MOLECULAR AND CELLULAR BIOLOGY, Feb. 1991, p. 773–784
0270-7306/91/020773-12$02.00/0

Vol. 11, No. 2

Extracellular Signals That Regulate Liver Transcription Factors during Hepatic Differentiation In Vitro

JEN-KUEI LIU, C. MICHAEL DiPERSIO, AND KENNETH S. ZARET*

Section of Biochemistry, Box G, Brown University, Providence, Rhode Island 02912

Received 13 August 1990/Accepted 8 November 1990

A complex cell culture environment has been shown to maintain the differentiated state of hepatocytes, yet the mechanisms by which environmental cues selectively maintain liver-specific gene transcription have been unknown. In this paper we show that the hepatic environment regulates the activities of at least three liver-enriched transcription factors, eE-TF, eG-TF/HNF3, and eH-TF, that activate the mouse serum albumin enhancer. eE-TF is a heat-stable factor that has a DNA-binding specificity similar to that of the liver transcription factor C/EBP, but is a distinct protein. eG-TF/HNF3 contributes to the liver-specific transcription of several other serum protein genes. eH-TF binds to a TGTTTGC sequence that occurs at regulatory sites of the albumin promoter, the hepatitis B virus enhancer, and other hepatic genes. eE-TF, eG-TF/HNF3, and eH-TF are regulated by different combinations of the following cell culture conditions: a hormonally defined serum-free medium; an extracellular matrix gel; and a transformation-competent simian virus 40 large T antigen. We propose a regulatory network model to explain how cues from the cell lineage and the extracellular environment coordinately help maintain the activities of transcription factors involved in hepatocyte differentiation.

Cell differentiation is mediated in part by transcription factors that are active in a subset of cell types and that interact in different combinations when brought together by binding to regulatory sequences in DNA (36, 39). Two central questions that remain are the following: what initiates the differential expression of such transcription factors, and how are their activities maintained in different cell types? Extracellular signals are presumed to play an important role in the activation of regulatory factors in development, and recent studies indicate that a number of factors remain active in a particular cell type, at least in part, as a result of autoregulatory control circuits (reviewed in reference 48). For example, the MyoD protein, which activates genes in the myogenic program (32), apparently maintains its own activity by stimulating transcription of the *MyoD* gene (52). However, the transcription of certain cell-specific genes can be perturbed selectively by altering the cell environment (see below), implicating exogenous signals in the postdevelopmental regulation of some cell-specific transcription factors. In the work described in this paper we investigated the role of the extracellular environment in the regulation of liver-enriched transcription factors.

When hepatocytes are disaggregated from the liver and cultured in serum-containing medium and on a plastic substratum, the cells assume a flat, extended morphology and the transcription of liver-specific genes, such as that for serum albumin, declines dramatically. By contrast, the transcription rates of common genes such as those for actin and tubulin remain constant (10, 11). However, when hepatocytes are cultured in a hormonally defined serum-free medium (SFM), the transcription of the albumin gene and other liver-specific genes is partially preserved (17, 26). Plating hepatocytes on extracellular matrices can cause the cells to exhibit a more differentiated, cuboidal morphology (46) and has recently been shown to help preserve albumin gene transcription (4a). Extracellular signals also have strong stabilizing effects on liver-specific mRNAs (26) and thereby further help to maintain hepatocyte differentiation.

To study the regulatory mechanisms underlying the transcriptional control, we created a conditionally transformed hepatocyte cell line, H2.35, that responds to the same extracellular cues as do nontransformed hepatocytes in culture. This cell line was derived (56) from adult mouse hepatocytes immortalized with simian virus 40 carrying a temperature-sensitive allele of large T antigen (*tsA*255 [7]). When H2.35 cells are cultured at the permissive temperature (33°C) in serum-supplemented medium and on a plastic substratum (dedifferentiating conditions), the cells assume a flat morphology and transcribe liver-specific genes such as the albumin gene at low rates. When cultured at the restrictive temperature (39°C) in hormonally defined SFM and on a collagen gel (differentiating conditions), the cells exhibit a cuboidal morphology, and the transcription of a subset of liver-specific genes, including that for albumin, is increased selectively (56). In transient-transfection experiments, we previously found that the albumin enhancer element, which is liver specific in transgenic mice (44), stimulated transcription from the albumin promoter in differentiated H2.35 cells, but not in dedifferentiated H2.35 cells (56). Thus, extracellular signals are required to activate this cell-specific enhancer.

In the work described in this paper, we identified liver-enriched proteins that bind to and activate the albumin enhancer; some of these proteins activate other liver genes. We have demonstrated that the extracellular environment plays a critical role in differentially regulating the activities of these transcription factors, thereby helping to maintain hepatocyte differentiation.

MATERIALS AND METHODS

Cell culture, transfection, and primer extension analysis. Culturing of H2.35 cells under differentiating and dedifferentiating conditions has been described previously (56). Transient transfections and primer extension analyses were

* Corresponding author.

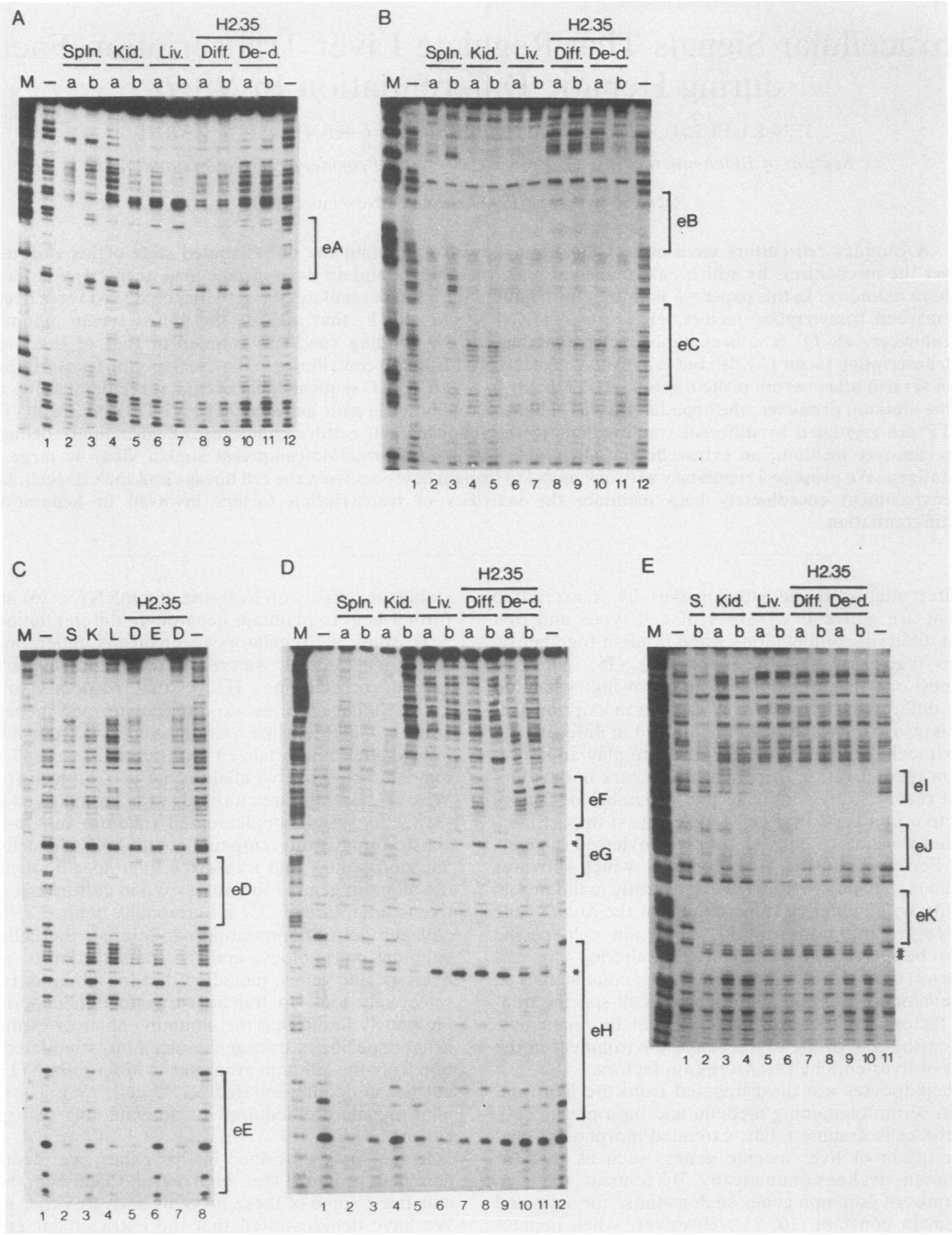

FIG. 1. DNase I footprint analysis of the albumin enhancer. DNA-binding reactions were with nuclear extracts from spleen (Spln., S), kidney (Kid., K), liver (Liv., L), differentiated (Diff., D), and dedifferentiated (De-d., E) H2.35 cells. Probe fragments were (A) *Nhe*I-*Pvu*II (1 to 140; see Fig. 2A for sequences); (B) *Dde*I-*Dde*I (111 to 206); (C) *Dde*I-*Dde*I (265 to 428); (D) *Dde*I-*Sau*3A (428 to 616); and (E) *Sau*3A-*Acc*I (616 to 825). All probes were labeled on the sense strand except the probe in panel A, which was labeled on the antisense strand. Positions of footprints are indicated by brackets. Protections above eA and eB cover pUC18 DNA; apparent footprints below eA were not observed on the other strand (data not shown). a and b indicate 20 and 50 μg of nuclear extracts, respectively; 32 μg of nuclear extracts was used in each binding reaction in panel C. Panel C shows the results with two independent preparations of differentiated H2.35 nuclear extracts. Lane M, purine cleavage ladder; lane –, no extract added. Asterisks indicate the positions of DNase I-hypersensitive cleavages induced by protein binding.

performed as described previously (56), except that 5×10^5 cells per 60-mm plastic dish or 2×10^6 cells per 60-mm collagen gel were transfected with 6 μg of pAT2, or an equimolar amount of each pAT2 derivative, and 75 ng of the pRT1 control plasmid.

