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Tissue-Specific Transcription of the Mouse α-Fetoprotein Gene Promoter Is Dependent on HNF-1

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Previous work identified four upstream *cis*-acting elements required for tissue-specific expression of the α-fetoprotein (AFP) gene: three distal enhancers and a promoter. To further define the role of the promoter in regulating AFP gene expression, segments of the region were tested for the ability to direct transcription of a reporter gene in transient expression assay. Experiments showed that the region within 250 base pairs of the start of transcription was sufficient to confer liver-specific transcription. DNase I footprinting and band shift assays indicated that the region between −130 and −100 was recognized by two factors, one of which was highly sequence specific and found only in hepatoma cells. Competition assays suggested that the liver-specific binding activity was HNF-1, previously identified by its binding to other liver-specific promoters. Mutation of the HNF-1 recognition site at −120 resulted in a significant reduction in transcription in transfection assays, suggesting a biological role for HNF-1 in the regulation of AFP expression.

The restriction of gene expression to specific tissues in a multicellular organism has been attributed to the interaction between *cis*-acting DNA elements and tissue-specific transcription factors (16, 22, 24, 26, 31, 45). *cis*-Acting elements have been identified close to genes as part of promoters and at varying distances from the gene as part of enhancers (2, 11, 32). In several instances, the same tissue-specific *cis*-acting element has been found in both positions, as is the case with the B-cell-specific octamer motif found in the promoter of the immunoglobulin light-chain gene and in the enhancer of the heavy-chain gene (26, 38, 40, 46). On the other hand, there are several promoter elements that have yet to be identified in enhancers. For example, recognition sites for the ubiquitous transcription factors Sp1 and NF-1 appear to be restricted to the region near the start of transcription (3, 21).

The regulatory sequences of genes expressed in liver have been intensively studied as a model for tissue-specific gene expression. These studies have resulted in the identification of several *trans*-acting factors that appear to be present at higher levels in liver than in other cell types (1, 16, 22, 39). The protein C/EBP, which was originally identified in rat liver extracts on the basis of its ability to bind the promoter of the herpes simplex virus (HSV) thymidine kinase (TK) gene and the enhancer of the Moloney leukemia virus (15), has since been shown to *trans*-activate the liver-specific albumin gene by interactions with both its promoter and enhancer (19, 25). Darnell and colleagues have identified two liver-enriched *trans*-acting proteins, HNF-3 and HNF-4, that bind to both enhancers and promoters of the transthyretin and α1-antitrypsin genes (5). In addition, the binding sites for another liver-specific *trans*-acting protein, HNF-1, appear to be restricted to the promoter regions of a large number of liver-specific genes, including the rat α-fetoprotein (AFP) gene (6, 7).

We wished to investigate the identity and functional significance of the proteins that interact with the mouse AFP gene. Given that transcription of this gene is repressed after birth in liver (43), it was possible that its transcriptional apparatus might be significantly different from the other liver-specific genes whose expression is not under developmental regulation. Previous experiments using transient expression assays had identified four elements that act in *cis* to mediate high-level transcription of the gene: three enhancer elements at 2.5, 5.0, and 6.5 kilobase pairs (kbp) of DNA upstream of the gene and a proximal domain within the promoter (12, 13). By transient expression assay, we have identified a region of the AFP promoter, between 100 and 130 bp upstream of the start of transcription, that contributes to the tissue-specific transcriptional regulation of the gene by binding to the factor HNF-1.

MATERIALS AND METHODS

Cells. HeLa and Hep3B cell lines were maintained as monolayer cultures in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. HepG2 cells were cultivated in a 1:1 mixture of Dulbecco modified Eagle medium and Ham F12 nutrient mixture supplemented with 10% fetal bovine serum and 10 μg of bovine pancreas insulin per ml.

DNA constructs for transient expression assays. A series of constructs containing BAL 31-derived 5′ endpoints in the AFP gene 5′ flanking region (37) were fused via a common 3′ *Taq*I site at the AFP mRNA start site to a *Taq*I site at the start of the HSV TK structural gene (28). The fusion genes, which contained 1.9 kbp of the TK gene and its 3′ flanking region, were inserted into pUC18 at the *Sal*I site by use of *Sal*I linkers. The chimeric constructions contained 604 (Δ1), 250 (Δ3), 118 (Δ7), and 85 (Δ7A) bp of the AFP 5′ flanking sequence driving transcription of the TK gene.

Transient expression assays. Transient expression assays were performed as described by Godbout et al. (12, 13). A total of 5×10^6 HepG2 cells, 10^6 HeLa cells, or 1.5×10^6 Hep3B cells were seeded on 100-mm-diameter tissue culture dishes. Calcium phosphate precipitates containing 15 μg of DNA per plate were incubated with the cells for 8 to 12 h. The cells were then washed, and the medium was replaced. At 48 h after transfection, cells were harvested, and RNA was extracted by the hot phenol method (36) and subjected

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to oligo(dT)-cellulose chromatography to enrich for polyadenylated species (1).

Analysis of RNA. A 5-μg sample of poly(A)$^+$ RNA from transfected cells was subjected to gel electrophoresis in 6% formaldehyde–1.5% agarose–MOPS (20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA [pH 7.0]) gels. After electrophoresis, the RNA was transferred to nitrocellulose (42) and hybridized to nick-translated probes in the presence of 50% formamide and 10% dextran sulfate (44). Probes used were a gel-purified 1.9-kbp *Pvu*II fragment of the TK gene and a 1.3-kbp *Hin*dIII fragment from rpL32/4A, a ribosomal protein gene (9).

