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Regulation of Hepatitis B Virus Expression in Progenitor and Differentiated Cell Types: Evidence for Negative Transcriptional Control in Nonpermissive Cells

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Mechanisms regulating cell type-specific gene expression are not completely understood. We utilized hepatitis B virus (HBV) enhancer I and preS1 promoter sequences, which exhibit cell type specificity, to analyze transcriptional control in pluripotential murine embryonic stem (ES) cells, bipotential HBC-3 progenitor liver cells, mature hepatocytes, and fibroblasts. In transient transfection assays, HBV sequences were most active in primary hepatocytes, followed by HBC-3 and ES cells, and became inactive in fibroblasts. Cotransfections with HNF-3 or C/EBP plasmids increased expression of HBV sequences in hepatocytes and HBC-3 cells. However, increased HBV expression was not observed in ES cells and HBV remained inactive in fibroblasts, suggesting different transcriptional controls, which was compatible with alterations in the abundance of endogenous transcription factors. Analysis in somatic hybrid cells created from NIH 3T3 fibroblasts and Hepa1-6 mouse hepatocytes with expression of albumin and selected hepatic transcription factors showed that HBV sequences were expressed weakly but without increased expression following transfection of HNF-1, HNF-3, and C/EBP plasmids. These findings indicated that repression of HBV in nonpermissive cells involved inactivation of transcription factor activity. Expression of HBV in stem cells is relevant for mechanisms concerning viral persistence and oncogenesis, as well as analysis of hepatocytic differentiation in progenitor cells.

Hepatitis B virus Transcription factor Gene expression Stem cell Liver

CELL differentiation is accompanied by regulated expression of specialized genes. During liver development, as well as during differentiation of progenitor liver cells, specific transcription factors are expressed in a hierachically coordinated fashion, which eventually confers unique gene expression patterns to mature hepatocytes (8,14,34). For instance, hepatocyte nuclear factor (HNF)-1α mRNA is first transcribed during liver development, with HNF-1α transcription being dependent upon HNF-4 as well as HNF-3 synthesis, whereas HNF-1β mRNA is detected earlier in primitive endoderm and mesoderm (9,30,46). Also, HNF-1α deficiency impairs the function of multiple organs, including the liver (37). In mature hepatocytes, HNF-3 activates transcription of a number of liver-specific genes and promotes cell differentiation in response to extracellular signals, although the transcription factor plays pleotropic roles in the development of endoderm-derived tissues and is clearly essential to life as shown in mutated mice (2,31). The CCAT/enhancer binding proteins (C/EBPα, β, and δ) belong to a later stage in the transcriptional heirarchy and first appear in the mouse on day 13 of gestation (29). C/EBPα is expressed abundantly in mature hepatocytes and is involved in maintaining cell differentiation states (39). Mice lacking C/EBPα

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die shortly after birth with impaired energy homeostasis and decreased glycogen and lipid storage in the liver (48). C/EBPβ also preferentially accumulates in the liver. Both C/EBPβ and δ are involved in the activation of the acute phase response and cytokine expression (1). A variety of additional less-well-studied transcription factors are also involved in regulating hepatic gene expression.

The cell type regulates transcription in both positive and dominant-negative fashions. While the nature of transcriptional upregulation has been under extensive study, gene repression mechanisms are less well understood (7,19), although chromatin-mediated gene repression is established as early as after the formation of a two-cell embryo (27). Cellular gene expression may be regulated at the level of nucleosome assembly, such as with histone deacetylation mechanisms (4). DNA methylation may be associated with the loss of transcriptional ability, although this mechanism has recently been proposed to apply most appropriately to the inactivation of undesirable foreign gene insertions (47).

In studies concerning cell type-specific regulation of gene expression, hepatitis B virus (HBV) sequences were restricted to epithelial cells in transgenic mice, which was independent of the chromosomal integration position of the transgenes (3). HBV contains four overlapping open reading frames, which transcribe the surface antigens (HBsAg, including preS1 and preS2 proteins), the core and e antigens, the x antigen, and the viral DNA polymerase (21). HBV expression is regulated by four promoters (for preS1, preS2, HBcAg, and HBxAg), two enhancers, of which enhancer I is better characterized, as well as a glucocorticoid-responsive element. Interestingly, both HBV enhancers contain domains capable of modulating cell type-specific HBV expression. For instance, HBV enhancer I contains binding sites for HNFs-1 and -4, as well as other transactivators, which upregulate HBV expression (18,33). Similarly, the preS1 promoter possesses binding sites for HNF-1 and HNF-3, which greatly enhance its activity in cells (16,24). The HBV core promoter also exhibits preferential activity in differentiated liver cells, which is partly conferred by HNF-4 binding (40).

