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Hepatic Transcription of the Acute-Phase α_1-Inhibitor III Gene Is Controlled by a Novel Combination of *cis*-Acting Regulatory Elements†

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mRNA coding for the abundant broad-range plasma proteinase inhibitor α_1-inhibitor III (α_1I3) was detected only in rat liver, while mRNA for the related proteins α_1-macroglobulin and α_2-macroglobulin was also found in a variety of nonhepatic tissues. *cis*-Acting control elements necessary for the hepatic transcription of α_1I3 were mapped by transfection and expression studies of control-region constructs in cultured hepatic and nonhepatic cells. The promoter-proximal 5′-flanking region contained four control elements, I to IV, located between −109 and −196 base pairs upstream of the transcriptional start site relevant for the hepatic transcription of this gene. Elements II and III were essential, and I and IV exerted strong modulatory effects. Elements I to III acted as positive regulators, and IV acted as a negative element. Element II contained the sequence TGGCA and is probably a binding site for a nuclear factor related to the known transcription factor NF1. The other three elements did not resemble consensus binding sites for known transcription factors that are involved in the hepatocyte-specific transcription of other well-characterized plasma protein genes, such as the prototype factor HNF-1. Thus, the α_1I3 gene achieves its highly hepatocyte-specific transcription through a novel combination of *cis*-acting control elements and *trans*-acting factors.

Acute-phase proteins are blood proteins whose concentrations in plasma are greatly altered in response to tissue damage and infections (14, 20, 47). The primary site of synthesis of these proteins is the liver, and the hepatic expression of the corresponding genes is controlled by mediators of inflammation, including glucocorticoids and peptide hormones such as interleukin-1, interleukin-6 (IL6), interferons, and tumor necrosis factor (TNF) (14, 20). The plasma concentrations of most of the acute-phase proteins are increased during an inflammatory response, but the concentrations of a smaller group of proteins, the so-called negative acute-phase proteins, are decreased. Representatives of this group are albumin, transthyretin, apolipoprotein A1, and α_1-inhibitor III (α_1I3) (4, 14, 32, 37, 39, 47).

α_1I3, a broad-range proteinase inhibitor, is the second most abundant plasma globulin in rats, with normal concentrations in the range of 6 to 10 mg/ml. α_1I3 belongs to the α-macroglobulin family of thiolester proteins that also includes α_1-macroglobulin (α_1M), α_2-macroglobulin (α_2M), and the complement components C3, C4, and C5 (4, 17, 52, 53). Unlike α_2M, which is strongly induced, and α_1M, which is only minimally increased, α_1I3 is strongly downregulated during an acute inflammatory response (32, 39). During the first few days of an acute inflammation, α_1I3 levels fall to 1 to 2 mg/ml, and during chronic inflammations they decline further to less than 0.5 mg/ml. Hepatic α_1I3 mRNA concentrations are lowered similar to α_1I3 plasma protein levels under acute-phase conditions (1, 4, 48), and the transcription rate of the α_1I3 gene was found to be decreased 12.7-fold 6 h after induction of an experimental acute-phase response, with a concomitant 16-fold decrease in α_1I3 nuclear precursor RNA concentrations (39).

α_1I3 cDNA clones have been isolated and characterized by several groups (1, 4, 48). From this analysis it became obvious that the α_1I3 mRNA population is composed of a mixture of closely related sequences, subsequently shown to be the products of a small α_1I3 gene family with approximately four to five closely related genes (4, 39). This microheterogeneity has since been detected at the plasma protein level (53). The developmental regulation of the α_1I3 gene is very different from that of its near relative, the α_2M gene. While the α_2M gene is expressed in fetal liver and shut off after birth to be reinduced during inflammations, the α_1I3 gene is silent in fetal liver, it is expressed at low levels at birth, and full adult levels are reached only approximately 20 days after birth (1).

Recently we analyzed signals that can reproduce the acute inflammatory regulation of the α_1I3 gene and lead to the negative regulation of α_1I3 in rat hepatoma cell lines (L. J. Abraham, A. D. Bradshaw, R. G. Fletcher, and G. H. Fey, Mol. Biol. Med., in press). The gene was downregulated by IL6 and glucocorticoids in the three cell lines FAZA, FT02B and FAO1. After treatment with different concentrations of IL6 alone or in combination with the synthetic glucocorticoid dexamethasone, the mRNA concentrations were decreased fourfold in a dose-responsive and time-dependent manner. Thus, the gene can be regulated by IL6 in hepatoma cells in a similar way as in the liver (Abraham et al., in press).

Hormone-mediated repression of transcription is an inter-

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esting and important biological process that is poorly understood in mammalian cells. The α_1I3 gene is an excellent example of a strongly and rapidly downregulated gene which can be used to study the responsible molecular mechanisms in greater detail. However, to understand the mechanism of downregulation of transcription, it is necessary to understand first the mechanisms responsible for the constitutively high hepatic transcription of this gene prior to the onset of an inflammatory response. Therefore, we have analyzed in this report the *cis*-acting control sequences and nuclear proteins binding to these sequences that are responsible for the normal hepatic transcription of the rat α_1I3 gene. Surprisingly, the results demonstrated that this gene achieves its highly specific hepatic transcription by using a combination of *cis*-acting control sequences that is different from those of most other well-known liver-specific plasma protein genes, suggesting that liver-specific transcription can be achieved by a variety of different combinations of *cis*-acting control elements and *trans*-acting factors.

