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Modulation of Liver-Specific Transcription by Interactions between Hepatocyte Nuclear Factor 3 and Nuclear Factor 1 Binding DNA in Close Apposition

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The liver-specific enhancer of the serum albumin gene contains an essential segment, designated eH, which binds the hepatocyte nuclear factor 3α (HNF3α) and ubiquitous nuclear factor 1/CCAAT transcription factor (NF1/CTF) proteins in tight apposition. We previously showed that activation of transcription by the eH site was correlated with an increase in intracellular HNF3α levels during the in vitro differentiation of the hepatic cell line H2.35. We now show that transfection of an HNF3α cDNA expression vector into dedifferentiated H2.35 cells is sufficient to induce transcription from the eH site. Mutational analysis of the enhancer demonstrates that NF1/CTF cooperates with HNF3α to induce enhancer activity. However, when the eH site is removed from the context of the enhancer, NF1/CTF can inhibit transcriptional activation by HNF3α. We conclude that the ternary complex of HNF3α, NF1/CTF, and the eH site forms a novel, composite regulatory element that is sensitive to the local DNA sequence environment and suggest that the transcriptional stimulatory activity of NF1/CTF depends on its higher-order interactions with other proteins during hepatocyte differentiation.

Cellular differentiation is governed by the binding of transcription factors to DNA in specific combinations, often leading to the formation of large arrays of protein-DNA complexes. Interactions between different transcription factors bound to adjacent sites can lead to striking changes in their respective activities. For example, the yeast protein MCM1 (PRTF) activates α-cell-specific promoters when bound adjacent to the α1 transcription factor, but MCM1 represses **a**-cell-specific promoters when bound next to the **a**2 protein (1, 29). In this report, we study the interactions between two liver transcription factors when they are tightly juxtaposed at a site on DNA and explore how the genetic context of the proteins' DNA binding sites governs their interactions during hepatic differentiation.

Serum albumin gene transcription is an excellent marker for liver differentiation in mammals because it is activated early during liver development (8, 49, 51, 53) and increases to a rate 1,000-fold greater in the liver than in other tissues (33, 44). Tissue specificity is controlled in part by an enhancer element that lies 10 kb upstream of the transcription start site and functions selectively in the liver of transgenic mice (43). The albumin enhancer contains three sites, designated eE, eG, and eH, that are essential for enhancer activity in various hepatocyte-derived cell lines, such as H2.35 cells (24, 25, 34). The eE site binds liver-enriched C/EBP-related proteins (6, 11, 56), and the eG site binds a family of transcription factors, hepatocyte nuclear factor 3α (HNF3α), HNF3β, and HNF3γ, expressed in liver and lung (31). The HNF3 family contains a conserved DNA binding domain found in the developmentally important *Drosophila fork head* protein (54). The eH site was defined by a large DNase I footprint with nuclear extracts from mouse liver and H2.35 cells (34, 58); only a portion of the eH sequence was protected in kidney and spleen extracts, suggesting the involvement of a tissue-restricted factor.

The eH site, like the albumin enhancer itself, responds to extracellular signals that control hepatocyte differentiation (13). When freshly isolated hepatocytes are cultured in the presence of serum and on a plastic substratum, they rapidly dedifferentiate, and the transcription of liver-specific genes declines dramatically (9). In H2.35 cells cultured under these conditions, the eH site footprinting activity is at low abundance, and neither the albumin enhancer nor multimers of the eH site stimulate the albumin promoter from 800 bp upstream. However, when hepatocytes are cultured in serum-free medium and on extracellular matrix gels, they maintain higher levels of liver-specific gene transcription (3, 7, 15, 17), and these conditions increase eH site footprinting activity and activate both the intact enhancer and multimers of the eH site in H2.35 cells (13, 34). By contrast, multimers of the enhancer eG HNF3 binding site function as enhancers in H2.35 cells even when the cells are cultured in serum and on a plastic substratum (13). Thus, understanding the control of the eH site would provide insight into the regulation of liver gene expression and differentiation.

In this study, we identify two transcription factors that bind simultaneously to the eH site, creating a composite regulatory element with properties that differ from those of either factor bound alone. These findings explain key features of the regulation of transcription by a liver-specific enhancer and further reveal how the genetic context of the factors' binding sites can govern whether one of the factors inhibits or augments liver-specific transcription.

MATERIALS AND METHODS

Preparation of nuclear extracts and in vitro translations. Mouse liver nuclear extracts were prepared by the method of Gorski et al. (21, 32), with minor modifications described elsewhere (38). H2.35 cell nuclear extracts were prepared for

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TABLE 1. Sequences of eH site oligonucleotides

Oligonucleotide	Sequence[a]
eH-WT	CCGAACGTGTTTGCCTTGGCCAGTTTTCCATGTACATGCA
eH-M4 (TGT3$^-$)	CCGAACGACCATCACTTGGCCAGTTTTCCATGTACATGCA
eH-M5 (NF1$^\pm$)	CCGAACGTGTTTGCCTTGGCCAGTTAGGTTGACACATGCA
eH-M7 (NF1$^-$)	CCGAACGTGTTTGCCTATATCAGTTTTAGCTGTACATGCA
eH-M4/M7 (TGT3$^-$, NF1$^-$)	CCGAACGACCATCACTATATCAGTTTTAGCTGTACATGCA

[a] Conserved TGT3 and NF1/CTF binding sequences are shown in bold; mutated sequences are underlined.

electromobility shift assays (EMSAs) as described previously (13); for immunoblots, nuclei were lysed in sodium dodecyl sulfate (SDS) sample buffer (47) and sonicated. HNF3α and nuclear factor 1 (NF1) proteins were translated in vitro from their respective cDNAs (30, 36) in micrococcal nuclease-treated reticulocyte lysates as recommended by the manufacturer (Promega Biotec).