Plasmid construction. The plasmids pAT2 and pRT1 have been described previously (56). The albumin enhancer from the *Nhe*I site to the *Ava*II site (see Fig. 2A; bp 1 to 830), or a 333-bp enhancer subfragment (see Fig. 2A; bp 337 to 669) was generated by the polymerase chain reaction and inserted into pAT2 adjacent to the albumin promoter at position −787, as described previously (57). Substitution mutations of the binding sites for eE-TF, eG-TF, and eH-TF were generated within either the 830-bp or the 333-bp enhancer fragment by using a polymerase chain reaction-based mutagenesis procedure described elsewhere (56). Top-strand sequences for each mutation were as follows (see Fig. 2A): eE site, GGATCCGAGT (bp 417 to 426); eG site, TCTAGAC CCG (bp 529 to 538); eH site, GTCGACAGCG (bp 575 to 584).

Preparation of nuclear extracts. Nuclear extracts were prepared from tissues essentially as described previously (35), except that the ammonium sulfate protein precipitates were resuspended into buffer D consisting of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 40 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.5 mM phenylmethylsulfonylfluoride, and 5 μg each of leupeptin, antipain, trypsin inhibitor, and benzamidine per ml.

Nuclear extracts were prepared from H2.35 cells cultured on 150-mm tissue culture plastic plates by transferring trypsinized cells to 30-ml Corex glass tubes with ice-cold phosphate-buffered saline (PBS) containing 0.35 M sucrose and 5 μg of each of the protease inhibitors listed above per ml and pelleted in a Sorvall HB-4 rotor at 15,000 rpm for 10 min at 4°C; the cells were washed twice more by the same procedure. Cells cultured on collagen gels were treated in situ with 1,000 U of collagenase (type VII; Sigma) per 150-mm plate in 2 ml of Dulbecco modified Eagle medium at 37°C for 5 min and collected by centrifugation; the cells were washed three times as described above. Nuclear proteins were isolated from cultured cells by the procedure of Dignam et al. (16), except that the nuclei were washed in the tissue homogenization buffer of Fritton et al. (19), to which sucrose was added to 0.35 M, and the extracted protein was precipitated with ammonium sulfate (0.36 g added per ml).

DNaseI footprinting assays. Nuclear extracts were preincubated in 30-μl reactions containing 10 mM Tris (pH 7.5), 40 mM KCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1% Ficoll, and 70 ng of nonspecific competitor poly(dI-dC) (Pharmacia) per 10 μg of nuclear extract. After 10 min at 23°C, about 1 fmol of filled-in, ^{32}P-end-labeled DNA probe was added and the incubations were continued for another 30 min. For competition binding reactions, the competitor DNA was added after the preincubation step and incubated for 10 min before addition of the probe. We added freshly diluted DNase I (2 μl; Worthington) and stopped the digestions and purified the DNAs as described previously (57). DNAs were resuspended into 100% formamide and electrophoresed on 6% polyacrylamide gels in 7 M urea. The dried gels were autoradiographed with intensifying screens at −80°C for 3 to 10 days.

Electromobility shift assays. Binding reactions were carried out essentially as described previously (35), except that they contained 7.5 μg of nuclear extract in a 15-μl solution containing 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1% Ficoll, with poly(dI-dC) (400 to 600 ng) as a nonspecific competitor and oligonucleotides (0.05 or 0.1 ng) that were labeled with ^{32}P-deoxynucleoside triphosphates by filling in ends with Klenow polymerase I. Double-stranded oligonucleotides used as probes or competitors were composed of the following mouse albumin sequences: pB (HNF1) site, TGGTTAATGATCTACAGT TA; CCAAT site, GGGGTAGGAACCAATGAAA; pD (C/E BP) site, ATGATTTTGTAATGGGGTAG; NF1 site, ACAA CTTTTTGGCAAAGAT, eE site, AACCTGCGTTACAGC ATCCACTCAGTAT; eG site, CCAGGGAATGTTTGTTCT TAAATACCATC; and eH site, CCGAACGTCTTTGCCTT GGCCAGTTTTCCATGTACATGCA. Oligonucleotides of HNF3 and HNF4 contain DNA sequences from −85 to −111 and −130 to −151, respectively, in the transthyretin promoter (12) and were provided by Rob Costa.

Methylation interference footprinting. ^{32}P-labeled oligonucleotides were partially methylated at nucleotides G and A with 1 μl of dimethyl sulfate in 200 μl of 50 mM sodium cacodylate (pH 8.0)–1 mM EDTA–1 μg of tRNA at 37°C for 2 to 3 min. Reactions were stopped by adding 50 μl of a solution of 1 M 2-mercaptoethanol–0.3 M sodium acetate (pH 7.0), the DNAs were precipitated twice with ethanol and sodium acetate, and the ethanol-washed pellets were resuspended into 0.1× TE. The oligonucleotides were then used in electromobility shift reactions, and the protein-DNA complexes and the free probes were excised. The DNA fragments were electroeluted from the gel pieces, cleaved at modified residues with 0.5 M piperidine at 90°C for 30 min, and then prepared for electrophoresis in 15% polyacrylamide–7 M urea gels. Equal counts per minute of all samples were loaded in each lane. After electrophoresis, the gels were rinsed with a solution of 20% methanol–10% acetic acid, dried, and exposed to X-ray film as above. Different exposures were analyzed by densitometry with an LKB laser scanner.

Western immunoblot assays. Nuclear proteins were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels (29) and either stained with Coomassie brilliant blue or electrophoretically transferred onto nitrocellulose filters (53). Immunoreactive material was visualized with an Immuno-Blot Assay Kit by using goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase, as described by the manufacturer (Bio-Rad) and Landschulz et al. (31).

RESULTS

Liver-enriched proteins binding to the albumin enhancer. Previous studies showed that an 830-bp *Nhe*I-*Ava*II subfragment of the mouse albumin enhancer (44) stimulated transcription when transfected into differentiated H2.35 cells (57). We therefore used a DNase I footprinting assay to identify protein-binding sites on this fragment by using nuclear extracts from mouse liver, kidney, and spleen. We examined the protections on both strands of the enhancer DNA and present data for one strand (Fig. 1). These studies revealed 11 binding sites for liver nuclear proteins, designated eA to eK (summarized in Fig. 2). Sites eA, eB, eD, and eK bound proteins that were present at similar levels in all extracts. Footprints eC and eE were detectable with liver and spleen extracts, but not with kidney extract, and footprint eF formed with liver (also see Fig. 8A) and kidney extracts, but not with spleen extract. Footprint eG was reproducibly observed only in liver extract and was accompanied by a characteristic DNase I-hypersensitive cleavage (asterisk). Spleen and kidney extracts each gave rise to a