Analysis of transfected DNA. Episomal DNA was isolated after transfection by the method of Hirt (20). After purification, equal portions of each preparation were digested with *Sal*I and *Bam*HI, analyzed by 1% agarose gel electrophoresis, blotted onto nitrocellulose, and hybridized to the TK gene probe (41).

Preparation of nuclear extracts. Nuclear extracts from 1×10^9 to 3×10^9 cells were made as described by Dignam et al. (8). Crude extracts were either frozen in liquid nitrogen or subjected to heparin-agarose affinity chromotography. Crude nuclear extract in buffer D (20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 100 mM KCl) was applied to a 100-ml column and then washed extensively with the same buffer. The protein was eluted in steps by washing the column with buffer D containing increasing concentrations of KCl.

Gel mobility shift assays. pUC18-based vectors containing DNA fragments for protein-binding assays were digested with *Hin*dIII and *Eco*RI, and the appropriate fragments were isolated from polyacrylamide gels. The fragments were end labeled by filling in the recessed ends with [^{32}P]dATP (Dupont, NEN Research Products) with the Klenow fragment of DNA polymerase (Boehringer Mannheim Biochemicals). Gel mobility shift assays were carried out essentially as described by Singh et al. (40). In a total volume of 25 μl, approximately 0.2 ng of labeled DNA was incubated with extract, 10 μg of poly(dI-dC) (Pharmacia, Inc.), and 10 μg of yeast tRNA in binding buffer (10 mM Tris hydrochloride [pH 7.5], 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol). After 30 min at 25°C, the reaction was analyzed by electrophoresis on a 1% agarose gel in 0.5× TBE (0.045 M Tris hydrochloride [pH 7.2], 0.045 M boric acid, 0.002 M EDTA) gel for 2.5 h at 100 V (12 V/cm). Competitor DNAs were either oligonucleotides or plasmids that had been digested with restriction endonucleases outside the sequence of interest and purified by phenol-chloroform extraction.

DNase I footprinting. DNase I footprint analysis was performed as described by Graves et al. (15). Plasmids were digested with *Hin*dIII and *Sac*I, and the fragments of interest were purified by gel electrophoresis. The Klenow fragment of DNA polymerase was used to asymmetrically label these fragments with [α-^{32}P]dATP. Approximately 2 ng of DNA was mixed with extract and 1 μg of poly(dI-dC) in DNase footprint buffer (25 mM Tris hydrochloride [pH 7.9], 6.25 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, 10% glycerol) in 45 μl and incubated at 4°C for 15 min and at 25°C for an additional 2 min. Then 5 μl of DNase (0.001 to 0.01 μg/μl) in 25 mM $CaCl_2$ was added to each tube, and the tubes were incubated for 1 min at 25°C. Digestion was stopped by addition of 100 μl of stop buffer (1% sodium dodecyl sulfate, 20 mM EDTA, 200 mM NaCl, 100 μg of tRNA per ml). Samples were extracted with phenol-chloroform (1:1) and precipitated with ethanol. Pellets were washed in 70% ethanol, dried, and suspended in loading buffer (80% formamide, 1× TBE, 0.05% bromphenol blue, 0.05% xylene cyanol). Samples were denatured at 90°C for 10 min and loaded onto 6% acrylamide–7 M urea–1× TBE gradient gels. After electrophoresis, gels were fixed in 10% acetic acid–10% methanol, dried, and autoradiographed. The sequence ladders used as markers were made by subjecting a portion of the probe to the base-specific cleavage reactions as described by Maxam and Gilbert (27).

Site-directed mutagenesis. Mutation of the factor-binding site was done as described by Zoller and Smith (47). The AFP sequence between bp −250 and +287 was cloned into M13mp10 via the artificial *Bam*HI site at bp −250 and the *Sac*I site at bp +287. An oligonucleotide corresponding to the sequence between bp −130 and −107 and containing the mutation TTAATTATTGGCA to TTATTATCAACA was synthesized. The oligonucleotide and a sequencing primer (5′-CCCAGTCACGACGTT-3′) were hybridized to the single-stranded bacteriophage DNA, and the second strand was synthesized by using the Klenow fragment of DNA polymerase. Bacteria were transformed, and the resulting plaques were screened by hybridization to the mutant oligonucleotide. The presence of the mutated sequence was confirmed by sequencing the single-stranded DNA (34), and the mutation was reinserted into the AFP-TK gene chimera, using *Bam*HI and *Spe*I sites in the AFP promoter.

RESULTS

Functional mapping of the AFP gene promoter. In previous studies, the immediate 5′ flanking region of the AFP mouse gene was subjected to deletion analysis to determine which sequences were required for its expression in a transient assay (12). These experiments indicated that in the presence of an AFP enhancer, only 85 bp of 5′ flanking DNA was required for high-level expression of an AFP minigene. However, the presence of the AFP enhancers and gene body raised the possibility that one or more elements outside the promoter region was masking the loss of promoter-specific elements. To overcome this concern, various lengths of the immediate 5′ flanking region of the AFP gene were fused to a neutral reporter gene, the HSV TK gene. The fusions were made at the *Taq*I sites at the transcriptional start sites in both genes (Fig. 1).

To test the ability of these promoter domains to direct transcription of the TK structural gene in a tissue-specific manner, each was transfected into HepG2, HeLa, and Ltk$^-$ tissue culture cells. HepG2 cells are a human hepatoma cell line (23) that has been shown to support the expression of many liver-specific genes, including AFP, in transfection assays (12, 13). HeLa and Ltk$^-$ cells are not of hepatic origin and generally do not transcribe endogenous or transfected liver-specific genes. Cells were harvested 48 h later, and poly(A)$^+$ RNA was analyzed for the presence of TK transcripts (Fig. 1). The RNA gels were also hybridized to an rp L32 cDNA to standardize the lanes for RNA loading. Transfection efficiency was monitored by measuring the levels of plasmid DNA in Hirt extracts.