We hypothesized that use of HBV enhancer I and preS1 promoter sequences, which undergo cell type-specific regulation, will help further understand gene regulation in cells. Analysis of tissue-specific regulation of gene expression is relevant for defining mechanisms in organ development and cell differentiation, as well as analyzing susceptibility of specific tissues to pathogens (e.g., during chronic viral persistence). Moreover, expressing therapeutic genes under control of heterologous promoters has captured interest for gene therapy, including restriction of toxic genes to cancer cells (34). The experimental design was to perform transient transfection assays for documenting which cells supported the activity of our HBV sequences, especially in the presence of cotransfected transcription factor plasmids. These systems allowed us to investigate transcriptional control in episomal locations, regardless of the state of the host chromatin, and obtain insights into transcription factor-related controls in intact cells.

MATERIALS AND METHODS

Cells

NIH 3T3 mouse fibroblasts and Hepa1-6 mouse hepatoma cells (15) were originally from American Type Culture Collection (CRL-6361). The cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. HBC-3 cells were derived from the E9.5 mouse liver and were maintained on $STON^+$-feeder cells in embryonic stem cell-qualified culture medium with FBS and antibiotics (GIBCO-BRL, Gaithersburg, MD) as described previously (42). For some experiments using electroporation for gene transfer, HBC-3 cells were cultured for 7 days on dishes coated with 0.1% gelatin in the presence of 5% dimethyl sulfoxide (DMSO) (Sigma Chemicals Co., St. Louis, MO). WW6 ES cells were provided by Dr. P. Stanley, Albert Einstein College of Medicine and were cultured on feeder cells as described previously (25). For electroporation, WW6 cells were cultured on 0.1% gelatin-coated dishes. Primary BALB/c mouse hepatocytes were isolated by collagenase perfusion of the liver and cultured in collagen-coated dishes as reported previously (23). The viability of primary hepatocytes was determined by trypan blue dye exclusion.

Generation and Characterization of Hepa1/3T3 Somatic Cell Hybrids

NIH 3T3 cells were transfected with a $Zeocin^R$ plasmid expressing luciferase and Hepa1-6 cells were transfected with Neo^R gene plasmid (Invitrogen Corp., San Diego, CA) using commercially available liposomes, followed by selection in 400 μg/ml each of Zeocin and G418, respectively. For cell fusion, cells grown to 60–70% confluency were kept without antibiotic selection for 1 day. Subsequently, 1×10^7 Zeo^{R+}-NIH 3T3 and Neo^{R+}-Hepa1-6 cells each were combined in 1:1 ratio and electrofused according to Radomska and Eckhardt (38). Fused cells were cultured in medium containing both Zeocin and G418 at

400 μg/ml each. Cell clones were screened by testing for the presence of luciferase activity, which was contributed by NIH 3T3 cells. To analyze DNA content, cell nuclei were isolated and stained with propidium iodide followed by flow cytometry. The cell karyotype was analyzed in the Cytogenetics Core of the Cancer Center with standard methods.

To determine retention of hepatocyte-specific gene expression in hybrid cells, albumin expression was measured by Western blotting. Cultured cells were washed three times in ice-cold PBS and lysed in triple-detergent buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% NP-40, 0.5% sodium deoxycholate, and 0.02% sodium azide) for 30 min on ice. The lysate was sedimented under 12,000 $\times g$ and 30-μg protein samples resolved by SDS-PAGE in a 10% gel. The proteins were transferred to nitrocellulose (HyBond ECL, Amersham Inc., North Chicago, IL), blocked, and probed with an anti-albumin antibody (Clone HSAII; Sigma) at 1:2000 dilution for 1 h at room temperature. After extensive washes, the membrane was incubated with peroxidase-conjugated anti-mouse IgG (Sigma) and binding detected by enzymatic chemiluminescence, using a commercial kit (Amersham Corp., North Chicago, IL).

Plasmids

The plasmid pGL2.HBpreS1P/HBVEnI-luc was constructed by cloning HBV preS1 promoter and HBV enhancer I sequences into the pGL2 basic luciferase expression plasmid (Promega Corp., Madison, WI), as reported previously (35). The plasmid contains a 912-base pair *Bam*HI fragment of the HBV enhancer I (base pairs 490–1402) and a 514-base pair *Bgl*II fragment of the preS1 promoter (base pairs 2425–2839) derived from the pCP10 HBV plasmid (17). The pGL2.basic plasmid (Promega) lacks regulatory elements. The pGL2.control plasmid contains a luciferase gene under the transcriptional control of simian virus 40 enhancer. To normalize for transfection efficiencies, the pSVβ-galactosidase plasmid (Promega) was used.