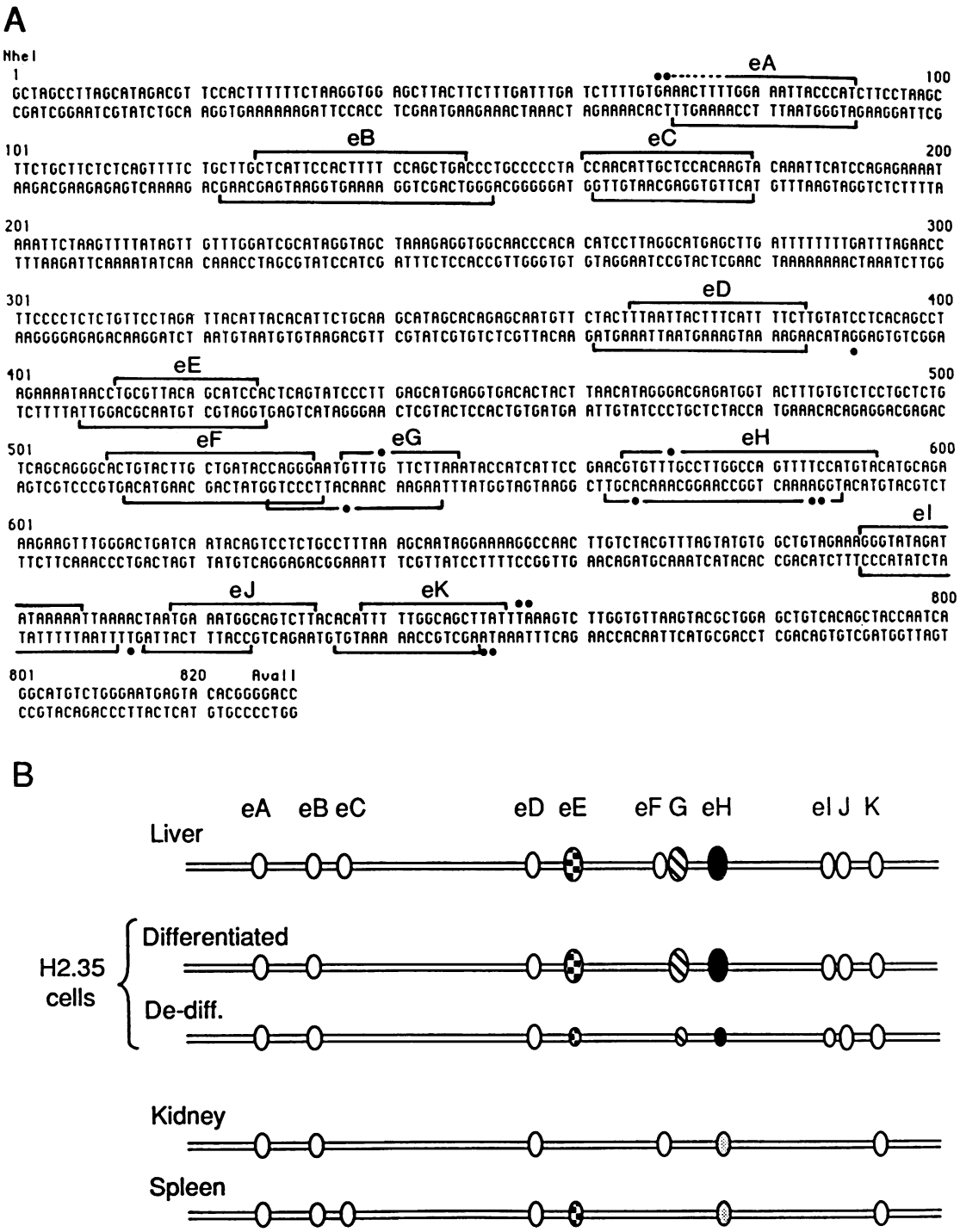

FIG. 2. Summary of DNase I footprinting analysis. (A) The footprinted DNA sequences with liver nuclear extract are indicated by brackets on the sequence from *Nhe*I (enhancer position 1) to *Ava*II (position 830). The *Nhe*I site is about 11.4 kb upstream of the transcription start site. Black dots indicate DNase I-hypersensitive cleavages induced by protein binding. (B) Protein-binding sites on the albumin enhancer with nuclear extracts from mouse tissues, differentiated, and dedifferentiated H2.35 cells. Ovals indicate sites of protein binding; shaded ovals indicate binding activities investigated in detail. The shaded ovals in the eH region from kidney and spleen indicate different DNA sequences protected compared with the liver eH footprint. The larger oval sizes for eE, eG, eH, and eI footprints in differentiated H2.35 cells indicate that the respective binding activities exist in greater amounts than in dedifferentiated H2.35 cells.

footprint that overlapped but did not encompass the liver eH footprint (Fig. 1D) and that lacked the hypersensitive cleavage near the edge of the eH footprint.

Herbst et al. (23) previously showed that the liver-enriched C/EBP protein (28) binds to the eE site (but see below). We have shown that a protein designated eH-TF binds to the TGTTTGC sequence within the eH site (57); this sequence occurs at regulatory protein-binding sites of the albumin promoter and other liver-specific genes (20, 49). In summary, we found a complex array of factors binding to the albumin enhancer; some are specific to liver (eG, eH, eI, and eJ) while others are present in liver and a subset of other tissues (eC, eE, and eF).

Induction of liver-enriched factors in differentiated H2.35 cells. Footprinting assays were performed with nuclear extracts from H2.35 cells cultured under differentiating and dedifferentiating conditions. As shown in Fig. 1 and summarized in Fig. 2B, H2.35 cells contained all of the footprinting activities seen in liver except eC; eF was detectable only at high protein concentrations in some preparations of differentiated cell extracts. The eD binding activity appeared similar in both differentiated and dedifferentiated H2.35 cells (Fig. 1C, lanes 5 to 7). In contrast, eE binding activity was readily detectable in two independent preparations of differentiated H2.35 cell nuclear extracts (Fig. 1C, lanes 5 and 7), but not in dedifferentiated H2.35 cell nuclear extracts (lane 6). Subsequent analysis showed the eE binding activity to be present at low levels in dedifferentiated H2.35 cell extracts (see Fig. 8, below). Binding activities specific to sites eG and eH were also induced significantly in differentiated H2.35 cells, compared with dedifferentiated cells (Fig. 1D, compare lanes 8 and 9 with lanes 10 and 11). The eG and eH footprints from H2.35 extracts were identical to those from liver, exhibiting the same patterns of DNase I protections and hypersensitive cleavages (Fig. 1D, asterisks) and were distinct from the cleavage patterns seen with extracts from other tissues that footprinted overlapping regions. Thus, binding activities specific to eE, eG, and eH were liver enriched and were inducible in H2.35 cells by culture conditions that maintain hepatocyte differentiation.

The eE, eG, and eH binding activities are essential for full enhancer function. To determine the relevance of the various binding sites, we tested deletions and clustered point mutations of the 830-bp *Nhe*I-*Ava*II enhancer fragment. The enhancer fragments were placed upstream of the albumin promoter in the vector pAT2, containing the thymidine kinase protein-coding sequence as a reporter (56), and were transfected into H2.35 cells cultured under differentiating conditions. An internal control plasmid containing the Rous sarcoma virus promoter fused to the thymidine kinase sequence (56) was cotransfected. Two days later, total RNA was isolated and transcripts that initiated from the different start sites of the promoters were quantitated simultaneously by primer extension. As shown previously (57), deletion of all sites that flank the central H2.35 cell footprints eD, eE, eG, and eH reduced enhancer activity only slightly (Fig. 3C, construct 5). This central 333-bp enhancer fragment, like the 830-bp *Nhe*I-*Ava*II fragment, was active only in differentiated H2.35 cells; deletion of the flanking sites did not activate the enhancer in dedifferentiated cells (Fig. 3A, lanes Dediff.).

Given that the eE, eG, and eH footprinting activities were induced in differentiated H2.35 cells, we next asked whether the sites were essential for enhancer activation. We used a new mutagenesis strategy involving the polymerase chain reaction to introduce 10-bp substitutions within each footprint site (57). A DNase I footprint titration assay demonstrated that each mutation destroyed its respective DNA-binding activity (Fig. 3B) (57). The effects of the mutations on enhancer function were tested in the context of both the 830-bp *Nhe*I-*Ava*II enhancer fragment and the 333-bp enhancer fragment; the results are summarized in Fig. 3C. Mutations of either the eG or eH sites destroyed the function of both the 830-bp and the 333-bp enhancer, when transfected into differentiated H2.35 cells (Fig. 3A, Differentiated lanes 3, 4, 7, and 8). The eE-site mutation had no effect on the 830-bp enhancer fragment, but it reduced the activity of the 333-bp fragment to about 25% of wild-type levels (Fig. 3A, Differentiated lanes 2 and 6). The lack of effect of the eE mutation on the 830-bp fragment indicated that binding sites excluded from the 333-bp fragment could compensate. None of the three mutations activated the enhancer in dedifferentiated H2.35 cells (data not shown), consistent with a positive regulatory role for the binding factors. We refer to these transcription factors as eE-TF, eG-TF, and eH-TF, and we next focus on their relationships to known regulatory proteins.

eG-TF appears identical to the hepatic transcription factor HNF3. We used oligonucleotide-binding sites for liver transcription factors as competitors in electromobility shift assays with an eG-site probe. Two binding complexes were seen after incubation of the eG probe with liver nuclear extracts; formation of both of these complexes were inhibited effectively by an excess of unlabeled eG-site oligonucleotide (Fig. 4, lanes 9 to 11). No competition was observed with binding sites for the liver-enriched factors C/EBP (28), HNF4 (12), NF-1, or eH-TF (57) (Fig. 4) (data not shown), but an HNF1 site (14) competed weakly (data not shown). Although the eG footprinted sequence exhibits some similarity to the HNF1 binding consensus (Fig. 5C) (13), 1 μl of purified HNF1 protein (a gift of D. Mendel and G. Crabtree) only weakly footprinted the eG site (Fig. 5A), whereas in a parallel reaction the same amount of HNF1 protein gave a strong footprint at the pB site (5, 34) of the albumin promoter (data not shown). Also, the migration of gel shift complexes formed with the eG probe was completely different from that of complexes formed with the pB probe (13) (data not shown); thus, eG-TF does not appear to be HNF1, although the proteins share DNA sequence recognition properties.

Experiments with an oligonucleotide recognized by the liver-enriched transcription factor HNF3, which is essential for the hepatic expression of the transthyretin and α_1-antitrypsin genes (12), suggested that the eG binding activity is HNF3. An HNF3 oligonucleotide from the transthyretin gene competed even better than the homologous eG oligonucleotide for binding of the eG factor in liver extracts (Fig. 4, lanes 13 and 14), and a labeled HNF3-site probe gave rise to the same gel shift pattern as did the eG probe (Fig. 4, lane 1). Both HNF3-site binding complexes were inhibited strongly by the homologous HNF3 oligonucleotide, whereas the eG oligonucleotide showed cross-competition with the lower complex strongly and the upper complex only moderately (lanes 2, 3, 5, and 6); no cross-competition was observed with the pD and HNF4 oligonucleotides (lanes 4, 7, and 8). The sequence of the eG site is highly A+T rich and shows some similarity to a transthyretin HNF3 binding site (Fig. 5C). We conclude that the predominant eG binding activity in liver is eG-TF/HNF3.