In HepG2 cells, the transcriptional activity of the smallest AFP promoter fragment tested, 118 bp, was equivalent to that obtained with the TK gene promoter alone. However, addition of the sequence between bp −118 and −250 resulted in 10-fold-higher levels of transcription (compare lanes 3 and 4 in Fig. 1A). Further addition of flanking sequences up to bp −1000 increased transcription only slightly (~50%). In non-hepatocytes, however, inclusion of the region between bp

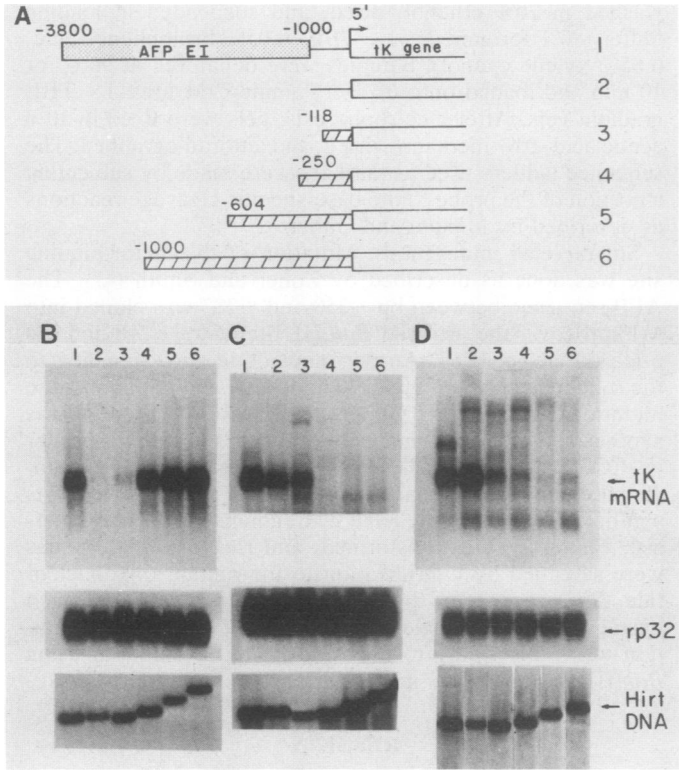

FIG. 1. Transient expression of AFP-TK gene fusions. (A) Fusion constructs. A series of constructs (3 to 6) were generated that contained from +1 of the AFP gene to the distances indicated upstream of the AFP gene (▨) ligated to the HSV TK gene body (□). These constructs, together with the TK gene and 200 bp of its own 5′ flanking DNA (construct 2) alone or ligated to the AFP enhancer I (construct 1), were transfected into HepG2 (B), HeLa (C), and Ltk$^-$ (D) cells. Poly(A)$^+$ RNA was isolated and analyzed on a denaturing agarose gel for the presence of TK mRNA (top panels) or the mouse rpL32 mRNA (middle panels) by hybridization to gene-specific probes. Hirt DNAs (20) were also prepared from these cells, digested with *Bam*HI and *Sal*I, and hybridized after agarose gel electrophoresis and transfer to nitrocellulose to a labeled TK gene fragment (lower panels). The lanes in all instances refer to the numbered constructs in panel A. The hybridization intensity in each set of gels was determined by densitometry, and the TK mRNA levels were corrected by using the rpL32 and Hirt DNA standards. The gels represent one of three comparable sets of experiments.

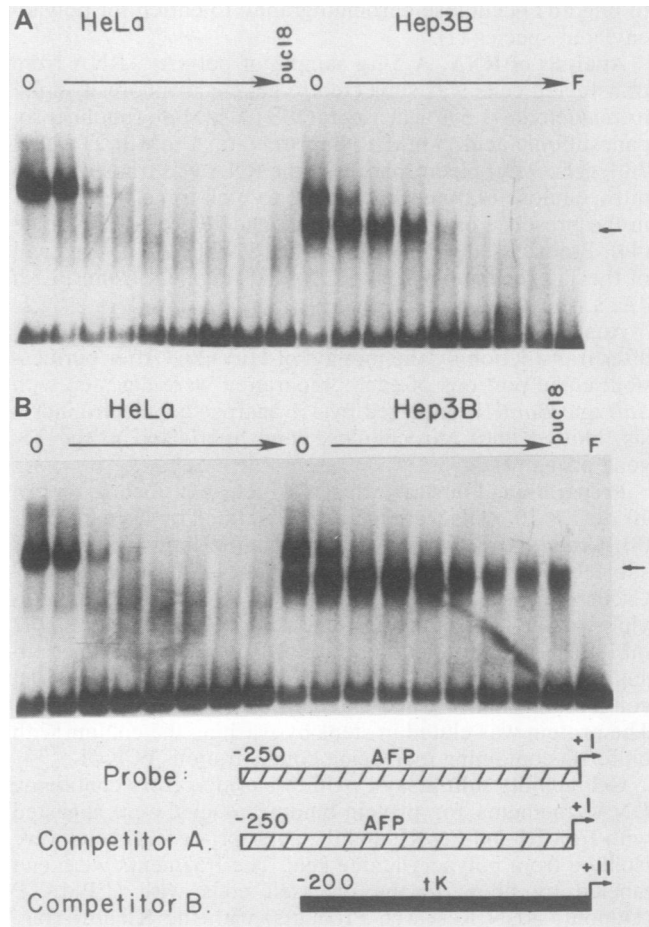

FIG. 2. Detection of proteins that interact with the AFP gene promoter. (A and B) Nuclear extracts from HeLa and Hep3B cells incubated with 0.1 ng of a labeled fragment derived from bp −250 to +1 of the AFP gene (lanes O). Unlabeled DNA composed of the same fragment (competitor A [A]) or the promoter region of the TK gene (competitor B [B]) were added in increasing concentrations from 0.1 to 100 ng, represented by the arrows above the lanes. pUC18 DNA (1,000 ng) was added as a competitor as indicated. F, Labelled fragment with no extract added. Incubations were analyzed on a nondenaturing agarose gel.