MATERIALS AND METHODS

Quantitation of α_1I3 mRNA in different rat organs. Fresh frozen rat organs were purchased from Pelfreeze Inc., and RNA was prepared by the guanidinium thiocyanate procedure followed by sedimentation through a cesium chloride cushion. RNA concentrations in each of the samples were determined both spectrophotometrically and by a pilot experiment in which hybridization to a radiolabeled rat rDNA probe was used (38). After evaluation of both of these concentration measurements, constant amounts of RNA from each of the organs were loaded in triplicate on nylon membranes and fixed by UV irradiation. Hybridization probes were 25-base-pair (bp) synthetic oligonucleotide probes specific for the so-called bait regions of each of the three macroglobulins, which show the highest degree of sequence specificity and avoid cross-species hybridization among the three closely related mRNA species. Hybridization in 5% sodium dodecyl sulfate (SDS)-containing buffers and wash steps were performed as described before (7). In addition, one series of dots was carried along in the main experiment and hybridized with a ^{32}P-labeled ribosomal cDNA probe (38).

Construction of luciferase fusion genes. Approximately 2.2 kilobases (kb) of 5′-flanking sequence from the α_1I3 gene was available from a previous study (39). Oligonucleotide-directed mutagenesis (30) was used to introduce an *Sst*I restriction site at position +58 relative to the transcription start site. The resultant α_1I3 5′ fragment extending from −2214 bp to the *Sst*I site was introduced into the *Sst*I site of the transcription vector p19LUC (54) to produce a fusion gene called A1I3(−2214), consisting of the 5′ noncoding sequences of the α_1I3 gene linked to the coding region of the firefly luciferase gene (13). A nested set of 5′ deletions were constructed either by making use of appropriate restriction sites, by removing unwanted sequences by exonuclease III digestion (26), or, in the case of A1I3(−48), by oligonucleotide-directed mutagenesis (30) to create a *Hin*dIII site at position −48. The 3′ deletion series was constructed by starting with appropriate α_1I3 promoter restriction fragments and, after addition of *Hin*dIII linkers, ligation into the *Hin*dIII site created in A1I3(−48).

Cell culture and transfection. The respective rat and human hepatoma cell lines FAZA (34) and Hep3B (28) and the human epithelial HeLa line were grown in a 1:1 mixture of Dulbecco modified Eagle medium and Ham F12 balanced salt solution. Media were supplemented with 10% fetal bovine serum and 100 U of penicillin and streptomycin per ml. Cells were cultured in 100-mm dishes in an atmosphere of 5% CO_2 at 37°C.

Transfection was carried out by a modified calcium phosphate precipitation technique (23). Cells were plated at a density adjusted so that they reached 60% confluence after overnight incubation. Prior to transfection, the cells were washed three times with HEPES-buffered saline (HBS; 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.3], 6.7 mM KCl, 142 mM NaCl). The DNA precipitate was formed by dropwise addition of 250 mM $CaCl_2$ to an HBS solution containing 7.5 mM Na_2HPO_4 and 20 μg of test plasmid DNA plus 1 μg of pRSVCAT DNA per ml (21). After incubation in fresh medium for 1 h, the cells were incubated for 6 h with the precipitate. The cells were then shocked with 25% glycerol (in HBS) for 2 min, washed three times with HBS, and incubated in fresh medium for 40 h. Plasmid DNA to be used for transfection was isolated as described before (2) and purified by two successive CsCl equilibrium centrifugation steps.

Luciferase assays were performed as described before (13). Following lysis and removal of the cell debris by centrifugation, constant amounts of protein (120 μg) were assayed for luciferase activity with a Monolight 2001 luminometer (Analytical Luminescence Laboratories, San Diego, Calif.). The relative efficiency of transfection in each experiment was monitored by assay for the production of constant amounts of chloramphenicol acetyltransferase used as an internal standard (51).

Preparation of nuclear extracts. FAZA and HeLa cell nuclear extracts were prepared from adherent tissue culture cells that had reached 90% confluence. The cells were detached from the dishes with trypsin-EDTA, and extracts were prepared by the method of Shapiro et al. (49). Fractionation of nuclear extracts was performed with heparin-Sepharose (Pharmacia) chromatography. Extracts prepared as above and dialyzed against 20 mM HEPES (pH 7.9)–20% (vol/vol) glycerol–0.2 mM EDTA–2 mM EGTA (ethylene glycol tetraacetic acid)–2 mM dithiothreitol (DTT) containing 50 mM KCl were loaded onto a heparin-Sepharose column equilibrated with the same buffer. After extensive washing, the nuclear proteins were eluted stepwise with 0.1 to 0.8 M NaCl in the same buffer but without KCl. After dialysis against 100 mM KCl in the same buffer, the different fractions were stored in liquid N_2 until needed.

DNase I footprinting. DNase I footprint reactions (16) were carried out with unfractionated nuclear extracts. The −225 to −81 fragment from the α_1I3 promoter region was isolated by cleaving the construct A1I3(−225) or the deletion construct A−81/−48 with *Hin*dIII, 3′-end labeling either the noncoding or coding strand, respectively, with the Klenow fragment of DNA polymerase I, and then recleaving with either *Ssp*I or *Eco*RI, followed by gel purification. Probe (1 ng) was then incubated in 20-μl reaction volumes containing 4 μg of poly(dI-dC) and either 10 or 50 μg of nuclear extract or 40 μg of bovine serum albumin (BSA; molecular biology grade; Boehringer Mannheim) in 20 mM HEPES (pH 7.9)–20% glycerol–0.2 mM EDTA–2 mM EGTA–2 mM DTT–60 mM KCl. After 10 min of incubation on ice and 40 min at 22°C, 20 μl of 20 mM $MgCl_2$–5 mM $CaCl_2$ was added, followed by an appropriate dilution of DNase I. After 120 s at room temperature, the reaction was stopped by the addition of 40 μl of stop buffer (1% SDS, 20 mM EDTA, 200 mM NaCl, 25 μg of tRNA per ml). The probe DNA was recovered by phenol-chloroform extraction and ethanol pre-