EMSAs. EMSAs were performed as previously described (13), with the following modifications. Nuclear extracts were added to binding reactions at 2 μg per reaction in the presence of 5 μg of bovine serum albumin and 80 to 100 ng of poly(dI-dC), depending on the particular extract preparation. After 10 min, oligonucleotide probe, with or without unlabeled competitor oligonucleotide, was added to the binding reaction, and incubation was continued for 20 min. One microliter of reticulocyte lysate translation product was added to binding reactions unless indicated otherwise. The reticulocyte lysate contained a nonspecific binding activity which was competed for by the inclusion of 35 to 150 ng of sonicated salmon sperm DNA per reaction. Binding reactions using purified NF1 were performed by using the amounts of protein and nonspecific competitor indicated in the figures and in the presence of 5 μg of bovine serum albumin.

Oligonucleotides and probes. Except for the TTR (mouse transthyretin gene) oligonucleotide (10) and eH-M4, the double-stranded oligonucleotides used were synthesized as complementary single strands spanning the sequences shown in Table 1 (top strand is shown). In addition to the sequences shown, double-stranded oligonucleotides contained 5′ extensions that created the sequence GCT at either end after fill-in with Klenow DNA polymerase. eH-M4 was created by annealing a 26-bp oligonucleotide containing the TGT3 sequence mutation to a 29-bp wild-type bottom strand and filling in the single-stranded regions with Klenow enzyme. Oligonucleotide probes were labeled by filling in 5′ extensions with [α-^{32}P]dATP or [α-^{32}P]dCTP and Klenow polymerase or by incubating filled-in oligonucleotides with [γ-^{32}P]ATP and polynucleotide kinase.

Antibodies and immunoblotting. Rabbit antibody that recognized the amino termini of HNF3α and HNF3β was generously provided by E. Lai (30). Rabbit antibody against bacterially expressed human NF1/cellular transcription factor 1 (CTF1) was a gift from N. Tanese and R. Tjian. For EMSAs, 1 μl of specific antiserum or normal rabbit serum was added to binding reactions as described above. For immunoblots, proteins were boiled in SDS sample buffer, electrophoresed in a reducing SDS-polyacrylamide gel, transferred to nitrocellulose filters, exposed to a 1:750 dilution of the NF1/CTF antiserum, and subsequently incubated with ^{125}I-protein A, using standard protocols (47).

Purification of NF1. A pig liver was minced and extensively washed in phosphate-buffered saline on ice, macerated with a tissue grinder, and homogenized in a blender in the tissue homogenization buffer of Fritton et al. (16) containing 0.45 M sucrose, 1 μg each of soybean trypsin inhibitor, leupeptin, and antipain per ml, 100 μM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Following homogenization, nuclei were pelleted through a 0.6 M sucrose cushion, resuspended, and washed once with tissue homogenization buffer. The nuclei were again collected by centrifugation, and the pellet was resuspended by Dounce homogenization in buffer C of Dignam et al. (12) containing protease inhibitors as specified above. After a 30-min incubation on ice, the nuclei were pelleted, and the protein in the supernatant was precipitated with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in the buffer D of Dignam et al. (12), and dialyzed against a large volume of the same buffer. Approximately 8 g of protein was recovered.

Two grams of the crude extract was fractionated on a 20-ml heparin-agarose column (IBF) and eluted with KCl in steps. The majority of the eH binding activity eluted at 400 mM KCl. After dialysis against buffer D, 24 mg of this fraction was passed over a 5-ml DEAE-Sepharose column; the 14-mg flowthrough fraction contained the bulk of the eH binding activity separated from a nuclease activity. The DEAE flowthrough (11.5 mg) was fractionated on a double-stranded DNA-cellulose column by step elution with KCl. The eH binding activity eluted as a sharp peak (880 μg) at the 400 mM KCl step. The enrichment of the NF1 DNA binding activity was approximately 400-fold.

Plasmid constructs. Single copies of double-stranded oligonucleotides (see Table 1) were inserted at a site 54 bp upstream of the albumin transcription start site in plasmid pAN6 (38), which contains albumin sequences up to the +8 position, fused to the neomycin resistance gene. The structures of all inserts were confirmed by DNA sequencing. The pRSV-tk internal transfection control plasmid has been described elsewhere (57). The HNF3α expression plasmid was created by inserting the 1.7-kb *Eco*RI cDNA fragment (30) into plasmid pRG, which contains the Rous sarcoma virus (RSV) long terminal repeat as a promoter and the bovine growth hormone polyadenylation site downstream (provided by Mitchell Reff, Smith Kline Beecham Laboratories). The NF1$^-$ version of the albumin enhancer in pAT2-NA (34) was created by using one of the eH-M7 oligonucleotides with the polymerase chain reaction-based method described in reference 58; DNA sequencing showed that the plasmid contained the expected M7 base pair changes except the A-to-C transversion in the second NF1 half-site (Table 1).