−118 and −250 caused a marked 20-fold reduction in transcription in HeLa cells but only slightly repressed transcription in Ltk$^-$ cells. These results suggested the presence of at least one regulatory element between bp −118 and −250 that was required for liver-specific transcription of the gene and may repress transcription in other cell types.

Identification of nuclear proteins that interact with the region between bp −118 and −250. To determine whether this region serves as a site for interaction with nuclear factors, the gel mobility shift assay was used (10, 40). Nuclear extracts from Hep3B cells (a human hepatoma cell line that transcribes both endogeneous [23] and exogenous [13] AFP genes) or HeLa cells were incubated with a ^{32}P-labeled fragment spanning the first 250 bp of DNA upstream of the start of transcription. The products were then analyzed by nondenaturing agarose gel electrophoresis. Two bands of slower electrophoretic mobility than the probe alone were observed with Hep3B extracts, whereas only one band was seen with HeLa cells extracts (Fig. 2). Both pUC18 and TK gene promoter sequences competed with the binding activities represented by the common band formed by Hep3B and HeLa extracts. However, only AFP promoter sequences could compete effectively for the binding activity in the lower band in Hep3B cell extracts, suggesting a more sequence-specific interaction.

The site for the Hep3B-specific protein-DNA interaction was localized by using unlabeled subfragments of the promoter as competitors in gel mobility assays. Effective competition was observed with only one fragment, containing sequences between bp −250 and −80, whereas fragments containing bp −600 to −154 and −118 to −52 had no effect (Fig. 3). Taken together, the data indicated that the region between 118 and 154 bp upstream of the mRNA cap site was required for factor binding to the probe.

The exact site for factor binding was identified by DNase I footprinting analysis, using Hep 3B extracts that had been enriched for DNA-binding proteins by heparin-agarose chromatography. This fraction contained 10% of the original

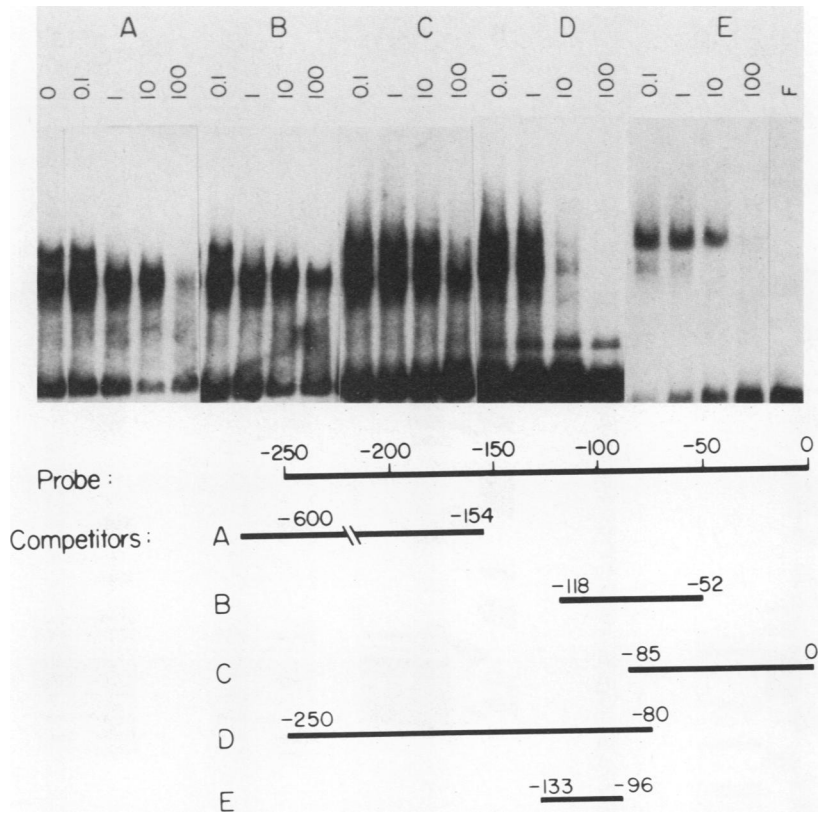

FIG. 3. Localization of the Liver-specific protein binding within the AFP Promoter. Nuclear extracts from Hep3B cells were incubated with 0.2 ng of labeled fragment consisting of bp −250 to +1 of the AFP gene (probe). Unlabeled isolated fragments (A to D) or a synthetic oligonucleotide (E) as shown in the diagram below the gel were added at increasing concentrations as indicated (in nanograms) along with 10 μg of poly(dI-dC) and 10 μg of tRNA per ml. The incubations were analyzed on a nondenaturing agarose gel. F, Incubation without extract.

protein and binding activity indistinguishable from that of crude extract, as judged by gel mobility shift assay (data not shown). The nuclear extract protected from DNase I digestion a region from bp −101 to −126 on the coding strand and from bp −102 to −126 on the noncoding strand (Fig. 4). An oligonucleotide from bp −96 to −133 that contained within it the protected sequence was synthesized and used as competitor in the gel shift assay. This sequence was an especially effective competitor for the lower Hep3B-specific band but competed less effectively for the upper band (Fig. 3E).