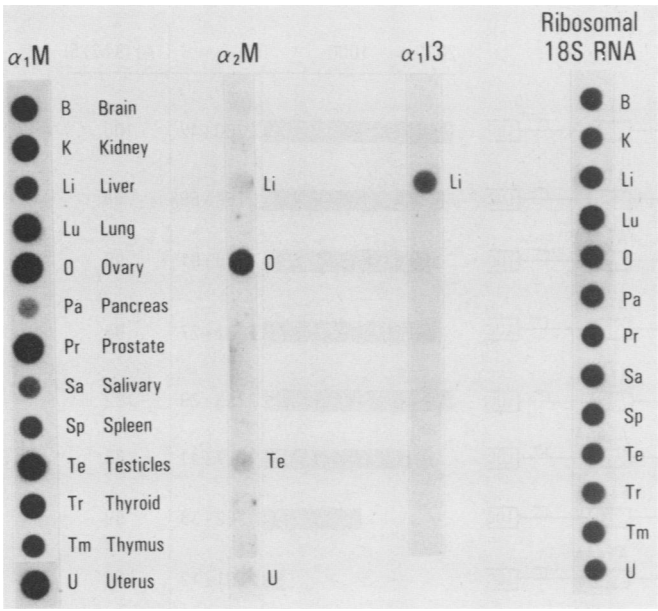

FIG. 1. Tissue-specific expression of the three rat α-macroglobulin genes. Total RNA was prepared from 13 different rat organs, and constant amounts of RNA were deposited on nylon filters. One set of filters each was hybridized with radioactively labeled oligonucleotide probes representing α_1M, α_2M, α_1I3, and a cDNA copy of 18S rat rRNA as a control for loading of constant amounts of RNA from each organ.

cipitation and analyzed on denaturing polyacrylamide gels along with probe DNA that had been subjected to the G+A cleavage reaction of Maxam and Gilbert (35). Binding reactions with purified transcription factor NF1 (27) (kind gift from C. Jones, Salk Institute) contained 4 μg of BSA and 20 ng of a double-stranded oligonucleotide (35-mer) from an unrelated downstream region of the α_1I3 gene. For competition experiments, 20 ng of cold specific oligonucleotide was included in the binding reaction instead of the nonspecific 35-mer.

RESULTS

Organ-specific expression of the three rat α-macroglobulin genes. Total RNA was prepared from 13 different rat organs and analyzed for expression of the three major rat α-macroglobulins α_1M, α_2M, and α_1I3 by hybridization with gene-specific synthetic oligonucleotide probes (4, 17; G. Eggertsen and G. H. Fey, unpublished data). α_1M mRNA was expressed in high concentrations in all 13 organs (Fig. 1), while α_2M mRNA was expressed in high levels only in the ovary. Traces of α_2M mRNA were also detected in the liver, the testes, and the uterus. From other studies it is known that the α_2M gene is expressed in liver, testes, uterus, placenta, and ovary and is subject to hormonal regulation by different characteristic hormones (6, 15, 25, 40). Thus, the trace levels visible in Fig. 1 are significant. Therefore, it was surprising that high levels of α_1I3 mRNA were detected only in the liver, suggesting that the α_1I3 gene exhibits unusually strong liver-specific expression. Further studies have demonstrated that this gene is expressed in the hepatocytes of the liver and preferentially in periportal hepatocytes (1a).

Delineation of the 5′ boundary of the transcriptional control region. To map the 5′ boundary of the region responsible for the hepatic expression of the α_1I3 gene, a deletion series extending from +58 bp to between −48 bp and −2.2 kb upstream of the transcription start site were constructed. These constructs were linked to the firefly luciferase reporter gene, and luciferase activity was measured after transient transfection into the rat hepatoma cell line FAZA. Constitutive expression of the α_1I3-luciferase fusion required at least 225 bp of 5′-flanking sequences (Fig. 2). Construct α_1I3(−186), containing only 186 bp of 5′ sequence, exhibited a 50% decrease in transcriptional activity. Shorter constructs showed a low transcriptional activity comparable to that seen with the shortest construct, α_1I3(−48), which contained only the TATAA box region. This level of activity was similar to that seen with the p19LUC vector, which contained no promoter sequences. Quantitation of the luciferase mRNA produced after transfection of three of the key constructs, A1I3(−2214), A1I3(−225), and A1I3(−118), by primer extension with a luciferase-specific oligonucleotide showed that the transcriptional start site used in FAZA cells was within 4 bp of that used in rat liver (data not shown).

Cell type-specific expression of the α_1I3 gene. The transcriptional activity of the α_1I3 promoter region was also tested in a number of other cell lines, including the human hepatoma Hep3B and HeLa cell lines (Fig. 3). Transfection of a range of α_1I3-luciferase fusions into Hep3B cells gave a transcription profile that was very similar to that seen with the rat hepatoma FAZA line and confirmed that the region important for transcription was contained within the first 225 bp upstream from the TATAA region. In contrast, when the same constructs were tested in HeLa cells, a low level of expression was observed that did not change significantly between the longest and the shortest construct. Preliminary results were also obtained with the rat fibroblast cell line FR3T3 and confirmed the results from the HeLa cell experiments (data not shown). Thus, the construct containing up to −225 bp of the α_1I3 promoter region carried sufficient information to mediate the cell type-specific expression of the α_1I3 gene in hepatic cells.