Transfection of H2.35 cells. H2.35 cells (6.5×10^5 to 10×10^5) were seeded onto 60-mm-diameter plastic dishes at 33°C in Dulbecco's modified Eagle's medium (GIBCO) containing 4% fetal bovine serum (HyClone), 2×10^{-7} M dexamethasone, and 100 U each of penicillin and streptomycin per ml. One day later, 3 μg of pAN6-based vector, 0.1 μg of pRT1, and 3 μg of pRG-HNF3α or pRG control vector, for a total of 6 to 8 μg of DNA per dish, were transfected into the cells

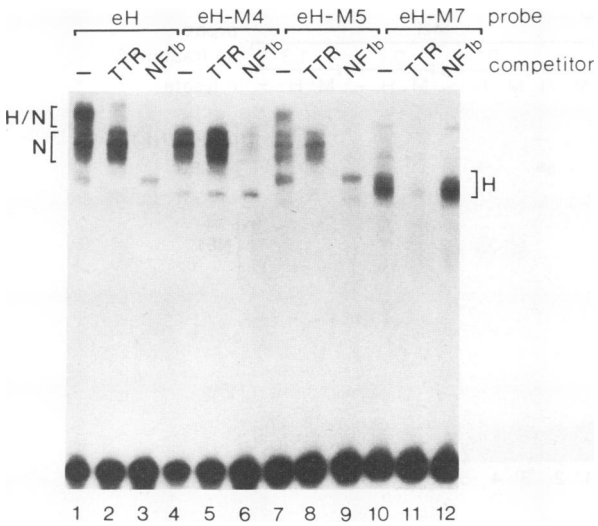

FIG. 1. Evidence that HNF3 and NF1/CTF form a ternary complex with the albumin enhancer eH site. EMSAs were performed with mouse liver nuclear extracts and wild-type and mutated eH site probes, as listed on the top row (see Table 1). The following unlabeled competitor oligonucleotides were added at a 75-fold molar excess (second row): TTR, HNF3 site (10); and NF1b, NF1/CTF site (2). Protein-DNA complexes H/N, N, and H are indicated at the sides of the autoradiograph; free probe migrated to the bottom of the gel. The specific activity of the eH probe was about half of that for the other probes. The band migrating slightly faster than H in lanes 4 to 6 is nonspecific.

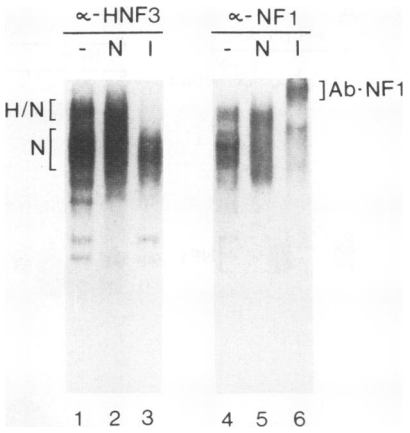

FIG. 2. Antibody (Ab) recognition of HNF3 and NF1/CTF proteins in the H/N complex. Binding reactions were performed as for Fig. 1 except that either immune serum (I) against HNF3α and HNF3β (lane 3) or against NF1 (lane 6) or nonimmune serum (N; lanes 2 and 5) was added to the binding reactions. Anti-HNF3 antibody (α-HNF3) was added to the binding reaction 9.5 min prior to the addition of probe; anti-NF1 antibody (α-NF1) was added 5 min after the addition of probe. Anti-HNF3 antibody disrupted the formation of the H/N complex, and anti-NF1 supershifted both H/N and N complexes. Free probe is not shown.

by the calcium phosphate procedure, using a kit from BRL/GIBCO. Alternatively, the pAT2-NA plasmids were transfected with pRT1, and 18 h later the cells were subcultured onto plastic and collagen gel substrata as described previously (13). Two days later, total cellular RNA was isolated and experimental and control transcripts were subjected to quantitative primer extension analysis, using radioactively labeled *neo*- and *tk*-specific primers simultaneously in the same reaction (34). Primer extension products were displayed on DNA sequencing gels with size standards consisting of dideoxy sequencing reactions initiated with the *neo* primer. Dried gels were exposed to Kodak XAR or Fuji RX film in the presence of intensifying screens. Different exposures of autoradiographs (12 to 72 h) were scanned with an LKB laser densitometer, test signals were normalized to the RSV internal control in the same lane, and data from different experiments were subjected to statistical analysis.

RESULTS

Distinct TGT3 and NF1 sequence motifs and binding activities at the eH site. In EMSA, an eH site oligonucleotide probe produces a complex pattern of bands with mouse liver nuclear extracts (13, 34) (Fig. 1, lane 1), suggesting that the site is bound by more than one protein. Inspection of the eH sequence revealed the TGT3 motif (50) (Table 1), TGTT TGC, which occurs in regulatory elements of diverse liver-specific genes (50, 57) and which, in some cases, binds HNF3 proteins (14, 40). Two base pairs downstream of TGT3 is the recognition sequence for the NF1/CTF family of transcription factors, $TGGCN_7CCA$ (5, 18, 36, 41, 46, 48). Mutational substitution of the TGT3 motif in eH (eH-M4) caused a loss of both the lowest-mobility species (designated H/N) and the highest-mobility species (designated H) of the EMSA pattern (Fig. 1, lane 4). A known HNF3 binding site from the mouse TTR gene (10) produced the same effect when used as a competitor in a binding reaction with the wild-type eH site oligonucleotide (lane 2). Mutational substitution of the 3′ half of the NF1/CTF consensus (eH-M5) led to a reduction of both the H/N complex and the central cluster of bands and to an increase in the H band (N; lane 7); an NF1/CTF binding site from the hepatitis B virus enhancer (NF1b [2]) produced the same effect when used as a competitor with the wild-type eH site (lane 3). Mutation of both NF1/CTF half-sites (eH-M7) completely eliminated the H/N and N complexes and led to a sharp increase in the H complex, which was competed for by an HNF3 site oligonucleotide. The increase in HNF3 binding to the eH-M7 oligonucleotide appears to be due to sequence changes in the NF1 half-site closest to the TGT3 motif, because the strong increase in binding was not observed with the eH-WT probe in the presence of the NF1/CTF competitor (lane 3). The eH-M7 sequence changes may affect the selectivity or affinity of the site for particular HNF3 family members (31). In conclusion, HNF3 and NF1/CTF proteins in liver nuclear extracts appear to bind to the TGT3 and NF1 motifs of the eH site, respectively.

