when compared with Colcat 6 ($n = 5$; $P < 0.05$) (Fig. 10). These experiments indicate that Col E-1 (-370 to -344 bp) is the main *cis*-acting element for LAP within -1,627 to +110 bp of the mouse collagen $\alpha_1(I)$ gene. This is the first characterized *cis*-acting element upstream of the 220-bp promoter of the mouse collagen $\alpha_1(I)$ gene.

Discussion

The *cis*- and *trans*-acting element(s) of the collagen $\alpha_1(I)$ gene necessary for the high level of expression in some cell types are not known. Positive and negative regulatory elements have been identified in the promoter and first intron of the collagen $\alpha_1(I)$ gene (23, 27, 29–32). The promoter and first intron are highly conserved among different species, including human, mouse, and rat (30, 31). Transgenic mice containing regions 5' of the human collagen $\alpha_1(I)$ promoter indicate that important upstream regulatory sequences are necessary for high levels of expression of this gene (27).

There are only four factors known to stimulate collagen gene transcription: lipid peroxidation (33, 34), acetaldehyde (35), a fibrogenic factor (36), and transforming growth factor β under some conditions (24) but not others (37). Identification of the transacting factors that are important in the regulation of colla-

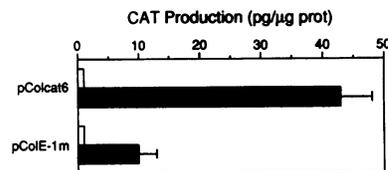


Figure 10. Transcriptional stimulation of the collagen $\alpha_1(I)$ by LAP is inhibited by mutation of the upstream element Col E-1. HepG2 cells were transfected for 72 h with chimeric reporter genes

as described in Methods. The chloramphenicol acetyl transferase (CAT) protein content was determined by ELISA, as described in Methods. Values are the mean \pm one-half the range of duplicate samples.

gen gene expression should provide insights into these important issues.

Several factors are known to interact with the collagen $\alpha_1(I)$ promoter (-220/+110) at four separate sites (13, 23). Two of these sites contain reverse CCAAT motifs and are conserved in human and rat sequences as well (23, 31). One factor that binds to these CCAAT *cis*-regulatory sequences is CBF (12). CBF is a heteromeric CCAAT binding protein isolated from rat liver nucleic proteins which in *in vitro* transcriptional assays acts as a transcriptional activator for both the collagen $\alpha_2(I)$ and $\alpha_1(I)$ genes (12). It would be of interest to assess the role of CBF in collagen gene regulation *in vivo*. Recently, it has been reported that CBF, NFY, and Rous sarcoma transcriptional virus factor 1 may be closely related factors (38). In fact, the sequence reported for NF-YB is identical to the "A" chain of CBF. Northern blots of various tissues suggest that the mRNA for NFY is ubiquitous (39).

In the normal liver, hepatocytes produce collagen (2, 3, 5) but the factors involved in the regulation of the collagen gene in hepatocytes are not known. In this study we have shown that LAP, a liver-enriched nuclear protein, binds to and directs transcription from the collagen $\alpha_1(I)$ promoter (-220/+110) in transfection experiments using a hepatic cell line (Fig. 11). Furthermore, we identified an upstream element that binds LAP, called Col E-1 (Fig. 11), which stimulates transcription in both the forward and reverse orientations when placed before either a heterologous or homologous promoter. Site-directed mutagenesis of footprint 1 within the collagen $\alpha_1(I)$ promoter or of the upstream element Col E-1 within the collagen $\alpha_1(I)$ gene (Colcat 6) markedly diminished transactivation by LAP of chimeric reporter genes containing these *cis*-acting elements. On the other hand, mutation of footprint 2 of the collagen $\alpha_1(I)$ promoter resulted in only a minor reduction of the transactivation by LAP of the chimeric genes containing -220 bp of the promoter. These experiments strongly support the notion that the binding of LAP to the promoter footprint 1 and upstream element Col E-1 are critical for the transcription of collagen $\alpha_1(I)$ gene by LAP. It is of interest that LAP does not bind to the consensus sequence oligonucleotides of SP1 or CTF/NF1, which have been described in FP1 and FP2 (reference 13; and K. Houglum, unpublished observations). In the hepatocyte, LAP is one of the

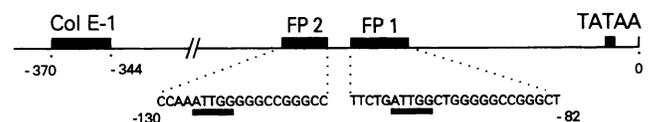


Figure 11. LAP binding sites on the 5' regulatory region of the collagen $\alpha_1(I)$ gene. The footprints of LAP (Col E-1, FP2, and FP1) are shown.

most abundant members of the C/EBP family of DNA-binding proteins (14) and is responsible, in part, for the high level of expression of liver-specific genes (14–17, 19) as well as the characteristic quiescent phenotype (18). LAP is present at high levels only in nuclear extracts from liver (14), and, in liver sections, is found only in hepatocyte nuclei by immunohistochemistry (18). It is not surprising the LAP may play a role in the expression of collagen from differentiated hepatocytes. Of particular interest is the striking homology between the albumin D site and an upstream element at –344 to –370 in the 5' region of the collagen $\alpha_1(I)$ gene (9 of 13 bp). This element is highly conserved in rat (10 of 13 bp) and in humans (10 of 13 bp, with a single base insertion) (31). This element, when multimerized and placed before both homologous and heterologous promoters, was able to direct high levels of CAT expression above the baseline promoter activity.

Unlike LAP, C/EBP α did not stimulate transcription from collagen $\alpha_1(I)$ chimeric reporter genes in transient cotransfection experiments. This CMV-C/EBP α mammalian expression vector was able to transactivate, as expected, an albumin promoter-CAT reporter gene as reported previously (25, 40). LAP shares extensive sequence homology in its DNA-binding and leucine zipper domains with C/EBP α and, as a consequence, these two proteins show an indistinguishable DNA-binding specificity and readily heterodimerize (14). However, we suggested that by virtue of their divergent NH₂-terminal domains LAP and C/EBP α would be likely to affect different target genes (14). Indeed, cotransfection of CAM-kinase II with LAP (but not with C/EBP α) stimulates transcription from a Ca²⁺-calmodulin-dependent protein kinase II responsive element. This effect is the result of phosphorylation of Ser²⁷⁶ (41) within the leucine zipper of LAP. In addition, LAP, but not C/EBP α , inhibits the progression of the cell cycle before the G1/S boundary in HepG2 cells (18).

It is of interest that in well-differentiated long-term primary rat hepatocyte cultures the expression of collagen $\alpha_1(I)$ gene parallels that of the albumin gene (9) and that LAP, an activator of many liver-specific genes (14–17, 41, 42), also stimulates the transcription from chimeric genes driven by collagen $\alpha_1(I)$ regulatory elements. Collectively, these experiments support the notion that hepatocyte collagen type I expression is part of a differentiated phenotype. Reports of the enhancement of differentiated characteristics of primary adult rat hepatocytes cultured on a collagen matrix (43, 44) lend further support to the biological association between collagen $\alpha_1(I)$ gene expression and hepatic cell differentiation. Whether collagen secreted by hepatocyte is part of an autocrine or paracrine feedback loop that sustains hepatocyte differentiation remains to be established.

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