Mapping the 3′ boundary of the upstream promoter element. A series of internal deletions were created that started at −48 and extended for increasing distances into the 5′ region (Fig. 4). These constructs were tested in the transient transfection assay in FAZA cells. The relative transcriptional activity of the constructs A1I3(−225) and Δ−81/−48 indicated that the region −81 to −48 did not contribute to the transcriptional activity of the α_1I3 gene. Unexpectedly, a deletion extending from −48 to −118 (Δ−118/−48) led to a 2.4-fold increase in transcription relative to the full-length construct. Further deletion from −48 to −150 abolished transcription almost entirely. When the same constructs were tested by transfection into HeLa cells, very low expression levels were observed. Significantly, however, an increase in activity was also seen in HeLa cells when the construct Δ−118/−48 was assayed.

To rule out positional effects caused by placing the upstream control region closer to the TATAA box, the regions of the deletion constructs that were 5′ to −48 were inverted (Fig. 4). The results of the transcription assay with the reverse deletion constructs (Fig. 4) confirmed the 3′ deletion results and indicated that the increase in transcription of the constructs Δ−118/−48 and Δ−118/−48R (i.e., reverse deletion of Δ−118/−48) compared with that in A1I3(−225) and A1I3(−225R), respectively, was not caused by moving the control region closer to the TATAA region. The results are consistent with the presence of a negative control element, functional in both FAZA and HeLa cells, that was contained

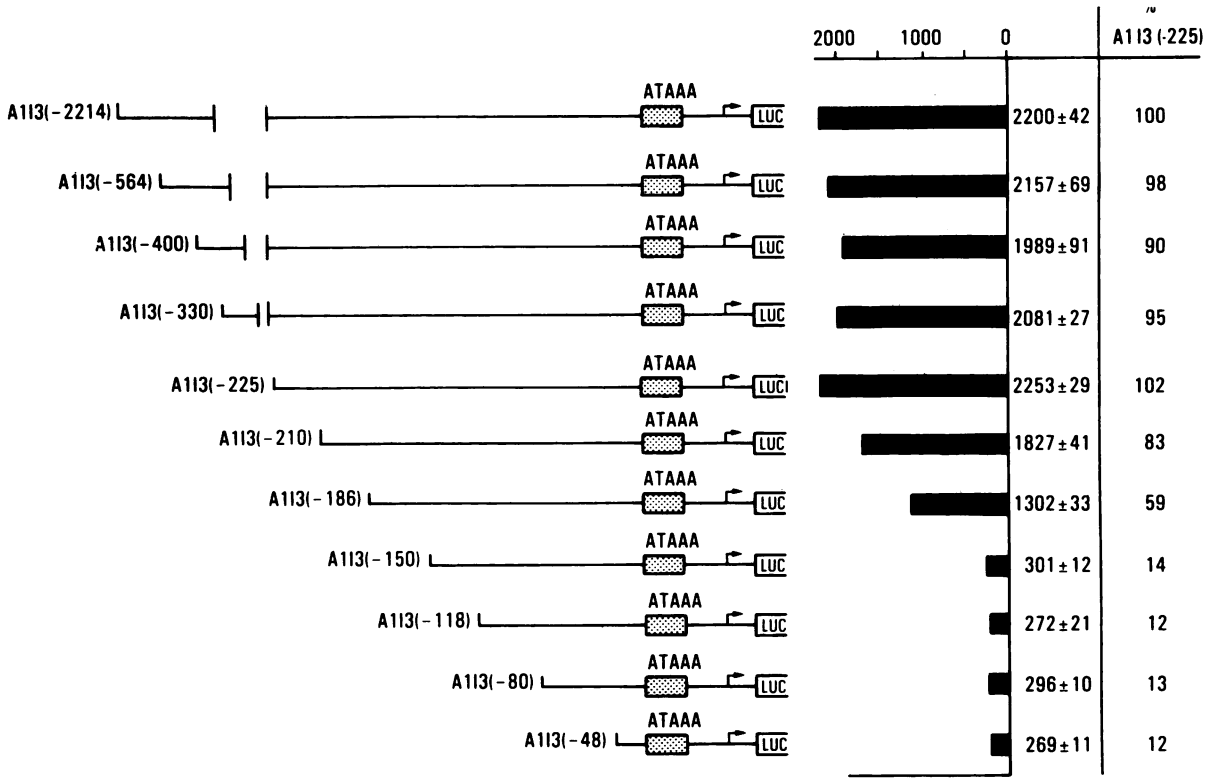

FIG. 2. Effect of 5′ deletions on the transcriptional activity of the α_1I3 promoter. A series of constructs containing from 2,214 bp [A1I3(−2214)] to 48 bp [A1I3(−48)] of α_1I3 5′-flanking sequences linked to the firefly luciferase reporter gene (LUC) were tested for transcriptional activity after transient transfection in FAZA cells. Luciferase activity was measured in cell extracts and normalized with respect to the chloramphenicol acetyltransferase activities measured in each extract. The relative luciferase activities presented are averages of five measurements ± 1 standard deviation.

within or overlapped the region −81 to −118. The 3′ reverse deletion results also showed that the high level of expression seen in FAZA cells with the entire construct was lost after deletion of the −48 to −150 region, indicating that the 3′ boundary of the positive control region maps between −118 and −150. The combined results of the 5′ and 3′ deletion studies indicate, first, that the region responsible for high-level hepatoma-specific expression of the α_1I3-luciferase fusion lies within a 107-bp region between −225 and −118 and, second, that a downstream negative element is present that is functional in both cell types.