Extracts were also prepared from mouse fetal liver, which expresses AFP, and from fetal brain, which does not. Similar DNase I footprints were generated by both of these extracts (Fig. 4) as well as by HeLa cell extracts (data not shown). Thus, all cell types, whether they expressed AFP or not, contained DNA-binding activities that recognized the same sequence in the AFP promoter.

This result could be explained by the presence of a ubiquitous protein represented by the common band of the gel shift pattern (Fig. 2). This binding activity could be separated from the Hep3B-specific band by heparin-agarose chromatography. Crude nuclear extract was applied to a heparin-agarose column and eluted first with 0.2 M KCl, followed by 0.3 M KCl. The gel mobility shift patterns obtained with the crude extract, the flowthrough, and the salt washes (Fig. 5) indicated that the activity constituting the upper band in Hep3B cells eluted primarily in the 0.2 M KCl fraction, whereas the Hep3B-specific component eluted at 0.3 M KCl.

When the 0.2 and 0.3 M KCl fractions were subjected to DNase I footprint analysis, they protected different overlapping regions (Fig. 5). The activity in the 0.2 M KCl wash footprinted the region between bp −111 and −130 strongly and the region from −130 to −134 weakly, whereas the DNA-binding activity in the 0.3 M KCl wash protected the region between bp −102 and −111 weakly, and the region from −111 to −127 more strongly. These results suggested that two different proteins recognized the AFP promoter at −120, only one of which showed liver specificity. They also serve to explain why all extracts, irrespective of tissue origin, contained an activity that footprinted the promoter.

The Hep3B-specific protein is HNF-1. The region of the AFP promoter recognized by the Hep3B-specific activity shows extensive dyad symmetry and includes consensus binding sites for the *trans*-acting factor NF-1 ($TGGN_6GCCAA$; [20]) at −119 to −105 and to the family of proteins that bind the motif CCAAT (29) (−121 to −117 on the opposite strand). In addition, there is an 11-of-13 match to a consensus sequence derived for the liver-specific transcription factor HNF-1 (GTTAATNATTAAC) (6, 7), from bp −128 to −116. HNF-1 was originally identified by virtue of its binding to the promoters of the α- and β-fibrinogen and α1-antitrypsin genes, which are transcribed in a liver-specific manner (7). Courtois et al. (6) have recently shown that HNF-1 binds to sites within the rat AFP gene promoter as well as promoters of the albumin, transthyretin, and hepatitis surface antigen genes.

To determine whether the binding we observe in Hep3B extracts is related to the rat liver HNF-1, an oligonucleotide containing the binding site for HNF-1 in the α-fibrinogen

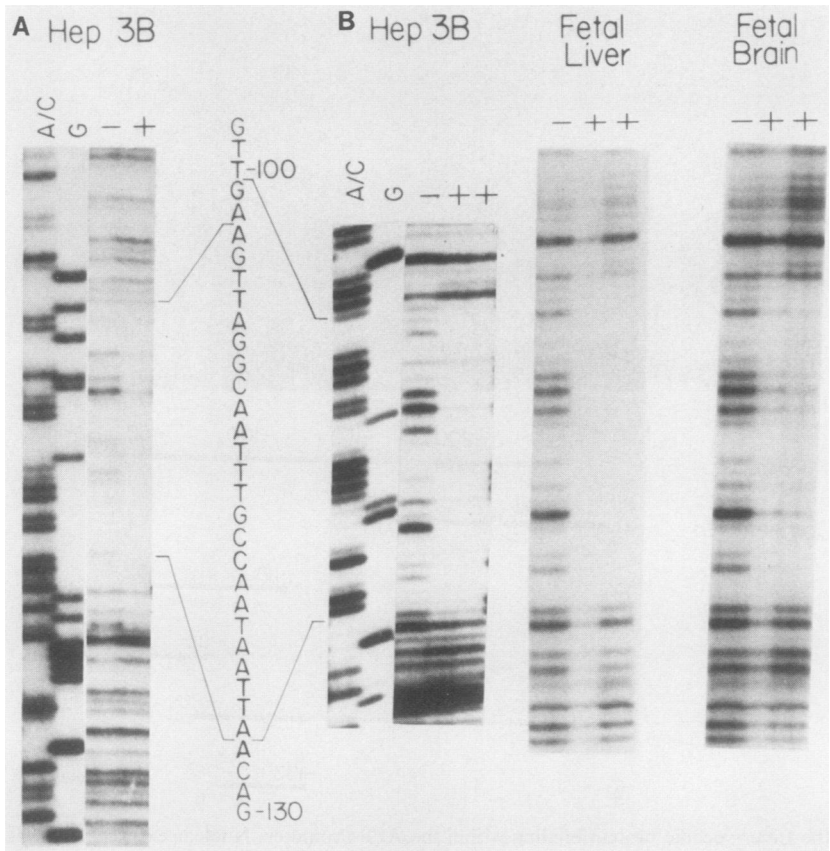

FIG. 4. DNase I footprinting of the protein-binding site in the AFP gene promoter. A restriction fragment composed of bp −250 to +1 of the AFP gene was labeled on either strand by T4 polynucleotide kinase (coding strand [A]) or by end filling with the Klenow fragment of DNA polymerase (noncoding strand [B]). The labeled DNA was incubated with a fractionated nuclear extract prepared from Hep3B cells (5 or 15 μg of the 0.3 M KCl wash of a heparin-agarose column) or crude nuclear extracts from fetal liver (3.2 and 9.6 μg) or fetal brain (7.2 and 21.6 μg) (+ lanes) or in buffer (− lanes), digested with DNase I, and analyzed on a denaturing acrylamide gel. Molecular markers were derived from the same fragments that had undergone treatment with NaOH (A/C) or dimethyl sulfate (G). The DNA sequence is that of the noncoding strand of the AFP gene promoter.

gene was synthesized (7). This oligonucleotide effectively competed with a labeled AFP promoter fragment for binding of the lower band in crude Hep3B extracts (Fig. 6), strongly suggesting that the binding was due to HNF-1 in the extract. The oligonucleotide was an efficient competitor for the AFP promoter for binding despite the fact that the oligonucleotide shared only 8 of 13 nucleotides within the HNF-1 consensus and less than 50% identity overall (see Fig. 6A). However, unlike other competitors tested, the HNF-1 oligonucleotide did not compete with the common band present in Hep3B and in HeLa cell extracts.