HNF3 and NF1/CTF proteins form a ternary complex with eH site DNA in liver nuclear extracts. To establish definitively that HNF3 and NF1 proteins form a ternary complex with the eH site in liver nuclear extracts, we added antisera specific for HNF3α and HNF3β (31), and for NF1/CTF, to eH site binding reactions. Anti-HNF3 antibody caused the selective loss of the low-mobility H/N complex without markedly affecting the central NF1-specific cluster of bands (Fig. 2, lane 3). Addition of anti-NF1/CTF antiserum caused the loss of the NF1 bands and the H/N band, concomitant with the formation of a supershifted band (designated Ab-NF1; lane 6). In contrast, the presence of normal rabbit serum in the binding reactions did not significantly alter the binding patterns (lanes 2 and 3). We conclude that HNF3 and NF1/CTF proteins in liver extracts can bind simultaneously to the eH site to form the H/N ternary complex.

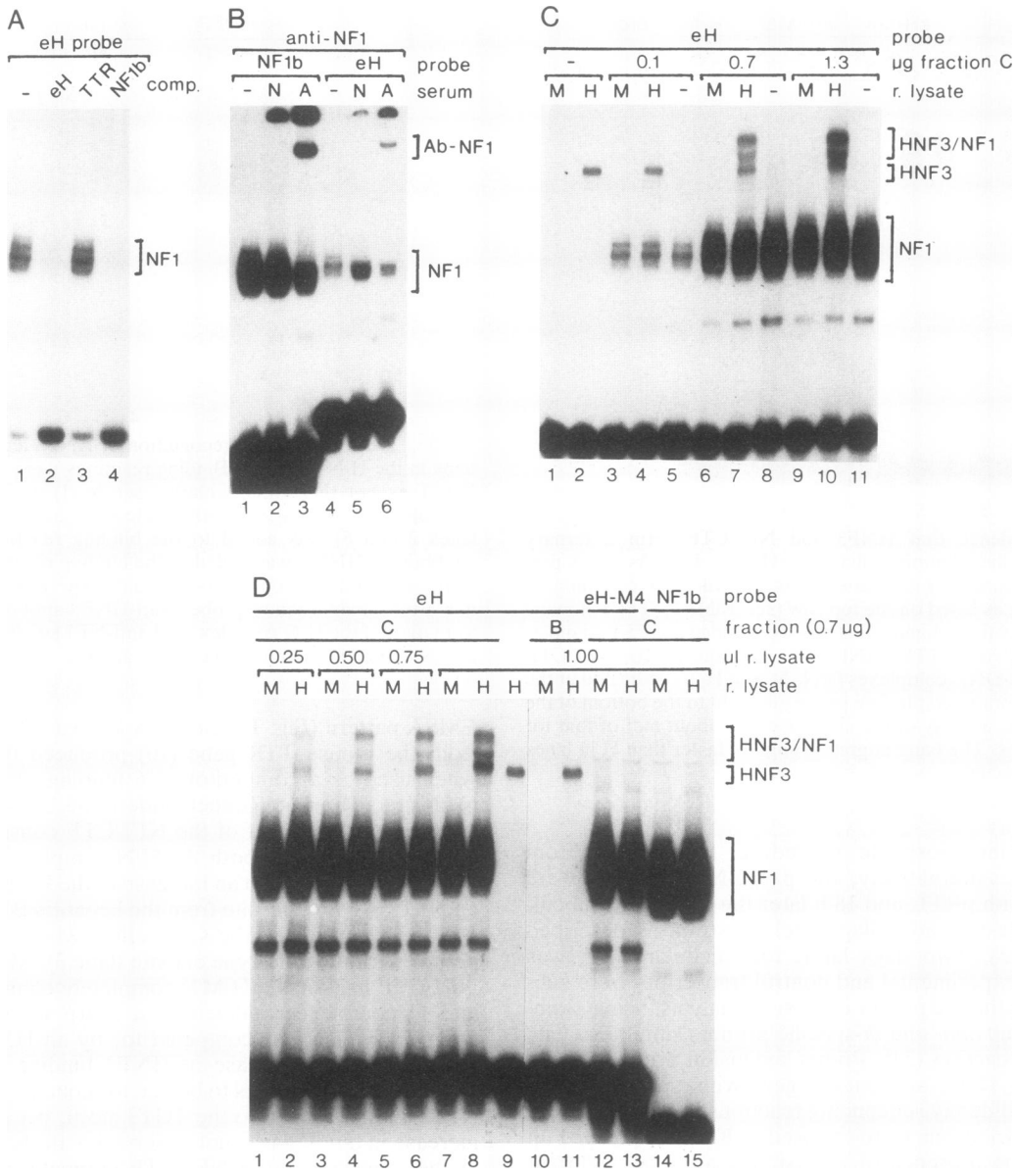

FIG. 3. Evidence that purified NF1/CTF and cloned HNF3α form a ternary complex with the eH site. (A and B) A highly enriched eH binding activity fractionated from porcine liver (fraction C) was used in EMSAs. (A) Competition assay using 25 ng of fraction C and 5 ng of poly(dI-dC) per reaction. (B) Antibody supershift experiment using 0.7 μg of fraction C, 150 ng of salmon sperm DNA, and no addition (−), 1 μl of preimmune serum (N), or 1 μl of anti-NF1 antiserum (A). The NF1b probe was labeled to a higher specific activity than was the eH probe. Ab, antibody. (C) Various amounts of the purified NF1/fraction C were mixed with HNF3α translated in a reticulocyte (r.) lysate (lanes H), with protein from a mock translation (lanes M), or with no addition (−) in the presence of the eH probe and 150 ng of salmon sperm DNA; bound complexes were resolved in an EMSA. The HNF3/NF1 complex discussed in the text is indicated at the right. (D) EMSA performed as described above with 1 μl of reticulocyte lysate and 0.7 μg of fraction C or of another fraction from the same column which contained no eH binding activity (fraction B).