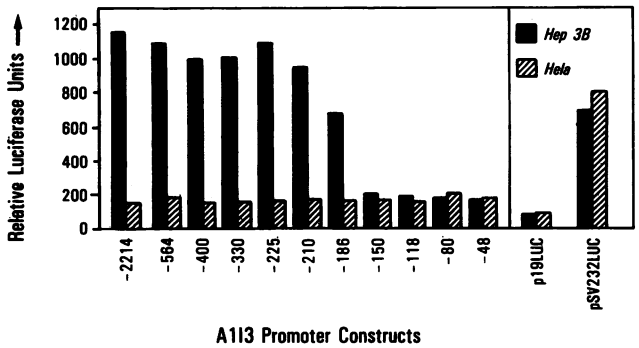

FIG. 3. Cell type-specific expression of α_1I3-luciferase fusions. The transcriptional activity of the 5′ deletion constructs was assayed after transfection into either the human heptoma cell line Hep3B or the human epithelial cell line HeLa. Luciferase units measured were compared with those obtained with the promoterless p19LUC and the pSV232LUC constructs.

Linker mutation analysis of the upstream promoter element. The longest 5′ construct retaining full transcriptional activity, A1I3(−225), was used to generate a series of *Sal*I linker scan mutations extending across the region that had been found to be essential for expression (Fig. 2). The transcriptional effects of the various mutations relative to the wild-type control, A1I3(−225) (Fig. 5), indicate that there are four important upstream promoter elements. Element I mapped to the mutation LM2. This mutation had a modulating effect on transcription and led to a 50% reduction in activity. The adjacent LM3 mutation also influenced the activity of the promoter, but to a lesser extent. The LM5 and LM6 mutations defining element II (−168 to −156) dramatically decreased the transcriptional activity of the α_1I3 promoter to a level comparable to that of the promoterless p19LUC vector alone. Mutation of a third region (element III), −143 to −130 (LM9), also reduced transcription to background levels. This third region may extend further to −149, as the LM8 mutation led to a partial transcriptional decrease. Element IV mapped to the LM10 mutation, which was responsible for a 2.2-fold increase in activity. The results from the 3′ deletion experiments indicated that a negative *cis*-acting element was contained within or overlapped the −118 to −81 region of the α_1I3 promoter. The activity of the linker mutation LM10 confirmed that a

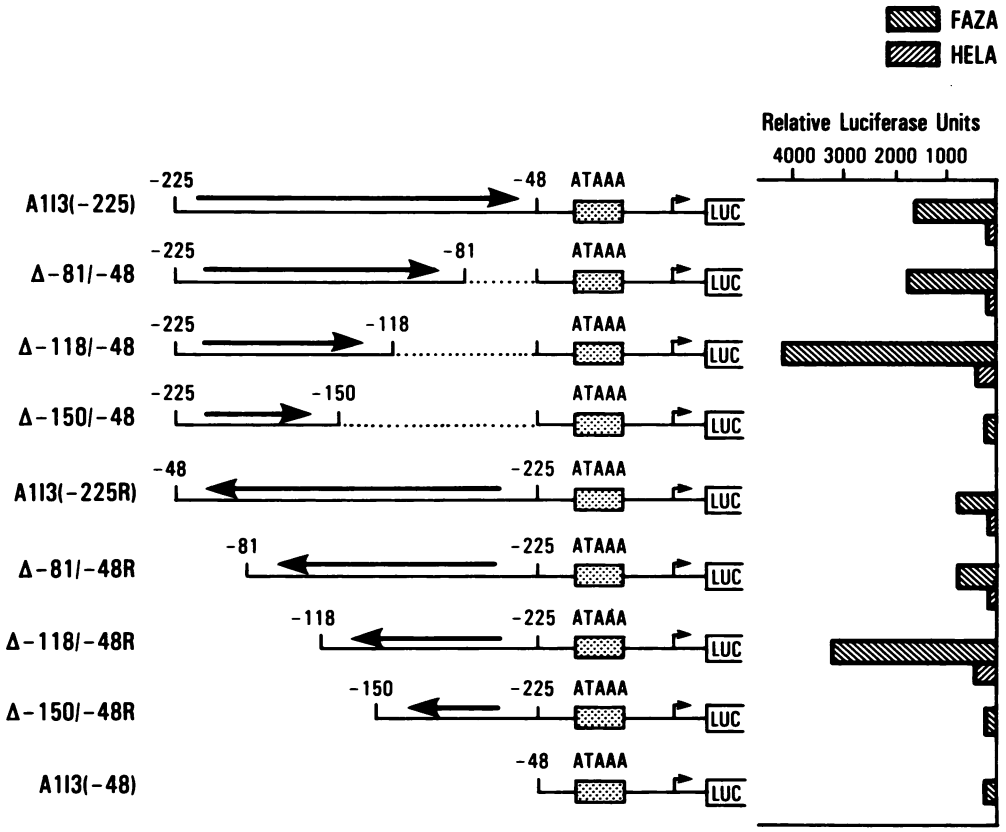

FIG. 4. Effect of 3′ deletions on the transcriptional activity of the α_1I3 promoter in FAZA and HeLa cells after transient transfection. The coordinates of the ends of the α_1I3 promoter regions contained in each construct are shown.

negative element was present in this region and indicated that the negative element extended from −81 to −129.

DNase I footprint analysis of the region −225 to −81. To identify nuclear proteins that bind to the four regions mapped as important for the transcriptional activity of the α_1I3 gene, DNase I footprint analysis of the −225 to −81 region of the promoter was carried out. A complex interaction of this region with nuclear proteins was observed (Fig. 6). Incubation of the DNA with nuclear extracts from FAZA cells led to four main regions of protection from DNase I digestion. These coincided with elements I to IV, demonstrated to be transcriptionally important in the linker mutation analysis (Fig. 6A and B). Similar regions of protection were seen with HeLa-derived extracts, but a number of strong hypersensitive sites at various positions indicated that the HeLa protein-DNA interactions may be different from those generated with FAZA nuclear extracts (Fig. 6).