Functional significance of the HNF-1-binding site. To determine whether disruption of the HNF-1-binding site was responsible for the loss of liver-specific transcription upon deletion of the AFP promoter to −118, a 3-bp substitution whereby the TGG at −119 was changed to CAA was incorporated into the promoter by site-directed mutagenesis (47). Sequencing confirmed the presence of the mutation and revealed that the mutated promoter also contained a one-base deletion of the A residue at bp −124, which must have occurred during the synthesis of the oligonucleotide.

Gel mobility shift assays were used to ensure that the mutated promoter had indeed lost its capacity to bind HNF-1. A 250-bp labeled fragment containing the mutated HNF-1 site formed only one complex with Hep3B extracts, which comigrated with the ubiquitous upper band found in Hep3B extracts (Fig. 6B). In addition, the mutated 250-bp promoter could not compete for binding of HNF-1 to the wild-type promoter. These experiments established that the mutations in the HNF-1-binding site had inactivated the ability of this site to bind the factor.

The effect of this specific mutation on transcription was then tested in transfection experiments into two liver-derived cell lines, Hep3B and HepG2. These experiments were done with the TK gene fusion containing 250 bp of AFP 5′ flanking DNA in which either wild-type or mutant HNF-1-binding sites were present. These fusions were compared with a gene fusion that contained only 85 bp of AFP 5′ flanking DNA and therefore lacked the HNF-1 site entirely (Fig. 1). The wild-type promoter directed transcription at high levels in both Hep3B and HepG2 cells (Fig. 6C). Deletion to bp −85 or mutation of the HNF-1 site in the AFP promoter reduced transcription three- to fourfold in a quantitatively similar manner in Hep3B cells. This result suggested that the decrease in transcription observed in Fig. 1 when the −118 promoter was compared with the −250 promoter was entirely attributable to the loss of the HNF-1 site. In HepG2 cells, on the other hand, the reductions in transcription from the two mutated promoters were not identical; rather, transcription from the mutated promoter was reduced 5-fold, whereas it was reduced 25-fold from the −85 promoter, suggesting that sequences in addition to the

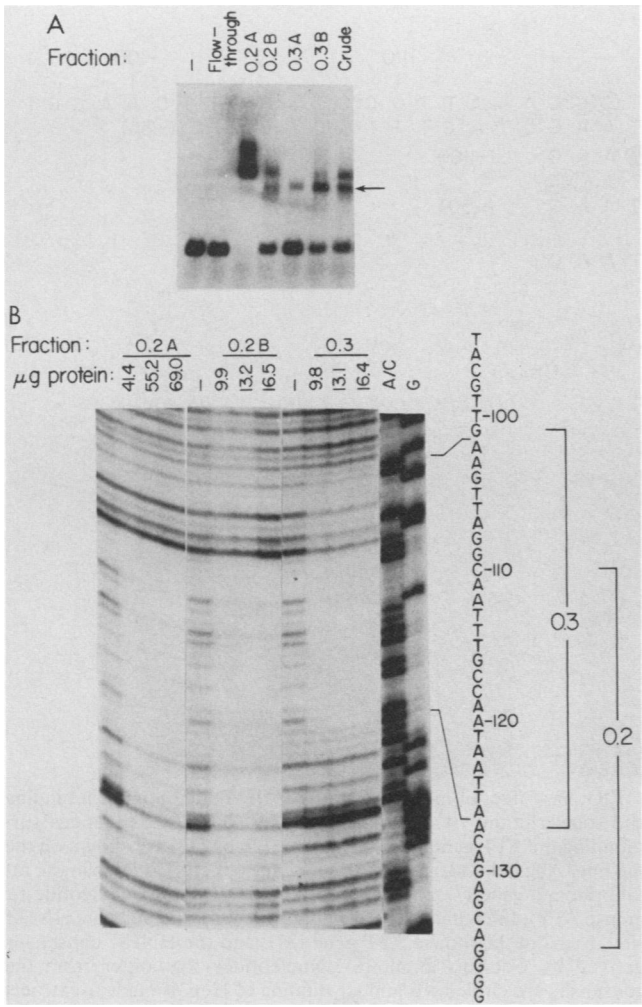

FIG. 5. Chromatographic separation of gel mobility shift bands. Hep3B crude nuclear extracts were applied to a heparin-agarose column and step eluted with 0.2 and 0.3 M KCl. Fractions 0.2A and 0.2B refer to the leading and trailing edges of the 0.2 M KCl wash; 0.3A and 0.3B refer to the leading and trailing edges of the 0.3 M KCl wash. The crude extract, the flowthrough of the heparin-agarose column, and the salt washes indicated were concentrated and incubated with a labeled fragment containing bp −250 to +1 of the AFP gene promoter. These were either analyzed by nondenaturing gel electrophoresis (A) or treated with DNase I and analyzed by denaturing polyacryamide gel electrophoresis (B). For the DNase I footprinting of the 0.3 M KCl wash, fractions 0.3A and 0.3B were pooled. The sequence to the right of the footprints is derived from the noncoding strand of the AFP promoter; brackets indicate the borders of the footprints of the two fractions.