Assembly of a ternary DNA binding complex with in vitro-translated HNF3α and partially purified NF1. To determine whether HNF3α and NF1 were the only binding proteins required to form the H/N ternary complex, we performed binding reactions with in vitro-translated HNF3α and partially purified NF1. NF1 was prepared from a porcine liver nuclear extract that had been sequentially chromatographed over heparin-agarose, DEAE-Sepharose, and double-stranded DNA-cellulose columns to obtain a fraction (designated C) that was highly enriched in an activity which binds the eH site. This activity was specifically competed for by eH and NF1b oligonucleotides but not by the TTR oligonucleotide (Fig. 3A), and identical binding patterns were produced with the eH and NF1b probes (Fig. 3B, lanes 1 and 4). The NF1 protein-DNA complexes migrated more rapidly than those seen in unfractionated material, suggesting that the protein underwent partial degradation during purification. Incubation of limited amounts of NF1/CTF antiserum with fraction C in the presence of the eH or NF1b probe produced a supershifted band (Ab-NF1) with a con-

current reduction in the NF1-like bands (Fig. 3B, lanes 3 and 6), indicating that at least the bulk of the binding activity is NF1/CTF.

Next, in vitro-translated HNF3α was mixed with the NF1-containing fraction C in the presence of excess eH probe. The appearance of a specific binding complex formed with the HNF3α translation product, and not with a mock-translated reticulocyte lysate, demonstrates definitively that HNF3α binds the eH site (Fig. 3C, lanes 1 and 2). Upon addition of increasing amounts of NF1, there was a progressive increase in the formation of a new, slowly migrating complex (HNF3/NF1; Fig. 3C, lanes 4, 7 and 10). Indeed, the amount of HNF3/NF1 complex eventually exceeded the amount of HNF3α-DNA complex that formed in the absence of NF1, indicating that the presence of NF1 increased the total amount of HNF3α bound. The formation of the HNF3/NF1 complex also increased progressively when increasing amounts of HNF3α were added to a constant amount of NF1 (Fig. 3D, lanes 2, 4, 6, and 8). The HNF3/NF1 complex was not detected when mock-translated reticulocyte lysate was added to the binding reaction (lanes M) or when HNF3α was mixed with protein eluted from the DNA-cellulose column at a different salt step from NF1 (fraction B; Fig. 3D, lane 11), indicating that production of this species depends on both NF1 and HNF3α. Formation of the complex also required the presence of binding sites for both factors, as no low-mobility species was detected when either the eH-M4 or NF1b oligonucleotide was used as a probe (Fig. 3D, lanes 13 and 15). We conclude that HNF3α and NF1/CTF together are sufficient to form a ternary complex with the albumin eH site.

NF1/CTF binding inhibits activation by HNF3α at the eH site in dedifferentiated H2.35 cells. We next wished to address why multimers of the eH site fail to stimulate transcription in H2.35 cells cultured under conditions that cause hepatocyte dedifferentiation. Under such conditions, HNF3α is present at a low concentration but it can still activate transcription from the albumin promoter when multimers of the eG HNF3α binding site are positioned 787 bp upstream of the transcription start site (13). Previously we showed that H2.35 cells express only HNF3α and not HNF3β or HNF3γ (13). Although H2.35 cells express less eH site binding factors than do liver cells (13), Fig. 4 shows that HNF3α and NF1/CTF proteins bind to the eH site in about the same proportion as seen with liver nuclear extracts and form the H/N ternary complex. Thus, it seemed likely that a detailed analysis of the interactions between the two proteins in H2.35 cells would shed light on the regulation of eH site activity.

To address this problem, we assessed the transcriptional stimulatory activity of a single copy of the eH site, and of eH binding site mutations, when placed directly upstream of the albumin TATA box; thus, TATA activity was solely modulated by eH site binding factors. This strategy allowed the direct examination of interactions between HNF3α and NF1/CTF and eliminated the effects of other albumin promoter-binding factors. A single eH site, or mutations thereof, was inserted 54 bp upstream of the transcription start site in plasmid pAN6, and the DNAs were transfected into H2.35 cells cultured in 4% serum, on a plastic substratum, and at the permissive temperature for the resident temperature-sensitive simian virus 40 (dedifferentiating conditions; [57]). The reporter plasmids were cotransfected with an internal control plasmid, pRT1, which contains the RSV long terminal repeat promoter driving the herpes simplex virus *tk* gene. After 2 days, total cellular RNA was isolated

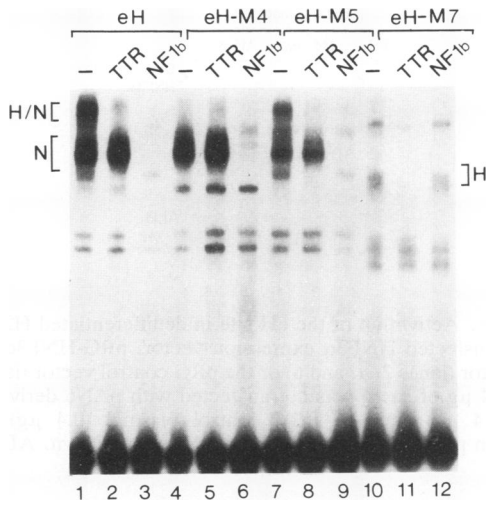

FIG. 4. HNF3α/NF1 ternary complex formation in nuclear extracts from H2.35 cells. EMSAs were performed with nuclear extracts from H2.35 cells cultured under dedifferentiated conditions (34). Probes and competitor DNAs were as for Fig. 1. The band migrating slightly faster than H in lanes 4 to 6 and the doublet below in all lanes are nonspecific.

and transcription from pAN6 and pRT1 was assayed simultaneously by primer extension, using labeled oligonucleotides specific for transcripts from each plasmid.