Methylation interference analysis with the eG probe showed that the formation of both eG-TF/HNF3 complexes from liver extracts could be blocked to similar extents by methylation of G residues at positions 536 and 540 (Fig. 5B,

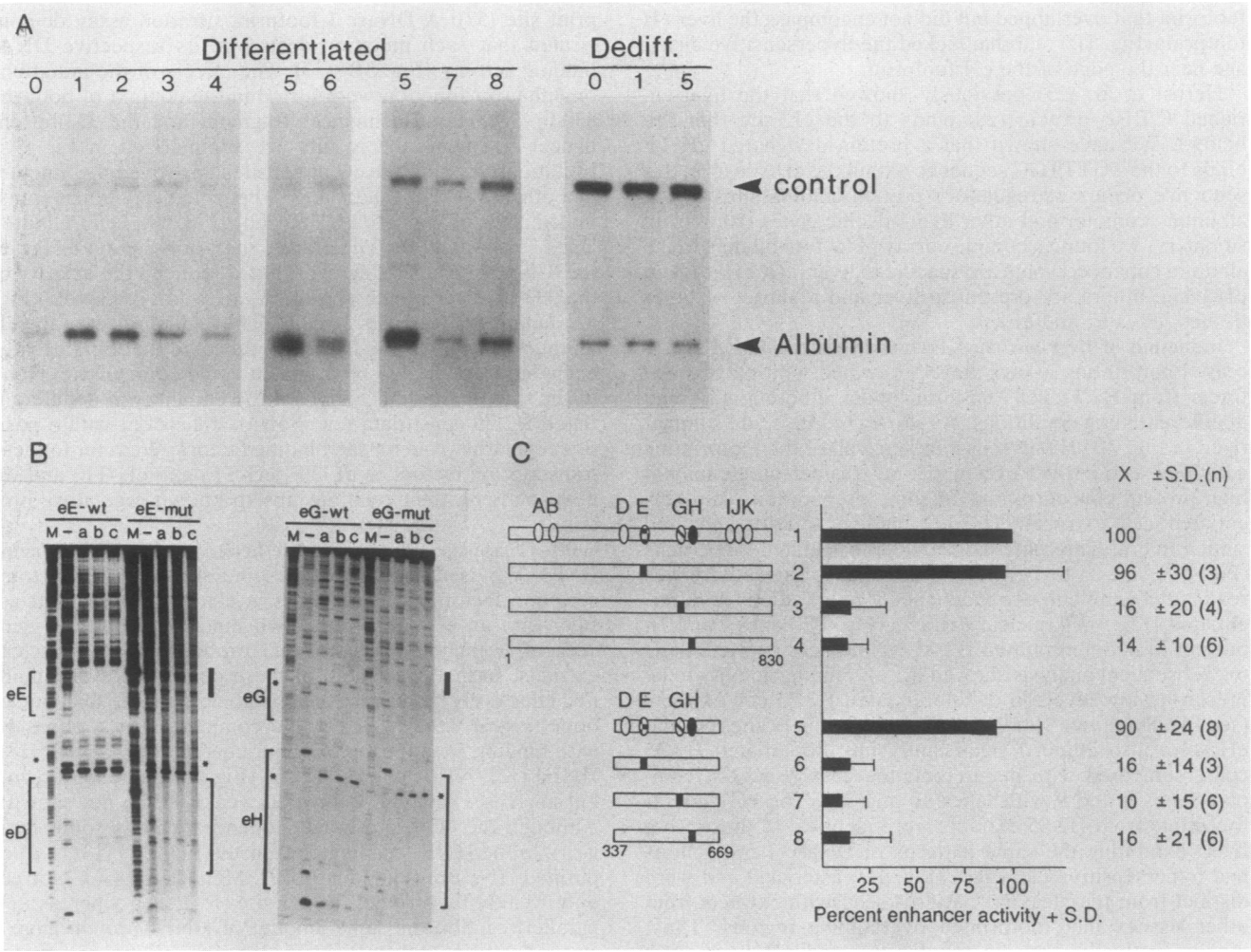

FIG. 3. Binding activities at eE, eG, and eH are necessary for full activity of the albumin enhancer. (A) Primer extension of RNAs from differentiated or dedifferentiated H2.35 cells transfected with the albumin promoter construct pAT2 (the promoter sequence is from −787 to +8 bp with respect to the transcription start) (lanes 0) or with pAT2-derived test plasmids containing wild-type or mutant enhancer segments fused to the albumin promoter (lanes 1 to 8); lane numbers correspond to the enhancer segments shown and numbered identically in panel C. The arrowhead marked Albumin shows the position of primer extension products from pAT2-derived plasmids; the arrowhead marked Control shows the extension products from the internal control plasmid, pRT1. (B) Footprinting of the wild-type (eE-wt, eG-wt) and mutant (eE-mut, eG-mut) eE and eG sites with differentiated H2.35 nuclear extracts. For eE-wt and eE-mut, lanes a, b, and c contain 20, 40, and 60 μg of nuclear extract, respectively. For eG-wt and eG-mut, lanes a, b, and c contain 15, 30, and 60 μg of nuclear extract, respectively. Black bars indicate the positions of the eE or eG site mutations, which are evident from the purine cleavage ladder. Other symbols are as in Fig. 1. The 10-bp mutation of the eH site was previously shown to prevent binding of eH-TF (57). (C) Summary of effects of eE, eG, and eH mutations on the albumin enhancer in differentiated H2.35 cells, assayed as in panel A. (Left) Open bars represent the 830-bp (segments 1 to 4) or 333-bp (segments 5 to 8) albumin enhancer segments. Ovals on segments 1 and 5 indicate binding activities detected in differentiated H2.35 cells (see Fig. 2B); black boxes (segments 2 to 4 and 6 to 8) indicate positions of mutated sequences. (Middle) Black bars indicate the enhancer activity of each derivative expressed as a percentage of the activity of the wild-type 830-bp segment, plus standard deviation (S.D.). (Right) Average percent enhancer activity (X) ± S.D.; n is the number of experiments performed.

lanes 2 and 3); thus, both complexes recognize similar features in the DNA. The more slowly migrating eG complex predominated in differentiated H2.35 cells (see Fig. 8, below) and exhibited a methylation interference pattern that was identical to that for the factor from liver nuclear extracts (Fig. 5B, lane 4). We conclude that the inducible eG binding activity in H2.35 cells is eG-TF/HNF3.

eE-TF is a distinct liver-enriched transcription factor with a DNA-binding specificity similar to C/EBP. In a footprinting assay, the eE-site binding activity was readily inhibited by an oligonucleotide of the pD site of the albumin promoter (6, 34), which binds the proteins C/EBP (28) and DBP (41); we then found that purified C/EBP protein could efficiently footprint the eE site (see Fig. 7B) (data not shown). Previous studies indicated that strong expression of C/EBP is limited to fully differentiated hepatocytes or adipocytes (2). To find whether C/EBP was the binding activity specific for the eE site in differentiated H2.35 cells, purified C/EBP protein and crude nuclear protein preparations from kidney, liver, and H2.35 cells were analyzed by Western blot analysis with two polyclonal antisera against different epitopes of C/EBP (a gift of B. Landschulz and S. McKnight [31]). As expected, specific signals from the 42-kDa C/EBP protein (Fig. 6B, lanes 5 and 6) and a 27-kDa degraded product (28, 31) were

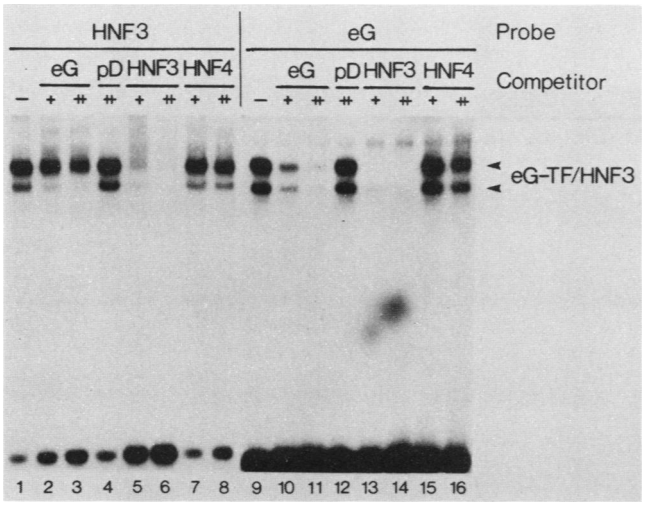

FIG. 4. eG-TF appears identical to HNF3. Radioactive oligonucleotide probes of a transthyretin promoter HNF3 binding site (lanes 1 to 8 [12]) or the eG site (lanes 9 to 16) were incubated with liver nuclear extract. Nonradioactive competitors comprising different binding sites of liver-specific factors were added: +, 30-fold molar excess of competitor; ++, 100-fold molar excess. pD, C/EBP site of the albumin promoter. The binding reactions were separated by electrophoresis in a native 8% polyacrylamide gel, and the gel was dried and exposed to X-ray film. Arrowheads indicate the specific binding complexes.