HNF-1 site at −120 may play a role in directing transcription. The effect of mutating the HNF-1-binding site in the AFP promoter was also assayed in HeLa cells, where it had no effect relative to the wild-type promoter (data not shown). This result is not surprising given that the mutation did not disrupt interactions with the ubiquitous protein, as measured by gel shift experiments (Fig. 6).

DISCUSSION

Transcription of the AFP gene is regulated by multiple elements, including three distal enhancers as well as a region proximal to the start of transcription (12, 13, 17). To understand the contribution of the proximal region to regulation of the gene, this region was separated from the enhancers and gene body and fused to the HSV TK structural gene. Testing sequential deletions of this domain by transient expression in several cell lines identified a region between −250 and −118 as required for transcriptional activity in HepG2 cells but not in HeLa and L cells (Fig. 1). A combination of DNase I footprinting and gel mobility shift assays showed that the region between −130 and −100 recognized two proteins in liver-derived nuclear extracts but only one protein in HeLa cell extracts.

To establish the functional link between protein binding and transcription, mutations that specifically abolished binding of the liver-specific protein were generated. These mutations were introduced into the vector without disrupting adjacent sequences, leaving the mutated site within its normal context. When tested by transfection, the mutations elicited a marked reduction in transcription in both Hep3B and HepG2 cells but not in HeLa cells. Thus, it was likely that the protein-DNA interaction at this site was mediating the liver-specific transcription. This conclusion is consistent with recent studies that demonstrate the presence of a DNase I hypersensitivity site at bp −120 in the endogenous AFP gene in fetal liver (14).

The most likely candidate for the observed binding activity is HNF-1. An oligonucleotide composed of an authentic HNF-1 site found in the α-fibrinogen gene promoter effectively competed for binding of the liver-specific protein to the mouse AFP gene (Fig. 6). HNF-1 was initially identified by virtue of its binding to the promoters of a number of liver-specific genes (4, 6, 7, 18, 30). Its role in directing liver-specific transcription has been established in several instances, as deletion of the HNF-1 recognition site in the promoters of several genes abolished expression of the genes in transient expression assays (7, 18, 30). Furthermore, a 13-bp oligonucleotide containing the *Xenopus laevis* albumin gene HNF-1 homolog was sufficient to confer liver-specific expression on the adenovirus 2 major late promoter TATA box (33). Finally, mutations that reduce factor binding also reduce transcription from linked promoters (25, 33).

Courtois et al. (6) compared 18 HNF-1 recognition sites to derive a 13-base-long consensus sequence of 5′-GTTAAT NATTAAC-3′, a perfect palindrome. The HNF-1-binding site at bp −120 in the mouse AFP gene differs from the consensus only at positions 11 and 12, substituting two G residues for two A residues. These two positions showed the most variability in the sequences examined (6).

The promoter of the rat AFP gene contains a second HNF-1 site between bp −61 and −37 (6) that binds purified HNF-1, although less avidly than the binding site further upstream at bp −120 (8). When the comparable region of the mouse AFP gene was used as a probe for binding to crude HepG2 or Hep3B extracts, no binding was observed (data not shown). In addition, this site did not compete effectively with the −120 site for binding to HNF-1 (Fig. 3, competitor C). However, a point mutation in this site in the rat AFP gene has a strong negative effect on transcription (17), and its deletion from the mouse gene abolishes transcription even in the presence of the enhancers (12). Thus, despite the apparent low affinity of HNF-1 for the proximal site in vitro, it likely plays an important role in vivo, in conjunction with the distal HNF-1 site.

HNF-1-binding sites have been found exclusively in the proximal promoters of liver-specific genes (6, 8). We have also found that none of the three AFP gene enhancers contains HNF-1 sites as well, as judged by their failure to