The presence of a single eH site weakly stimulated transcription from the TATA element in dedifferentiated cells, about 1.7-fold (n = 4, standard deviation [SD] = 0.73; Fig. 5B, lane 2). The eH-M4 mutation, which disrupts the binding of HNF3α, caused the loss of transcriptional stimulation (lane 3). Long exposures of the autoradiographs indicated that the eH-M4 segment, which retains the NF1/CTF binding site, neither activated nor repressed basal TATA activity

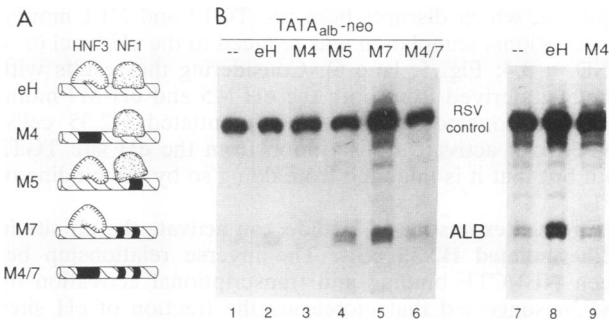

FIG. 5. Evidence that NF1/CTF inhibits activation by HNF3α when the eH site is positioned near a TATA element. (A) eH oligonucleotides and the ability of HNF3 and NF1 to bind. Black segments indicate positions of mutated base pairs. (B) Primer extension products of total cellular RNA isolated from H2.35 cells cultured under dedifferentiating conditions (34) and transfected with plasmid pAN6 (lane 1) or derivatives containing the wild-type (lane 2) or mutated (lanes 3 to 6) eH site (4 μg) and with the internal control plasmid pRT1 (0.4 μg). Extension products from transcripts of pAN6 (ALB) and pRT1 (RSV control) are indicated between the autoradiographs. Lanes 7 to 9 depict a longer exposure of lanes 1 to 3; the exposure in lane 2 is for less time than in other lanes, normalizing for the RSV control signal. The eH-M7 mutation leads to novel transcription start sites, one of which comigrates with the RSV control signal.

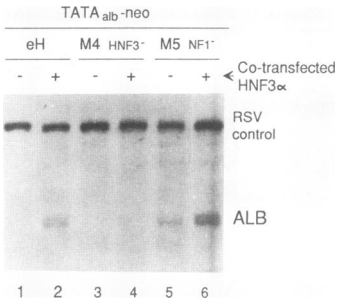

FIG. 6. Activation of the eH site in dedifferentiated H2.35 cells by cotransfected HNF3α expression vector. pRG-HNF3α expression vector (lanes 2, 4, and 6) or the pRG control vector (lanes 1, 3, and 5) (4 μg of each) was cotransfected with pAN6 derivatives as shown (4 μg) and the RSV control plasmid (0.4 μg). Primer extension products of the resulting RNAs are shown. ALB, albumin.

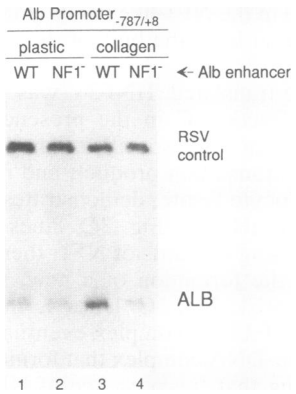

FIG. 7. Evidence that NF1/CTF binding to the eH site is required for the activity of the albumin (Alb) enhancer. H2.35 cells were transfected on plastic substrata with pAT2-NA (34) containing the wild-type albumin enhancer (WT) or with pAT2-NA/M7, containing a mutation of the NF1 site (NF1$^-$), and the control plasmid pRT1. After 18 h, the cells were released from the plates and subcultured either on plastic or on collagen gels. Two days later, RNA was isolated from the cells and subjected to the primer extension assay.

(lane 9). Strikingly, the eH-M5 mutation, which reduces binding of NF1/CTF but not of HNF3α, increased transcription 2.5-fold more than did eH-WT (n = 4, SD = 0.28; lane 4). The eH-M7 mutation, which eliminates NF1 binding, further increased transcription from the normal transcriptional start site, and from various other sites up to 15 nucleotides upstream, a total of 3.5-fold more than did eH-WT (n = 3, SD = 1.8; lane 5). Most of these start sites are evident on long exposures of the wild-type template (lane 7). An abundant primer extension product arises from a start site 15 bp upstream of the normal site and comigrates with the pRT1 product (data not shown); the use of these upstream initiation sites may be controlled by an A/T-rich region 15 bp upstream of the albumin TATA box in plasmid pAN6-eH-M7.

It is possible that the different transcription start sites with the eH-M7 plasmid could be due to the creation of a binding site for an unexpected protein, although no novel binding activities were discovered in an EMSA (Fig. 1 and 4, lanes 9). To address this possibility further, we tested the eH-M4/7 sequence, which disrupts both the TGT3 and NF1 motifs; transcriptional stimulation was reduced to the eH level (n = 2, SD = 0.4; Fig. 5, lane 6). Considering the results with templates derived from both the eH-M5 and eH-M7 mutations, we conclude that in dedifferentiated H2.35 cells, HNF3α can activate transcription from the eH site TGT3 motif but that it is inhibited from doing so by the binding of NF1.