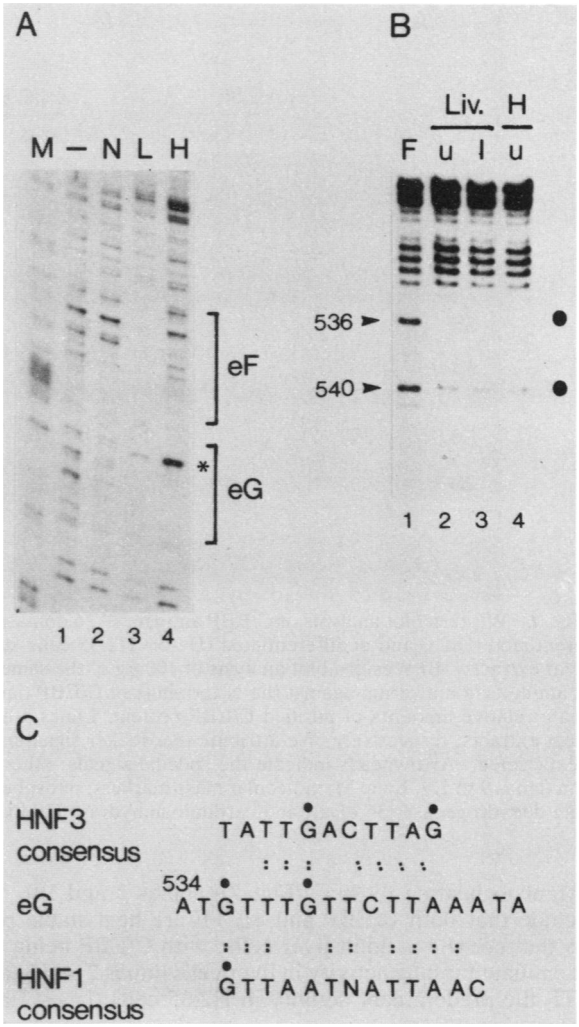

FIG. 5. HNF1 and eG-TF/HNF3 binding to the eG site. (A) Purified HNF1 binds weakly to the eG site. Purified HNF1 (N), liver (L), and differentiated H2.35 (H) nuclear extracts (40 μg) were incubated with a *Dde*I-*Sau*3A (428 to 616) enhancer subfragment that was labeled on the sense strand; they were then treated with DNase I. The digest products were analyzed on a sequencing gel and autoradiographed. Other lanes: M, purine cleavage ladder; –, no protein added. Brackets indicate the positions of footprints. (B) Methylation interference footprint of the sense strand of the eG-site oligonucleotide. Partially methylated probes were incubated with liver (lanes 2 and 3) and differentiated H2.35 nuclear extracts (lane 4) and applied to a polyacrylamide gel. Specific binding complexes were excised from the gel, cleaved at methylated Gs, applied to a sequencing gel, and autoradiographed. Lanes: F, free probe from the preparative gel; u, upper gel shift complex seen in Fig. 4; l, lower gel shift complex. Closed circles indicate Gs that interfered with formation of complexes when methylated. (C) Summary of methylation interference footprint on the eG DNA sequence. Also shown is the dimethyl sulfate interference pattern on an HNF3 footprint from the transthyretin promoter, considered the HNF3 consensus (12). The solid circle on the HNF1 consensus is a G that is highly conserved (13) and whose methylation interferes with HNF1 binding (5).

detected in the liver nuclear extract (Fig. 6B, lane 7, arrowheads), but not in the kidney extract (lane 8). Surprisingly, no specific signals were detected in nuclear extracts from either differentiated (lane 4) or dedifferentiated (lane 3) H2.35 cells, although the eE footprinting activities in liver and differentiated H2.35 cell extracts were of comparable abundance (Fig. 1C, lanes 4, 5, and 7). We also probed H2.35 cell nuclear extracts that had been incubated with purified C/EBP protein at 23°C for 30 min before the gel was loaded. The specific signals of C/EBP protein were readily detectable in the mixtures (Fig. 6B, lanes 1 and 2), indicating that the absence of C/EBP protein in H2.35 nuclear extracts was not due to selective degradation. From these and other Western blot studies with ^{125}I-protein A (data not shown), we estimate that the amount of C/EBP is at least 15-fold lower in H2.35 cells than in liver cells. The differentiated hepatoma cell line, HepG2, also has very low levels of C/EBP (18). We conclude that the inducible eE binding activity in H2.35 cells has a sequence specificity similar to that of C/EBP, but is a distinct protein.

To ask whether eE-TF and C/EBP coexist in liver, we performed electromobility shift assays with an eE-site oligonucleotide. Liver nuclear extract gave rise to a slowly migrating complex (eE-TF) and a cluster of more abundant, faster-migrating complexes (Fig. 7A, lane 3) which could be resolved into discrete bands on lighter exposures. The faster-migrating cluster comigrated with the position of a complex with purified C/EBP protein (Fig. 7A, compare lanes 1 and 3). Differentiated H2.35 nuclear extracts revealed primarily the slowly migrating cluster and some faster-migrating bands (Fig. 7A, lane 7). All of the signals could be specifically inhibited by the homologous eE oligonucleotide and not an HNF1 site (Fig. 7A, lanes 5, 6, and 9). We designate the more slowly migrating band as eE-TF because it was the predominant and consistent binding activity in differentiated H2.35 cells (Fig. 7A, lane 7; Fig. 8, eE-TF panel); the faster-migrating bands from H2.35 extracts could be due to partial degradation of eE-TF or to different proteins. The DNA-binding activities of C/EBP from liver and of eE-TF from both liver and H2.35 cells were

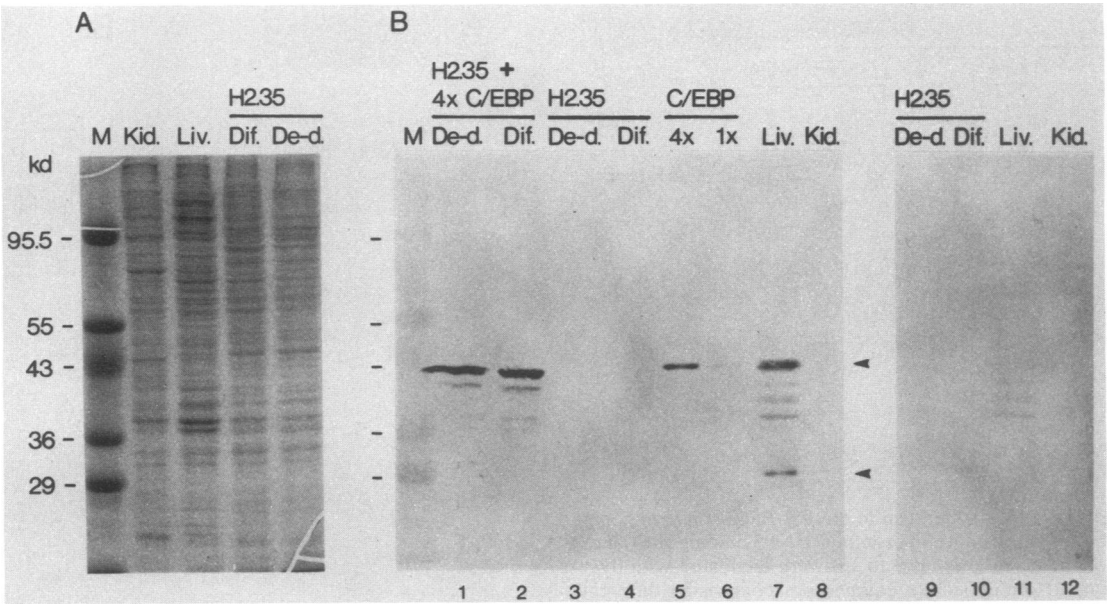

FIG. 6. Western blot analysis of C/EBP antigen. (A) Coomassie blue-staining patterns of nuclear proteins from kidney (Kid.), liver (Liv.), differentiated (Dif.), and dedifferentiated (De-d.) H2.35 cells separated on an SDS-polyacrylamide gel, demonstrating the integrity of the nuclear extracts. (B) Western blot analysis of 100 μg of the same nuclear proteins as in panel A and recombinant C/EBP protein probed with α-14 amino acid antiserum against the N terminus of C/EBP (lanes 1 to 8) (31) or with nonimmune rabbit serum (lanes 9 to 12). 1× and 4× indicate relative amounts of purified C/EBP protein. Lanes 1 and 2 are mixtures of C/EBP with dedifferentiated and differentiated H2.35 nuclear extracts, respectively. We attribute the weaker signal in lane 5, compared with lanes 1 and 2, to nonspecific loss of dilute C/EBP in the experiment. Arrowheads indicate the specific signals; other bands are nonspecific signals that were detectable with nonimmune rabbit serum (lanes 9 to 12). Lane M, molecular mass markers: phosphorylase *b* (95.5 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa), and carbonic anhydrase (29 kDa).

resistant to heating to 90°C (Fig. 7A, lanes 2 and 10). We conclude that both C/EBP and eE-TF are heat-stable proteins that coexist in adult liver cells, with C/EBP being the predominant binding activity in liver cells (lanes 2 and 3) and eE-TF the predominant activity in H2.35 cells (lanes 7 and 10).

We next used methylation interference and binding competition assays to try to distinguish properties of eE-TF and C/EBP. Methylation of Gs at positions 413 and 415 on the top strand of the eE-site probe prevented the binding of purified C/EBP and of eE-TF from liver or H2.35 extracts (Fig. 7B, lanes 2 to 4), whereas methylation at position 421 enhanced the binding of purified C/EBP, but not of eE-TF. Methylation of the G residue at position 419 on the bottom strand interfered with binding by both factors, affecting eE-TF to a greater extent (lanes 7 to 9) (as confirmed by scanning densitometry). In a competition assay, the eE site competed about equally for binding of either eE-TF or C/EBP to the labeled eE probe (Fig. 7A, lanes 5 and 9), but the pD site from the albumin promoter was a weaker competitor of eE-TF binding than was the eE oligonucleotide (lanes 4, 5, 8, and 9). Similarly, binding of eE-TF to the pD probe was weaker than binding to an eE probe labeled to the same specific activity (compare lane 13 with lane 3 and lane 17 with lane 7), whereas C/EBP bound with similar affinity to eE and pD probes (Fig. 7B, compare lane 1 with lane 11 and lane 2 with lane 12). In conclusion, eE-TF and C/EBP recognize very similar features of the major groove of DNA, but their binding specificities are distinguishable.

eE-TF, eG-TF/HNF3, and eH-TF are regulated by different signal transduction pathways. The induction of eE-TF, eG-TF/HNF3, and eH-TF during H2.35 cell differentiation occurs under a complex set of culture conditions; namely, on a collagen gel substratum, in hormonally defined SFM, and at the restrictive temperature for simian virus 40 large T antigen (39°C). We next asked whether individual culture conditions could selectively alter the levels of the respective DNA-binding activities.