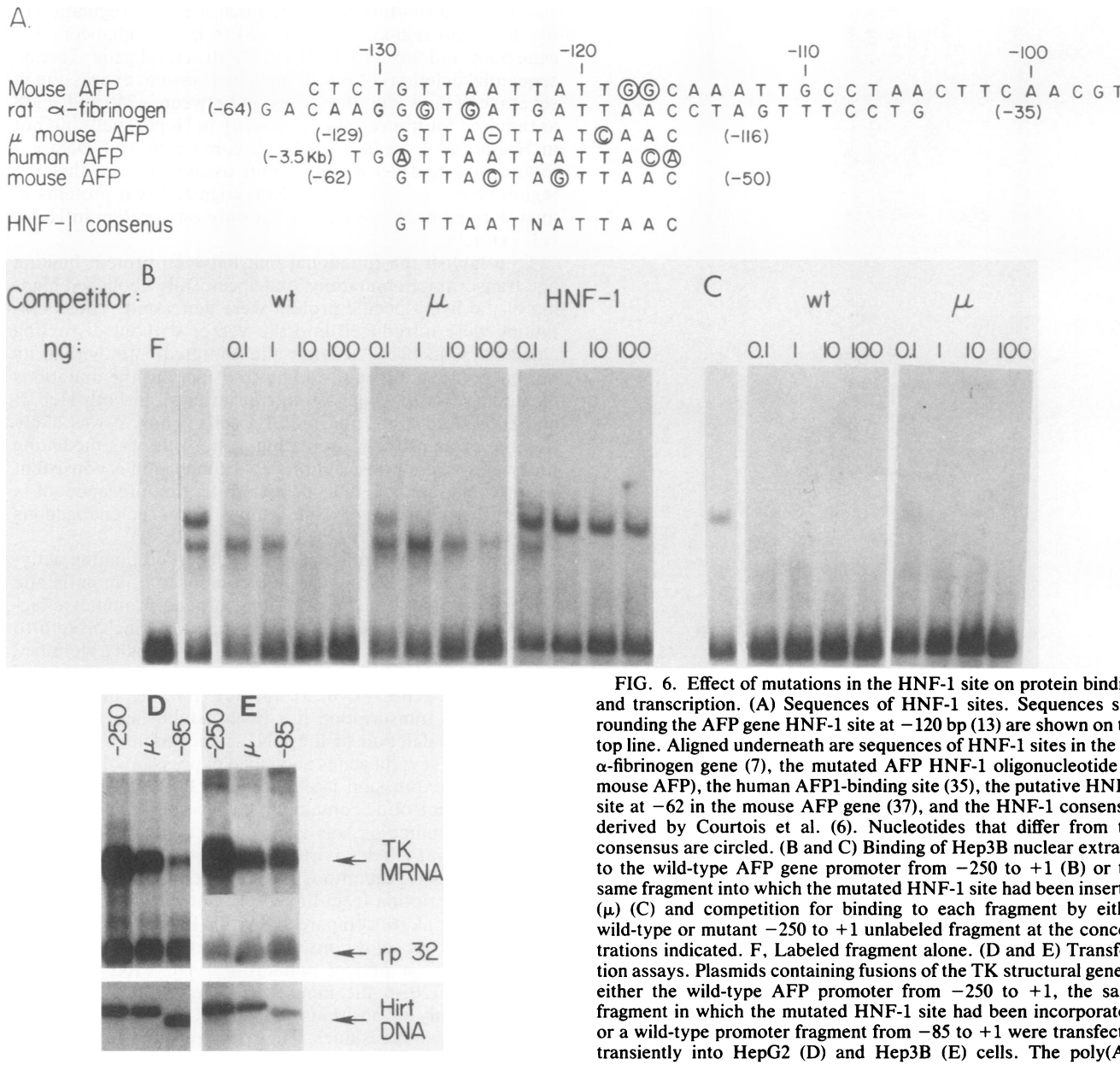

FIG. 6. Effect of mutations in the HNF-1 site on protein binding and transcription. (A) Sequences of HNF-1 sites. Sequences surrounding the AFP gene HNF-1 site at −120 bp (13) are shown on the top line. Aligned underneath are sequences of HNF-1 sites in the rat α-fibrinogen gene (7), the mutated AFP HNF-1 oligonucleotide (μ mouse AFP), the human AFP1-binding site (35), the putative HNF-1 site at −62 in the mouse AFP gene (37), and the HNF-1 consensus derived by Courtois et al. (6). Nucleotides that differ from the consensus are circled. (B and C) Binding of Hep3B nuclear extracts to the wild-type AFP gene promoter from −250 to +1 (B) or the same fragment into which the mutated HNF-1 site had been inserted (μ) (C) and competition for binding to each fragment by either wild-type or mutant −250 to +1 unlabeled fragment at the concentrations indicated. F, Labeled fragment alone. (D and E) Transfection assays. Plasmids containing fusions of the TK structural gene to either the wild-type AFP promoter from −250 to +1, the same fragment in which the mutated HNF-1 site had been incorporated, or a wild-type promoter fragment from −85 to +1 were transfected transiently into HepG2 (D) and Hep3B (E) cells. The poly(A)$^+$ RNAs were analyzed for TK mRNA and rpL32 mRNA and Hirt DNA was analyzed as described in the legend to Fig. 1. Gels represent one of four comparable sets of transfections.

compete for HNF-1 binding in gel mobility shift assays (data not shown). Thus, HNF-1 appears to be a transcription factor, like NF-1 and Spl, that is restricted to sites close to the transcriptional start site of genes. A possible exception to this rule has been reported in the human AFP gene. Sawadaishi et al. (35) have recently shown that a liver-specific protein found in human hepatoma cells (AFP1) binds both to the human AFP gene enhancer and to a sequence in the promoter located between bp −169 and +29. Although the recognition site for this protein is A+T rich, like the HNF-1 site, it is unclear whether the protein is HNF-1. An oligonucleotide composed of the enhancer-derived AFP1 sequence competes for binding of HNF-1 to the mouse AFP gene with a 10-fold-lower efficiency than to the bona fide HNF-1 sites in the α-fibrinogen or mouse AFP genes. It should also be noted that the AFP1 site in the human AFP enhancer is not conserved in the mouse gene (13).

A second protein or protein complex that could be separated from HNF-1 by heparin-agarose chromatography was present in both Hep3B and HeLa cell extracts. DNase I footprinting of the separated activities indicated that the protein fractions protected different overlapping regions of the promoter. The observation that this binding was competed by many DNAs, including pUC18, made an assignment of its identity impossible. We also cannot attribute any transcriptional significance to this binding, since the mutation that was generated in the HNF-1 site did not affect its binding activity. However, the fact that the disruption of HNF-1 binding was reflected in a loss in liver-specific transcription argues strongly that in vivo HNF-1 preferentially occupies this site.

A unique property of the AFP gene is its dramatic repression shortly after birth (43). The finding that the AFP gene

promoter responds to a transcription factor that is actively involved in the expression of a variety of liver genes expressed in the adult argues that HNF-1 cannot be mediating this event. This conclusion is reinforced by recent studies using transgenic mice, which have mapped the element responsible for repression upstream of the HNF-1-binding site, between bp −250 and −800 (J. Vacher and S. M. Tilghman, submitted for publication). This negative element acts as a silencer, and its deletion results in constitutive high-level expression of the AFP transgene throughout the adult life of the animal. It will be important to understand how the silencer can interfere with the continued presence of HNF-1, as well as the proteins that interact with the enhancers, in the adult liver.

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