Inspection of element II revealed a TGGCA motif known to be a binding site for the CTF/NF1 family of nuclear factors (3, 31, 42, 50). DNase I footprint analysis with the highly purified transcription factor NF1 revealed a protection of the region −165 to −158, which contained the TGGCA motif (Fig. 6C). This protection could be relieved by competition with either an oligonucleotide, B3, that covered this region of the α_1I3 promoter or the TGGCA-containing oligonucleotide E from the mouse albumin promoter, which has been shown to bind NF1 (31, 33).

DISCUSSION

Recently, considerable information has been accumulated on the role of *cis*-acting DNA elements in the liver-specific expression of genes. In a number of cases, the proximal promoter elements located in the first 100 to 200 bp upstream from the start site are crucial for the selectivity of expression in hepatic cell types (9, 22, 36, 43). These elements are recognized by *trans*-acting factors, and although the precise mechanism is not fully understood, it appears that gene expression is the result of the combined effects of a set of *trans*-acting factors with multiple *cis*-acting elements (33). These *cis*-acting elements interact with the nuclear factors to participate in stabilizing the transcriptional complex. Formation of the complex is thought to result from specific DNA-protein and protein-protein interactions. Data from the study of the *cis* and *trans* elements of many genes suggest that the expression of a specific gene may be the result of a unique combination of a large set of *trans*-acting factors, some of which appear to be tissue specific and some of which are ubiquitous.

We have shown that in the α_1I3 5′-flanking region, the minimal information necessary for tissue-specific expression is contained in a short segment of DNA within the first 225 bp upstream from the transcription initiation site. Regions further upstream have little effect on transcription. Data from the 3′ deletion experiments mapped the extent of the upstream regulatory region to between −225 and −81. Linker scanning in this region allowed the delineation of four regulatory elements. Two of these *cis*-acting elements were essential for transcriptional activity of the α_1I3 promoter. The other two elements served a modulatory role: the upstream element was able to enhance the transcription rate, and the proximal element had a negative effect on transcription.

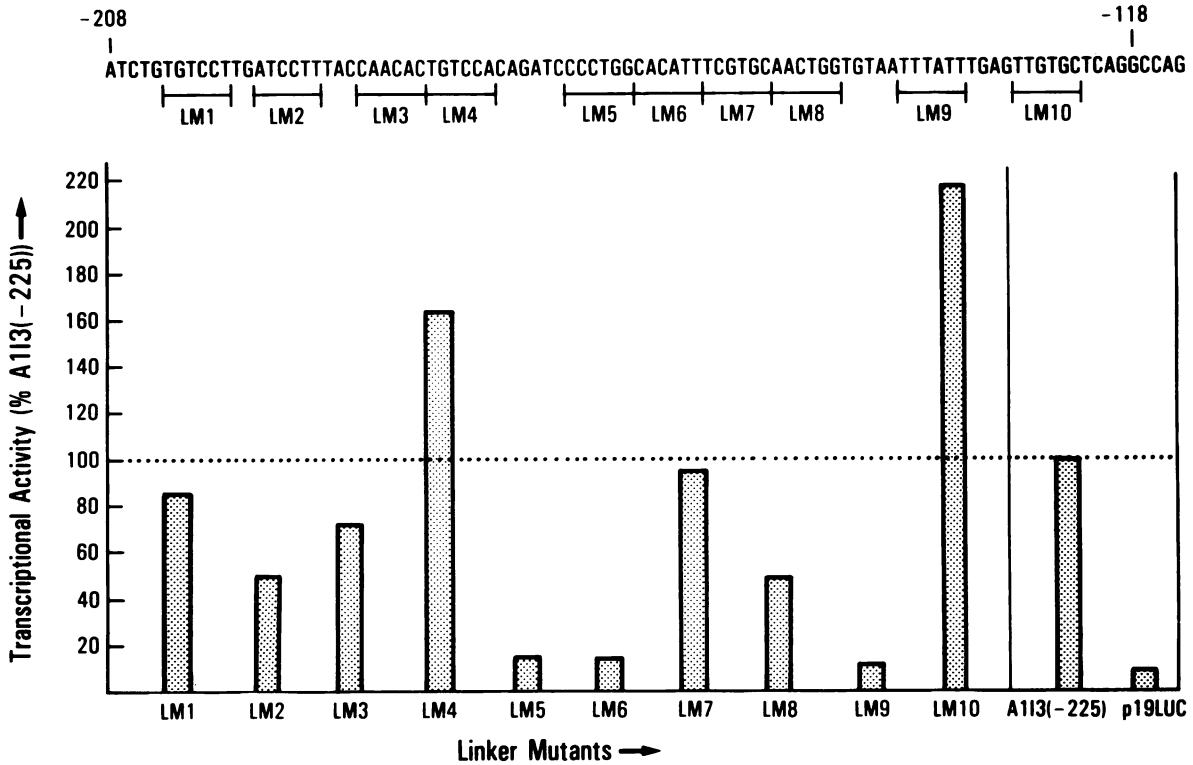

FIG. 5. Linker mutational analysis of the region −225 to −118 bp of the α_1I3 promoter. A series of *Sal*I substitutions in the upstream promoter region of the A1I3(−225) construct were produced as indicated below the sequence. The effect of each mutation was assayed after transient transfection in FAZA cells. The luciferase activities are expressed as a percentage of the activity obtained with the wild-type construct A1I3(−225). The level of the promoterless vector p19LUC is also shown.

Preliminary analysis of nuclear factors that interacted with the four regulatory elements indicated a complex interaction between specific DNA-binding proteins and the four elements I to IV (Fig. 6). Mutations in the region between −196 and −181 indicated that this region contained a *cis*-acting sequence (element I) that was responsible for a twofold increase in transcriptional activity. This region corresponds to the sequence ATCCTTTACCAACAC.