As a control, we first tested the binding activity of a tissue-ubiquitous factor that binds to the CCAAT sequence in the albumin promoter (6, 34). As shown in the three independent electromobility shift experiments in Fig. 8, this binding activity was at virtually identical levels in H2.35 cells under all culture conditions tested.

The gel shift patterns of eG-TF/HNF3 in H2.35 nuclear extracts are shown in Fig. 8; the top arrowhead indicates the predominant binding activity. The eG-site binding activity in H2.35 cells was induced about twofold relative to the CCAAT factor by simply changing the plastic substratum to a collagen gel matrix (Fig. 8, lanes 1 and 2; summarized in Fig. 9A) or induced threefold by shifting the temperature to 39°C (Fig. 8, lanes 1 and 4). eG-TF/HNF3 was not induced by SFM at 33°C (lane 3), nor did SFM further induce the factor at 39°C (lane 8). However, eG-TF/HNF3 was induced more by both a collagen gel matrix and 39°C than by either condition alone, with (lanes 7 and 9) or without (lanes 10 to 13) SFM. Thus, a collagen matrix and inactivation of large T antigen appeared to independently and additively increase eG-TF binding activity. Figure 9A summarizes this and other electromobility shift experiments in which binding activities were titrated (data not shown).

In contrast to the situation for eG-TF/HNF3, neither a collagen gel matrix (Fig. 8, lanes 2 and 11), the presence of SFM (lane 3), nor 39°C (lanes 4 and 12) alone was sufficient to induce eE-TF or eH-TF binding activities (the apparent induction of eH-TF in lane 4 was not reproducible in titration

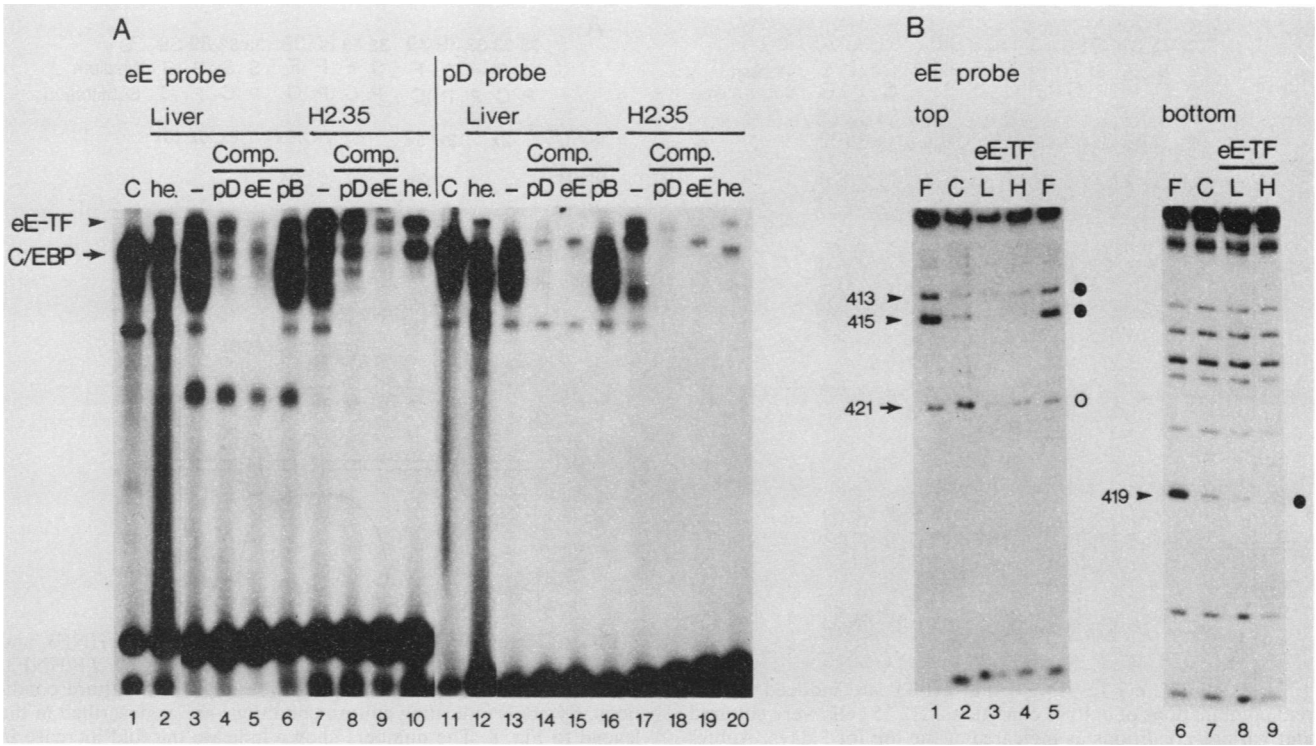

FIG. 7. Binding activities to the albumin enhancer eE and promoter pD sites. (A) Radioactive probes of the eE site (lanes 1 to 10), or the pD site (lanes 11 to 20) were incubated with purified C/EBP (C), with liver and differentiated H2.35 cell nuclear extracts, or with heated (he.) extracts as shown. A 100× molar excess of competitors (Comp.) was used as indicated. The pB oligonucleotide binds HNF1 (14). Binding reactions were separated by electrophoresis in a native 8% polyacrylamide gel and autoradiographed. Arrowhead, eE-TF complex; arrow, C/EBP complex. (B) A methylation interference footprinting was performed with the eE oligonucleotide labeled on the top strand (lanes 1 to 5) or the bottom strand (lanes 6 to 9). Partially methylated probes were incubated with purified C/EBP (C), liver (L), and differentiated H2.35 cell (H) nuclear extracts and then applied to a polyacrylamide gel. Specific C/EBP and eE-TF binding complexes and free probe were excised from the gel, cleaved at methylated Gs, resolved on a sequencing gel, and autoradiographed. Symbols: ●, methylation sites that interfered with the binding of eE-TF and C/EBP to the probe; ○, methylation site that enhanced the binding of C/EBP, but not of eE-TF. F, free probe isolated from the preparative gel. Numbers at the side indicate positions of the nucleotides (Fig. 2A).

experiments). The combinations of SFM plus 39°C (lane 8) and of a collagen gel matrix plus 39°C (lane 13) were also not sufficient for induction of eE-TF or eH-TF; both a collagen gel matrix and SFM (lanes 5, 7, and 9) were required for the induction of these binding activities. When H2.35 cells were cultured on a collagen gel matrix and in SFM, eE-TF and eH-TF were induced to similar extents at either 33 or 39°C (lanes 7 and 9), indicating that under such conditions induction of large T antigen had no effect. These results were obtained in the three independent experiments whose results are shown in Fig. 8 and in titration experiments not shown, and are summarized in Fig. 9A. We conclude that the binding activities of at least three liver-enriched transcription factors, but not of the ubiquitous CCAAT factor, were induced selectively by different combinations of culture conditions that together promote hepatocyte differentiation.

DISCUSSION

We demonstrated previously that the liver-specific albumin enhancer could be activated by shifting the H2.35 cell line to culture conditions that maintain hepatocyte differentiation (56). We now show that extracellular cues regulate at least two factors, eG-TF/HNF3 and eH-TF, that have a limited cell type distribution and that play key roles in determining the hepatocyte-specific transcription of a number of genes (12, 49, 57); the role of the inducible eE-TF in controlling other hepatic genes remains to be established. The increase in DNA-binding activities and transcriptional activation by these cell type-differentiating factors could reflect changes in either their abundance or function. In either case, we propose that their activities are maintained in part by the continuous presence of exogenous stimuli. Although it is likely that we have examined only a subset of the relevant transcription factors, our findings provide a framework for understanding part of the reason why hepatocytes cultured in vitro, where the natural cell environment is lost, dedifferentiate rapidly. We predict an analogous situation for the regulation of transcription factors in other cell types that selectively lose differentiated functions when they are removed from their natural tissue context (4, 33, 45). In fact, the interplay between intrinsic programming and response to the extracellular environment may be critical for virtually all differentiating cells (24). For example, the pituitary-specific transcription factor GHF1 has been shown to be regulated by cyclic AMP (37). Our results also suggest that viral transforming proteins may act in part by obstructing the signal transduction pathways which maintain the activity of tissue-specific transcription factors.