Increased expression of HNF3α can activate the eH site in dedifferentiated H2.35 cells. The inverse relationship between NF1/CTF binding and transcriptional activation by HNF3α suggested that increasing the fraction of eH sites occupied by HNF3α alone, by increasing the cellular concentration of the protein, should increase transcriptional activity. This model could account for the increased activity of multimers of the eG and eH sites, and the albumin enhancer itself, during hepatic differentiation, when the concentration of HNF3α increases (13, 34) but that of NF1/CTF does not (see below). To test this model directly, we transfected H2.35 cells cultured under dedifferentiating conditions with the HNF3α expression vector pRG-HNF3α, or the pRG plasmid lacking the HNF3α insert, and with a subset of the pAN6 plasmids and pRT1. Cotransfection of pRG-HNF3α reproducibly caused a 4.1-fold stimulation (n = 3, SD = 1.4) of pAN6-eH transcription, whereas the pRG control vector had no effect (Fig. 6, lane 2). The stimulatory effect of HNF3α was dependent upon an intact TGT3 motif (eH-M4; lane 4) and persisted when NF1 binding was weakened in eH-M5 (3.5-fold; n = 2, SD = 1.8; lane 6). These findings demonstrate that the eH TGT3 motif represents a functional binding site for HNF3α and that increased expression of HNF3α is sufficient to activate the albumin eH site in dedifferentiated H2.35 cells.

NF1/CTF binding is required for albumin enhancer activity in differentiated H2.35 cells. The aforementioned results suggest a simple model for the role of NF1 during hepatocyte differentiation. In dedifferentiated cells, bound NF1 inhibits activation by HNF3, blocking enhancer activity that could be mediated by low amounts of liver-enriched transcription factors. During differentiation, increased expression of HNF3α increases the chance that the eH site is occupied by this factor alone, contributing to an active enhancer. Therefore, disruption of the NF1 site in the intact enhancer should lead to greater transcriptional stimulatory activity. To test this hypothesis, we introduced the eH-M7 sequence into an 830-bp enhancer segment that contains the essential eE, eG, and eH sites described above (34). This NF1$^-$ version of the enhancer and the wild-type segment were placed 787 bp upstream of the albumin transcription start site in the test plasmid pAT2 (34). These plasmids and the pRT1 control were transfected into H2.35 cells cultured on a plastic substratum; the cells were subsequently subcultured onto plastic and collagen gel substrata; the latter condition activates the albumin enhancer (13).

These experiments led to an unexpected finding. As seen in Fig. 7 (lane 2), mutation of the NF1 binding site did not lead to activation of the albumin enhancer in H2.35 cells cultured on plastic. This finding suggests that in dedifferentiated cells, there may be insufficient amounts of HNF3α or other enhancer binding proteins for enhancer activity. As expected, the wild-type enhancer stimulated the albumin promoter in the cells cultured on collagen (lane 3). However, contrary to the prediction of the model described above, mutation of the NF1 binding site did not increase enhancer activity; rather, enhancer activity was abolished (lane 4). Thus, in the context of the albumin enhancer, binding of

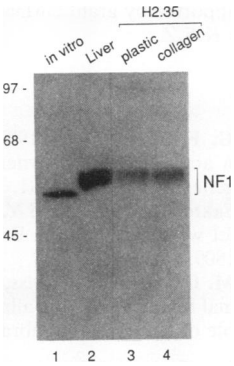

FIG. 8. Immunoblot analysis of NF1/CTF proteins. Proteins from an in vitro transcription/translation reaction with an NF1/CTF cDNA (36) (lane 1) or 75-μg amounts of protein from liver nuclear extracts (lane 2) and from nuclear extracts of H2.35 cells cultured on either plastic (lane 3) or collagen gel (lane 4) substrata were separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose, NF1/CTF proteins were visualized with an NF1/CTF-specific antibody and ^{125}I-protein A.

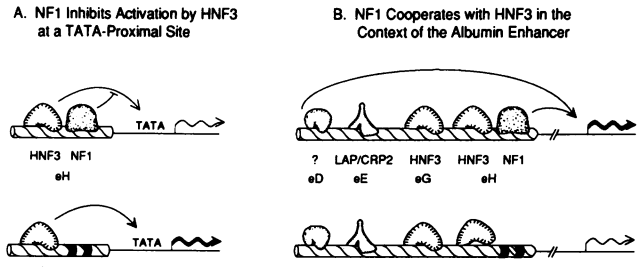

FIG. 9. Model for transcription factor interactions at an isolated eH site (A) or in the context of the albumin enhancer (B). The large arrow in panel B indicates that the factors work cooperatively to enhance transcription from the promoter.

NF1/CTF is required for transcriptional induction during hepatic differentiation.

Considering that different NF1/CTF species might possess distinct regulatory properties during differentiation, we next wished to determine whether different NF1/CTF family members are expressed in H2.35 cells on plastic versus collagen gel substrata. When nuclear proteins were analyzed by immunoblotting with the NF1/CTF-specific antibody, the detected antigens in H2.35 cells and mouse liver migrated together as a smear (Fig. 8, lanes 2 to 4), which could be resolved to three bands in some experiments. NF1/CTF proteins from all cellular samples migrated more slowly than NF1 translated in a reticulocyte lysate (lane 1), suggesting that the protein is modified posttranslationally in vivo. No change in the distribution or amount of the NF1/CTF species was evident when H2.35 cells were shifted from plastic to a collagen gel substratum. While these experiments could not detect subtle changes in NF1/CTF species, they support the hypothesis that it is the induction of HNF3 during hepatic differentiation, not of NF1/CTF, that is a critical switch for enhancer activation.

DISCUSSION

Regulation of transcription by the albumin enhancer eH site provides a useful model for dissecting the mechanisms of hepatocyte differentiation. The eH site is essential for the activity of the enhancer, by itself it functions only in hepatocyte-derived cells, and it is sensitive to cues that activate the transcription of a variety of liver-specific genes (13, 34). We found that the eH site binds and is activated by the liver transcription factor HNF3α, which accounts for the site's liver-specific activity. We also found that the eH site binds members of the NF1/CTF family of ubiquitous transcription factors. The interaction between HNF3α and NF1 at the eH site creates a composite regulatory element that permits the modulation of liver-specific transcription.