The sequence CCCTGGCACATT, located between −168 and −157 (element II), was found to be essential for hepatic expression. Mutations in this region were sufficient to completely abolish activity. This region contains a TGGCA motif that has been shown for other liver genes to bind members of the CTF/NF1 family of transcriptional activators. NF1-like activities, recognizing the half CTF/NF1 site TGGCA, have been detected in rat liver nuclear extracts (3, 5, 31, 36, 42, 50). Recently, a cDNA clone for a TGGCA-binding activity in rat liver has been isolated (41). Comparison of the sequence with the corresponding cDNA sequence coding for NF1 from HeLa cells (45) shows that the two proteins are highly conserved in the region corresponding to the DNA-binding domain but diverge in the remaining regions. It is probable that this divergent region is involved in interactions with other components of the transcriptional apparatus. In this way, *trans*-acting factors from different tissues, while having the same DNA-binding activities, can interact in different ways with other nuclear factors involved in the expression of a gene. Whether the various interactions result in stabilization of the transcriptional complex will depend on the combinations of factors present in a particular cell type and the ways in which they interact rather than whether they simply bind to a recognizable *cis*-acting DNA element. This may be the case with the α_1I3 gene, where interactions of the TGGCA-binding protein in FAZA cells with the other transcription factors binding to the upstream sequences lead to increased transcriptional activity. In contrast, binding of NF1 in HeLa cells to the TGGCA motif may not lead to transcriptional activity, possibly because NF1 may not be able to interact profitably with the other bound factors.

The other site (−146 to −134) that was found to be essential for hepatic transcription, element III, contained the sequence GGTGTAATTTATTT (Fig. 7). This element superficially resembles a number of other A+T-rich motifs found to be important in liver-specific expression of many genes, such as fibrinogen, albumin, α-fetoprotein, transthyretin, and α_1-antitrypsin (10, 11, 24, 29). The liver-specific factor HNF-1 recognizes these A+T-rich elements, but a close comparison of element III and the HNF-1 consensus site (GTTAATNATTAAC) suggests that element III may represent a separate but related *cis*-acting DNA element that is essential for hepatic expression of α_1I3 (Fig. 7). Element III is unlikely to bind HNF-1, because the two nucleotides G and C located at the extremes of the consensus sequence are essential for HNF-1 binding and are not conserved in element III. We have also noticed that this element has an 8-bp identity with an A+T-rich region in the human α-fetoprotein gene that has been shown to have enhancer activity (46). This so-called AFP1-binding site contains a half HNF-1 site that overlaps with the element III homology (Fig. 7). Further work is under way to elucidate the relationships between the factors that bind to these sequences.

The negative proximal element IV was mapped to between −81 and −129. However, it is likely that the relevant control

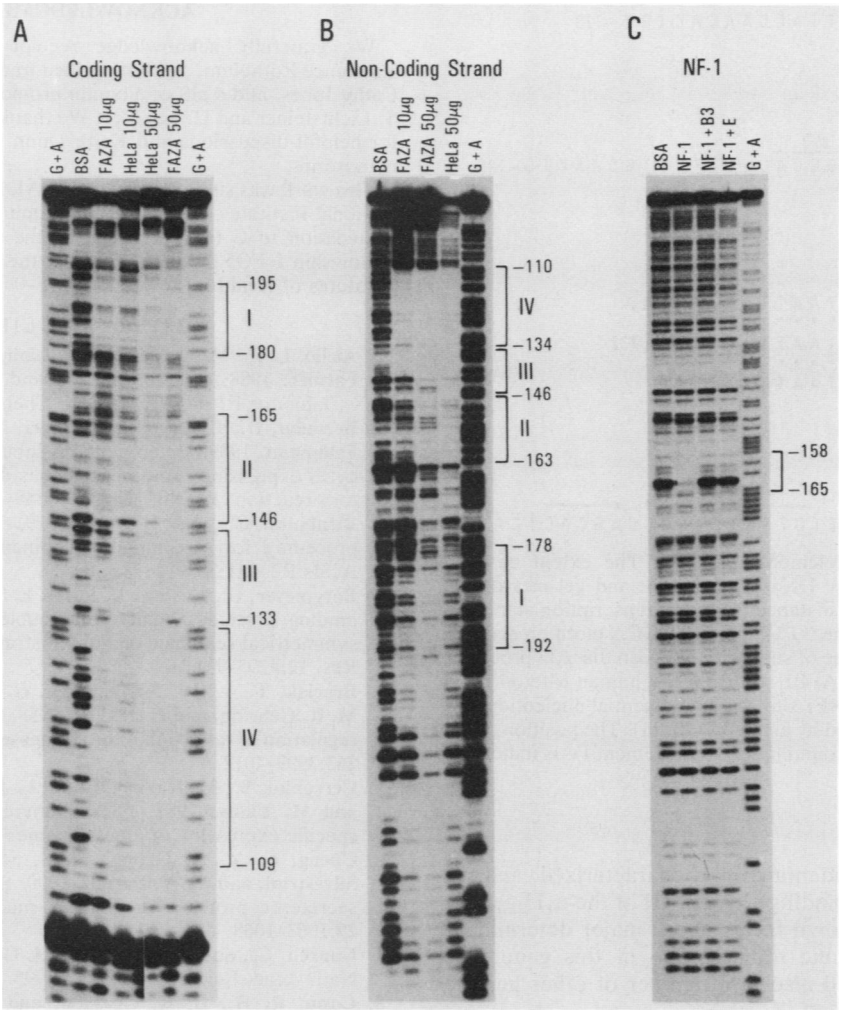

FIG. 6. DNase I footprint analysis of the α_1I3 upstream promoter elements. G+A chemical cleavages of the end-labeled probes were used as sequence markers (35). The extent of protection of elements I to IV by FAZA nuclear proteins is indicated. (A) Footprint on the coding strand of the α_1I3 promoter region with FAZA and HeLa cell nuclear extracts. (B) Footprint on the noncoding strand with FAZA and HeLa cell extracts. (C) Footprint of purified NF1 on the noncoding strand. Bracket indicates the region of protection. The DNAs used for competition were the B3 oligonucleotide of the α_1I3 gene or the site E oligonucleotide from the mouse albumin promoter (31).