We found that eG-TF/HNF3 was induced under different culture conditions from those under which eH-TF and eE-TF were induced (summarized in Fig. 9A), suggesting

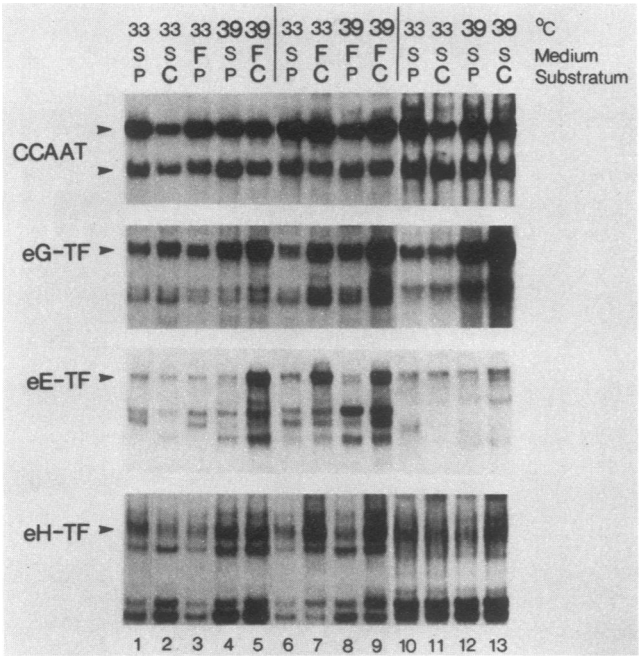

FIG. 8. eE-TF, eG-TF/HNF3, and eH-TF are induced by different combinations of culture conditions. H2.35 cells were cultured under various conditions as indicated at the top for 3 days. Abbreviations: S, Dulbecco modified Eagle medium plus 4% fetal calf serum and 10^{-7} M dexamethasone (56); F, hormonally defined SFM (17) plus 10^{-7} M dexamethasone; P, plastic substratum; C, collagen gel substratum. Nuclear extracts were prepared from cells and incubated with radioactive oligonucleotide probes of binding sites for the albumin CCAAT factor (from the albumin promoter), eG-TF, eE-TF, and eH-TF. The binding reaction was performed on ice for CCAAT factor and at room temperature for the others. The binding reactions were separated by electrophoresis in a 6% polyacrylamide gel in 0.5× TBE buffer for CCAAT factor and in a 8% polyacrylamide gel in 1× TBE buffer for the others. Arrowheads point to the complexes observed most consistently for each probe, although all bands shown can be inhibited specifically. The vertical lines separate groups of extracts prepared at different times.

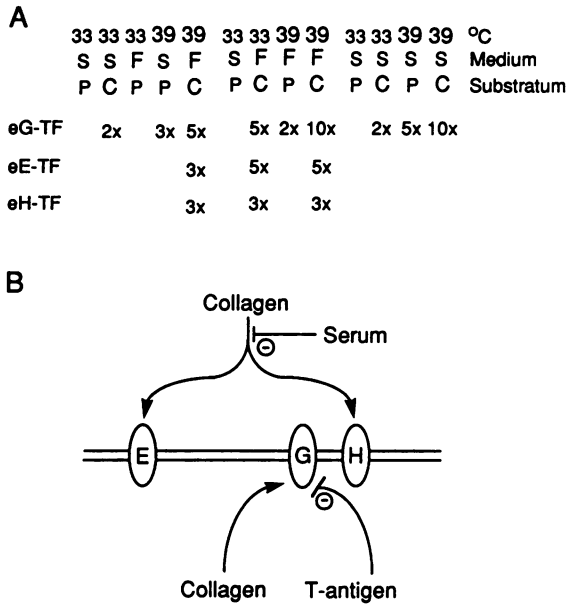

FIG. 9. Summary of the induction of eE-TF, eG-TF/HNF3, and eH-TF. (A) Quantitation of the induction of eE-TF, eG-TF/HNF3, and eH-TF binding activities under different H2.35 culture conditions. Symbols indicating culture conditions are as described in the legend to Fig. 8. The numbers shown indicate the fold increase in binding activity of each factor, with respect to the CCAAT factor, on the basis of densitometry scanning of different autoradiographic exposures of the films shown in Fig. 8. The quantitations take into account other experiments (not shown) in which specific binding activities were tested over different amounts of nuclear extract. (B) Diagramatic summary of the influence of a collagen substratum, serum, and large T antigen on the induction of eE-TF, eG-TF/HNF3, and eH-TF. − means that the effect is negative. The details of the model are discussed in the text.

that the factors are regulated by different signal transduction pathways; a working model for the regulatory network is shown in Fig. 9B. The DNA-binding activities of eE-TF, eG-TF/HNF3, and eH-TF were detectable in dedifferentiated H2.35 cells, perhaps reflecting basal levels of expression that occur in a cell lineage-specific fashion. The fact that each respective binding site was necessary for activity of the 333-bp enhancer in differentiated H2.35 cells indicates that the factors must work together; binding sites remaining on the mutated enhancer fragments were insufficient. Thus, although the increase in the individual DNA-binding activities seems modest (3- to 10-fold), their simultaneous induction could play a significant role in promoting hepatocyte differentiation.

The eE-TF and eH-TF DNA-binding activities increased only when the cells were cultured simultaneously in a hormonally defined SFM and on a collagen gel matrix. We favor the interpretation that the presence of serum is inhibitory to the transcription factors, because earlier studies showed that the addition of serum to hepatocytes cultured in SFM caused liver-specific gene transcription to decline (26). Since we have not yet performed a similar mixing experiment, we cannot exclude the possibility that the increase in abundance of eE-TF and eH-TF is due to the positive action of hormones and growth factors present in SFM.

The DNA-binding activities of eE-TF, eH-TF, and eG-TF/HNF3 were regulated by cell contact with the extracellular matrix. Although the extracellular matrix has been shown to promote the differentiation of a variety of cell types (reviewed in references 3, 38, and 55), the underlying mechanisms were unknown. The fact that eG-TF/HNF3 was modestly induced by culturing H2.35 cells on a collagen gel alone, whereas eE-TF and eH-TF required both collagen and SFM for induction, suggests that there may be multiple mechanisms for matrix responsiveness. The identification of albumin enhancer-binding factors as targets for matrix-promoted differentiation should help elucidate the relevant signal transduction pathways.

Other hepatocyte-derived cell lines created with the simian virus 40 *tsA*255 mutant (reviewed in reference 8), like H2.35 cells, are temperature sensitive for liver-specific functions. The *tsa*255 defect can be rescued (30) by a region of the large T-antigen gene necessary for ATPase activity, viral DNA replication, and cellular transformation (9, 50). We found that the level of eG-TF/HNF3, as assessed by its DNA-binding activity in nuclear extracts, was reduced by large T antigen in H2.35 cells cultured at the permissive temperature. Other examples in which T antigen affects the activity of transcription factors are known: an inhibitory complex forms between T antigen and transcription factor

AP2 (40); the expression of factor Sp1 is stimulated (47); and HiNF-D is aberrantly expressed (25). Whether T antigen affects albumin transcription factors by the same direct mechanism as established for AP2, or indirectly via association with antioncogenic proteins such as p53 (54) or the *rb* gene product (15) remains to be established. Given that HNF3 activates a number of liver-specific genes (12) and is sensitive to large T antigen, our findings may explain in part how oncogenic transformation causes hepatocyte dedifferentiation.

The transcription factor eE-TF has a DNA-binding specificity very similar to but distinguishable from that of C/EBP. Two other transcription factors have recently been described with binding specificities similar to C/EBP; DBP, which is expressed later in liver development than C/EBP and which activates the albumin promoter (41); and NF-IL6, whose mRNA is highly expressed in many adult mouse tissues during the acute-phase response and which regulates the interleukin-6 gene (1). Both of these proteins possess some structural similarity to C/EBP; their relation to eE-TF is under investigation. Although both eE-TF and C/EBP were detectable in adult liver, only eE-TF was detectable in H2.35 cells, implying that the factors are regulated differently. If two different factors that bind the same site interact differently with other regulatory proteins (see, e.g., reference 51), dramatically different transcriptional responses could occur depending upon which binding activity is dominant at the moment.

Extracellular signals not only may be critical for maintaining the activity of liver-enriched transcription factors, but they also may be required for their initial expression. The transcription of many hepatic genes is activated at different developmental stages (43), and all liver-specific genes that have been studied are regulated by multiple liver-specific transcription factors (reviewed in reference 27). Specific combinations of factors that respond to disparate extracellular signals could give rise to the complex timing of transcriptional activation seen in hepatic development. The discovery of homeotic gene products in *Drosophila melanogaster* and *Caenorhabditis elegans* that resemble growth factors with transmembrane domains (21), growth factor receptors (22), and extracellular matrix proteins (42) and that respond to cell-cell interactions, demonstrates that cell differentiation can be highly dependent upon exogenous signals. It is therefore possible that some of the signals that maintain the activities of the transcription factors we have studied are also important for activating the factors during mouse development.

ACKNOWLEDGMENTS

We thank Bill Landschulz and Steve McKnight for providing purified C/EBP protein and antibodies; Dirk Mendel and Gerald Crabtree for purified HNF1 protein; Rob Costa for the HNF3 and HNF4 oligonucleotides; and John Burch, David Jackson, and Patrice Milos for valuable comments on the manuscript.

The research was supported by grants to K.S.Z from the National Institutes of Health (GM36477) and the Searle Scholars Program/ Chicago Community Trust.

REFERENCES

1. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9**:1897–1906.
2. **Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight.** 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev. **3**:1146–1156.
3. **Bissell, M. J., H. G. Hall, and G. Parry.** 1982. How does the extracellular matrix direct gene expression? J. Theor. Biol. **99**:31–68.
4. **Carey, D. J., M. S. Todd, and C. M. Rafferty.** 1985. Schwann cell myelination: induction by exogenous basement membrane-like extracellular matrix. J. Cell Biol. **102**:2254–2263.