At an isolated eH sequence next to the TATA element, NF1/CTF inhibits transcriptional activation by HNF3α (Fig. 9A). To our knowledge, this is the first instance in which NF1-related proteins have been shown to negatively regulate transcription. It is unlikely that they do so, in this case, by directly occluding the binding of TATA-binding protein because the NF1 binding site is upstream by four turns of the DNA helix and because the NF1 binding site alone (pAN6-eH/M4) did not reduce basal TATA activity. The lack of effect of eH multimers distal to TATA in HeLa cells (13), which lack HNF3, further demonstrates that NF1 does not generally repress transcription of the albumin promoter. Multimers of the albumin enhancer eG site, which bind only HNF3, function as an enhancer at a distal position in dedifferentiated cells, whereas multimers of the eH site do not (13); hence, NF1 may also be inhibitory to HNF3 at isolated eH elements distal to TATA. The increased activity of pAN6-eH in dedifferentiated cells when an HNF3α expression vector is cotransfected likely reflects increased occupancy of the eH site by HNF3α. Given the tight juxtaposition of HNF3 and NF1/CTF at the eH site, we suggest that at an isolated eH site (Fig. 9A), NF1/CTF can directly inhibit a transcriptional activation domain of HNF3 (39).

By contrast, at the albumin enhancer, NF1/CTF and HNF3 are among a host of different proteins that form an active complex in differentiated cells (Fig. 9B). The on-or-off nature of the albumin enhancer (34) suggests that complex formation, transcriptional stimulation, or both is a cooperative process dependent upon the binding of NF1/CTF. During hepatic differentiation, increased binding by HNF3 and other proteins at the enhancer may introduce protein-protein interactions that preclude inhibition of HNF3 by NF1/CTF and that utilize an inherent transcriptional stimulatory property of NF1/CTF (37).

The modulation of cell-type-specific transcription by NF1/CTF proteins, as seen at the albumin eH site, may prove to be a relatively widespread phenomenon, as other tissue-specific enhancers containing NF1 binding sites have been described (19, 23, 27, 52) and in one case an increase in the expression of an NF1 mRNA has been correlated with the activation of an enhancer in differentiating cells (23). Western immunoblot analysis failed to detect changes in the abundance or migration of NF1/CTF proteins when H2.35 cells were induced to differentiate on a collagen gel substratum. We doubt that covalent alteration of an NF1/CTF family member during differentiation is sufficient to explain the difference in the protein's function at the enhancer versus an isolated eH site, because preliminary studies show that NF1 binding to an isolated eH site inhibits HNF3 in both differentiated and dedifferentiated cells (26).

However, NF1/CTF proteins are products of a multigene family (18, 37, 41, 46, 48), and it is possible that the binding of a particular positive-acting isoform is stabilized by protein-protein interactions at the albumin enhancer, while a different, negative-acting isoform binds the isolated eH site.

For example, the Oct-2 protein does not normally activate a U2 small nuclear RNA promoter, but the Oct-2B variant protein, which contains a different carboxy-terminal domain, recognizes the same sequence as does Oct-2 and can activate the promoter (51a). Similarly, specific domains of retinoic acid receptors confer interactions with adjacent binding proteins in one genetic context but not another (38a). The system that we have described should allow mapping of NF1/CTF protein domains that confer sensitivity to the genetic context of eH site.

The TGT3 class of HNF3 binding sites, like that found at the eH site, is often found in close association with binding sites for other transcription factors (4, 14, 20, 22, 35, 42, 50, 55, 58). The TGT3 motif does not resemble the TTR consensus from the TTR gene promoter (10); however, TGT3 and TTR sites can compete with one another in binding assays (this report; 34, 40), suggesting that HNF3 proteins have a single DNA recognition domain. Preliminary experiments indicate that HNF3 dissociates significantly faster from TGT3 elements than from the TTR site (26). Hence, occupancy of TGT3 sites by HNF3 could be more sensitive to variations in the abundance of HNF3 than is occupancy of TTR sites. In vitro reconstitution experiments of the H/N complex suggested that the binding of HNF3α and NF1/CTF may be cooperative at the eH site. The combination of a relatively weak HNF3 binding site and cooperative interaction with NF1 could make the eH site, in the context of the albumin enhancer, a highly sensitive switch for responding to the increase in HNF3α concentration that occurs during hepatocyte differentiation.

HNF3 binding at other TGT3 sites may also be more readily subject to modulation by other proteins bound nearby. For example, a TGT3 sequence occurs within a glucocorticoid response element (GRE) 2.5 kb upstream of the rat tyrosine aminotransferase gene (TAT [28]). Binding of the steroid receptor to the GRE in vivo appears to be transient, and it causes the subsequent binding of a liver-enriched factor to an overlapping TGT3 sequence within the GRE (45). On the basis of the similarity of DNase I hypersensitivities over the TGT3 sequence in footprinting assays of the eG site, the eH site (34, 58), and the TAT −2.5 site (45), we suggest that the factor binding to the TAT sequence is HNF3. The interactions between HNF3 and the glucocorticoid receptor provide an explanation for the liver-specific activity of the TAT hormone response element.

These studies demonstrate that the assembly of transcription factors at a complex regulatory sequence creates a local environment that influences the functional properties of the bound proteins. To understand the role of the proteins in gene regulation, it therefore becomes imperative to assess the function of transcription factors bound to their natural sequence context and not solely to isolated elements. Our comparison of the interaction between NF1/CTF and a liver-specific factor in isolated and natural contexts contributes to an understanding of how tissue-specific regulatory elements, such as the albumin enhancer, are constructed from segments that bind ubiquitous and tissue-restricted proteins.

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