sequence lies at the extreme 5′ end of this region, as the LM10 mutation was able to abolish the negative effect on transcription. Footprint analysis showed strong protection of region −134 to −109, which covers the LM10 region. This protected region contains a centrally located inverted repeat sequence, GTGCTcaggccAGCAC (Fig. 7), that may represent the negative transcriptional element. Negative *cis*-acting DNA elements have been described only recently in mammalian genes. Negative elements, for instance, have been identified in the α-fetoprotein, beta interferon, and apolipoprotein B and CIII genes (12, 18, 19, 43). Comparison of these elements with element IV of the α_1I3 gene does not reveal any obvious sequence similarities. It is intriguing that a gene such as α_1I3 that is expressed at such high levels in the liver should be under the control of a negative *cis*-acting element. One possible reason for this type of control may be that an upper limit of expression needs to be defined as well as the lower level. Consequently, α_1I3 promoter control relies on a complex interaction between both the positive and negative *cis*- and *trans*-acting elements that defines the rate of initiation of transcription. We do not know whether this negative element IV plays a role in the transcriptional downregulation of the α_1I3 gene by mediators of inflammation (39; Abraham et al., in press). Element IV was identified here as an element participating in the control of the normal high-level expression of the α_1I3 gene in hepatic cells.

An unexpected result of this study is that the strong hepatic transcription of the α_1I3 gene is achieved without the participation of the *cis*-acting control elements utilized in other major plasma protein genes that are known to be determinants of liver-specific transcription. Albumin, fibrinogen, α_1-antitrypsin, transthyretin, and many other genes expressed in hepatocytes utilize the factors HNF1, HNF3, HNF4, C/EBP, and combinations of these factors and the corresponding binding sites (8, 10, 11, 24, 29, 31, 42). Some of these factors, such as HNF1, are themselves limited to liver cells, and this fact facilitates our understanding of liver-specific transcription (10, 11). These elements are not used in the α_1I3 gene, and we have therefore concluded that liver-specific transcription can be generated by a variety of different combinations drawn from a reservoir of *cis*- and *trans*-acting nuclear factors. The relevant factors have not

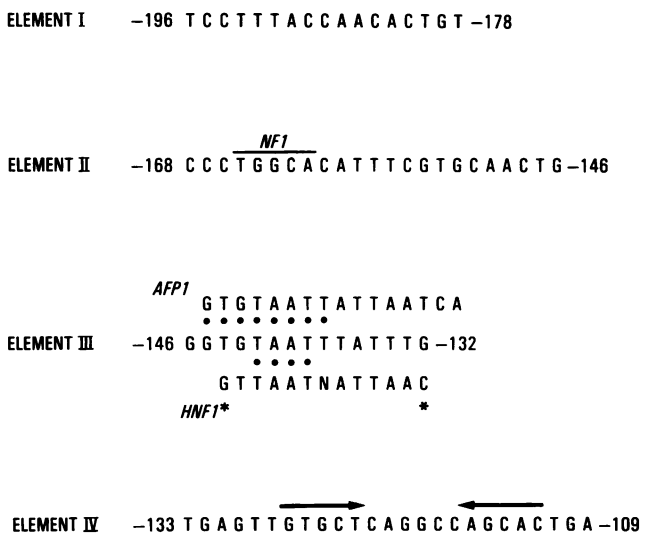

FIG. 7. Sequences of elements I to IV. The extent of each element, as determined by DNase I footprint and gel retardation analysis, is defined by the distance from the transcription start site. Shown is the position of the NF1-binding TGGCA motif on element II. Also shown is the degree of similarity between the α_1I3 promoter element III and both the AFP1 site from the human α-fetoprotein (46) and the consensus HNF1 site; the two essential nucleotides for HNF1 binding are indicated by asterisks (10, 11). The position of the inverted repeat sequence found in negative element IV is indicated.

yet been exhaustively identified and characterized, and we believe that the factor binding element III of the α_1I3 gene is probably a so-far-unknown factor and a major determinant of the hepatocyte-specific transcription of this gene. We expect to find it utilized also in a number of other hepatic genes.

An interesting problem for the future is to investigate whether a correlation exists between the developmental stage of the onset of transcription of a hepatic gene and the combination of control elements used by this gene. If such a correlation existed, it might explain why albumin and other genes that are expressed in fetal liver use one set of elements including HNF1 and why α_1I3 and other genes that are expressed only after birth use a different combination. The α_1I3 gene is an informative example of the class of genes that are only expressed after birth.

The preferential expression of the α_1I3 gene in hepatic cells has recently been confirmed by additional studies in primary cultures of rat liver cells (44; K. S. Koch, X. P. Lu, D. A. Brenner, G. H. Fey, A. Martinez-Conde, and H. L. Leffert, *in Modern Cell Biology*, in press). The α_1I3 promoter region was linked either to the luciferase reporter, as described here, or to a β-galactosidase reporter gene. The recombinant molecules were transfected into primary cultures containing hepatocytes and other cell types dispersed from rat livers with collagenase. Strong preferential expression of the constructs in hepatocytes over other cell types was observed.

Thus, the α_1I3 gene promoter may become a useful tool for future studies requiring a strong promoter preferentially expressed in rat hepatocytes that can be downregulated by a defined set of agents, including inflammatory cytokines such as IL6 (Abraham et al., in press).

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