4a. **Caron, J. M.** 1990. Induction of albumin gene transcription in hepatocytes by extracellular matrix proteins. Mol. Cell. Biol. **10**:1239–1243.

5. **Cereghini, S., M. Blumenfeld, and M. Yaniv.** 1988. A liver-specific factor essential for albumin transcription differs between differentiated and dedifferentiated rat hepatoma cells. Genes Dev. **2**:957–974.
6. **Cereghini, S., M. Raymondjean, A. G. Carranca, P. Herbomel, and M. Yaniv.** 1987. Factors involved in control of tissue-specific expression of albumin gene. Cell **50**:627–638.
7. **Chou, J. Y.** 1983. Temperature-sensitive adult liver cell line dependent on glucocorticoid for differentiation. Mol. Cell. Biol. **3**:1013–1020.
8. **Chou, J. Y.** 1989. Differentiated mammalian cell lines immortalized by temperature-sensitive tumor viruses. Mol. Endocrinol. **3**:1511–1514.
9. **Clark, R., K. Peden, J. M. Pipas, D. Nathans, and R. Tjian.** 1983. Biochemical activities of T-antigen proteins encoded by simian virus 40 A gene deletion mutants. Mol. Cell. Biol. **3**:220–228.
10. **Clayton, D. F., and J. E. Darnell, Jr.** 1983. Changes in liver-specific compared to common gene transcription during primary culture of mouse hepatocytes. Mol. Cell. Biol. **3**:1552–1561.
11. **Clayton, D. F., A. L. Harrelson, and J. E. Darnell, Jr.** 1985. Dependence of liver-specific transcription on tissue organization. Mol. Cell. Biol. **5**:2623–2632.
12. **Costa, R. H., D. R. Grayson, and J. E. Darnell, Jr.** 1989. Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and α1-antitrypsin genes. Mol. Cell. Biol. **9**:1415–1425.
13. **Courtois, G., S. Baumhueter, and G. R. Crabtree.** 1988. Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. Proc. Natl. Acad. Sci. USA **85**:7937–7941.
14. **Courtois, G., J. G. Morgan, L. A. Campbell, G. Fourel, and G. R. Crabtree.** 1987. Interaction of a liver-specific nuclear factor with the fibrinogen and α1-antitrypsin promoter. Science **238**:688–692.
15. **DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston.** 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell **54**:275–283.
16. **Dignam, J. D., R. M. Lebowitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
17. **Enat, R., D. M. Jefferson, N. Ruiz-Opazo, Z. Gatmaitan, L. A. Leinwand, and L. M. Rei.** 1984. Hepatocyte proliferation *in vitro*: its dependence on the use of serum-free hormonally defined medium and substrata of extracellular matrix. Proc. Natl. Acad. Sci. USA **81**:1411–1415.
18. **Friedman, A. D., W. H. Landschulz, and S. L. McKnight.** 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev. **3**:1314–1322.
19. **Fritton, H. P., A. E. Sippel, and T. Igo-Kemenes.** 1980. Nuclease-hypersensitive sites in the chromatin domain of the chicken lysozyme gene. Nucleic Acids Res. **11**:3467–3485.
20. **Godbout, R., R. S. Ingram, and S. M. Tilghman.** 1988. Fine-structure mapping of the three α-fetoprotein gene enhancers. Mol. Cell. Biol. **8**:1169–1178.
21. **Greenwald, I.** 1985. *lin-12*, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. Cell **43**:583–590.

22. **Hafen, E., K. Basler, J.-E. Edstroem, and G. M. Rubin.** 1987. *Sevenless*, a cell-specific homeotic gene of Drosophila, encodes a putative transmembrane receptor with a tyrosine kinase domain. Science **236**:55–63.
23. **Herbst, R. S., N. Friedman, J. E. Darnell, Jr., and L. E. Babiss.** 1989. Positive and negative regulatory elements in the mouse albumin enhancer. Proc. Natl. Acad. Sci. USA **86**:1553–1557.
24. **Herskowitz, I.** 1989. A regulatory hierarchy for cell specification in yeast. Nature (London) **342**:749–757.
25. **Holthuis, J., T. A. Owen, A. J. van Wijnen, K. L. Wright, A. Ramsey-Ewing, M. B. Kennedy, R. Carter, S. C. Cosenza, K. J. Soprano, J. B. Lian, J. L. Stein, and G. S. Stein.** 1990. Tumor cells exhibit deregulation of the cell cycle histone gene promoter factor HiNF-D. Science **247**:1454–1457.
26. **Jefferson, D. M., D. F. Clayton, J. E. Darnell, Jr., and L. M. Reid.** 1984. Posttranscriptional modulation of gene expression in cultured rat hepatocytes. Mol. Cell. Biol. **4**:1929–1934.
27. **Johnson, P. F.** 1990. Transcriptional activators in hepatocytes. Cell Growth Differ. **1**:47–52.
28. **Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight.** 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1**:133–146.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Lai, C.-J., and D. Nathans.** 1975. A map of temperature-sensitive mutants of simian virus 40. Virology **66**:70–81.
31. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2**:786–800.
32. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. Cell **58**:823–831.
33. **Li, M. L., J. Aggeler, D. A. Farson, C. Hatier, J. Hassel, and M. J. Bissel.** 1987. Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells. Proc. Natl. Acad. Sci. USA **84**:136–140.
34. **Lichtsteiner, S., J. Wuarin, and U. Schibler.** 1987. The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. Cell **51**:963–967.
35. **Liu, J.-K., Y. Bergman, and K. S. Zaret.** 1988. The mouse albumin promoter and a distal upstream site are simultaneously DNAaseI hypersensitive in liver chromatin and bind similar liver-abundant factors in vitro. Genes Dev. **2**:528–541.
36. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**: 1237–1245.
37. **McCormick, A., H. Brady, L. E. Theill, and M. Karin.** 1990. Regulation of the pituitary-specific homeobox gene GHF1 by cell-autonomous and environmental cues. Nature (London) **345**:829–832.
38. **McDonald, J. A.** 1989. Matrix regulation of cell shape and gene expression. Curr. Opin. Cell Biol. **1**:995–999.
39. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
40. **Mitchell, P. J., C. Wang, and R. Tjian.** 1987. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**:847–861.
41. **Mueller, C. R., P. Maire, and U. Schibler.** 1990. DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. Cell **61**:279–291.
42. **Norton, P. A., R. O. Hynes, and D. J. G. Rees.** 1990. *Sevenless*: seven found? Cell **61**:15–16.
43. **Panduro, A., F. Shalaby, and D. A. Shafritz.** 1987. Changing patterns of transcriptional and post-transcriptional control of liver-specific gene expression during rat development. Genes Dev. **1**:1172–1182.
44. **Pinkert, C. A., D. M. Ornitz, R. L. Brinster, and R. D. Palmiter.** 1987. An albumin enhancer located 10 kb upstream functions along with promoter to direct efficient, liver-specific expression in transgenic mice. Genes Dev. **1**:268–276.
45. **Reid, L. M., and D. M. Jefferson.** 1984. Culturing hepatocytes and other differentiated cells. Hepatology **4**:548–559.
46. **Reid, L. M., M. Narita, M. Fujita, Z. Murray, C. Liverpool, and L. Rosenberg.** 1986. Matrix and hormonal regulation of differentiation in liver cultures, p. 225–248. *In* A. Guillouzo and C. Gugen-Guillouzo (ed.), Research in isolated and cultured hepatocytes. John Libbery Euratext Ltd./INSERM.
47. **Saffer, J. D., S. P. Jackson, and S. J. Thurston.** 1990. SV40 stimulates expression of the *trans*-acting factor Sp1 at the mRNA level. Genes Dev. **4**:659–666.
48. **Serfling, E.** 1989. Autoregulation—a common property of eukaryotic transcription factors? Trends Genet. **5**:131–133.
49. **Shaul, Y., and R. Ben-Levy.** 1987. Multiple nuclear proteins in liver cells are bound to hepatitis B virus enhancer element and its upstream sequences. EMBO J. **6**:1913–1920.
50. **Soprano, K. J., N. Galanti, G. J. Jonak, S. McKercher, J. M. Pipas, K. W. C. Peden, and R. Baserga.** 1983. Mutational analysis of simian virus 40 T antigen: stimulation of cellular DNA synthesis and activation of rRNA genes by mutants with deletions in the T-antigen gene. Mol. Cell. Biol. **3**:214–219.
51. **Stern, S., M. Tanaka, and W. Herr.** 1989. The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature (London) **341**:624–630.
52. **Thayer, M. J., S. J. Tapscott, R. L. Davis, W. E. Wright, A. B. Lassar, and H. Weintraub.** 1989. Positive autoregulation of the myogenic determination gene MyoD1. Cell **58**:241–248.
53. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
54. **Wang, E. H., P. N. Friedman, and C. Prives.** 1989. The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T antigen. Cell **57**:379–392.
55. **Watt, F. M.** 1986. The extracellular matrix and cell shape. Trends Biochem. Sci. **11**:482–485.
56. **Zaret, K. S., C. M. DiPersio, D. A. Jackson, W. J. Montigny, and D. L. Weinstat.** 1988. Conditional enhancement of liver-specific gene transcription. Proc. Natl. Acad. Sci. USA **85**: 9076–9080.
57. **Zaret, K. S., J.-K. Liu, and C. M. DiPersio.** 1990. Site-directed mutagenesis reveals a liver transcription factor essential for the albumin transcriptional enhancer. Proc. Natl. Acad. Sci. USA **87**:5469–5473.