Upregulatability of the HBV reporter plasmid was tested by cotransfection with specific transcription factor plasmids. Plasmids expressing HNF-3α and β, and C/EBPα and β were driven by the cytomegalovirus (CMV) promoter/enhancer and were kindly provided by either Dr. G. Crabtree (Stanford University) or Dr. R. Costa (University of Chicago). For analyzing Hepa1/3T3 somatic hybrid cells, the luciferase reporter was removed from the HBV plasmid by digestion of the pGL2-based plasmid with *Hin*dIII and *Bam*HI, followed by insertion of the lacZ gene, which was removed from the pSVβ-galactosidase plasmid with *Hin*dIII and *Bam*HI digestion. This provided us with pGL2.HBpreS1P/HBVEnI-β-gal plasmid to measure gene expression in fusion Hepa1/3T3 cells, which already contained the luciferase gene.

Cell Transfections

Cultured cells were dissociated with 0.01% trypsin/EDTA and resuspended at 5×10^6 cells per ml in FBS-containing medium. Primary hepatocytes were used immediately after cell isolation. Reporter plasmid (10 μg) and transcription factor plasmid (1 μg) (1:10 ratio) in 25 μl were placed in electroporation cuvettes (Invitrogen Corp., Carlsbad, CA). Cells in 400 μl were added to cuvettes with incubation on ice for 5 min, followed by electroporation with 260 V/960 μF in the BioRad apparatus (BioRad Labs., Hercules, CA). Cells were cultured in dishes, typically for 48–96 h (HBC-3 cells) before analysis of reporter gene expression.

Analysis of Reporter Gene Activity

Luciferase activity was measured in cell lysates with a commercial kit (Promega), according to the manufacturer, and expressed as arbitrary light units. LacZ activity was assayed by incubating aliquots of cell lysates in 120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, and 100 mM β-mercaptoethanol with 1.33 mg/ml *o*-nitrophenyl β-D-galactopyranoside substrate (Sigma), followed by spectrophotometry at 420 nm in a microplate reader.

Analysis of Cellular Transcription Factor Activity

Gel shift assays were performed with commercial systems (Promega). Sets of consensus and mutant oligonucleotides included those for testing AP1, SP1, C/EBPs, HNF-3, and HNF-4 transcription factor activities and were from commercial sources (Santa Cruz Biotechnology, Santa Cruz, CA, or Promega Corp). Nuclear extracts were made from cells plated at density of 6×10^6 cells per 100 mm dish. Cells were scraped with a rubber policemen and washed with 40 mM Tris-HCl, pH 7.6, 14 mM NaCl, and 1 mM EDTA. Cell membranes were disrupted in 2 ml hypotonic buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM spermidine, 1 mM DTT, 1 mM PMSF, and 1 μg/ml each of leupeptin, aprotinin, and antipain (Sigma) for 10 min at 4°C, followed by 60–100 strokes in a dounce homogenizer. Cell disruption was monitored microscopically and nuclei were pelleted at $800 \times g$ for 5 min. The nuclear proteins were extracted in 50 μl hypertonic extraction

buffer containing 30 mM HEPES, pH 7.9, 25% glycerol, 450 mM NaCl, 0.3 mM EDTA, 6 mM DTT, 12 mM $MgCl_2$, 1 mM PMSF, and 1 μg each of leupeptin, aprotinin, and antipain for 45 min. After centrifugation at 40,000 × *g* for 30 min at 4°C, the supernatant was recovered and protein concentration determined with the Bradford assay (BioRad).

Probe preparation involved annealing of single-stranded oligonucleotides at 65°C for 15 min followed by slow cooling to room temperature. For radiolabeling, annealed oligonucleotides were incubated with 2 units of DNA polymerase I and 20 μCi each of [^{32}P]dCTP, [^{32}P]dATP, [^{32}P]dTTP, and [^{32}P]dGTP (3000 Ci/mmol, Amersham) in 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 7.5 mM DTT at 37°C for 60 min. After phenol extraction, unincorporated nucleotides were removed with G-25 sephadex columns (Microspin™, Pharmacia Biotech., Uppsala, Sweden). The specific activities of the labeled probes were ~5×10^7 cpm per μg.

DNA binding was in 15-μl reactions with 2 ng annealed, double-stranded oligonucleotide probes, 1 μg poly(dI:dC)/poly(dI:dC), and 6 μg nuclear protein extracts in 25 mM HEPES, pH 7.9, 18% glycerol, 100 mM NaCl, 0.25 mM EDTA, 5 mM DTT, 9 mM $MgCl_2$, 0.75 mM PMSF, and 0.75 μg each of leupeptin, aprotinin, and antipain. After incubation for 20 min at room temperature, DNA binding was analyzed by electrophoresis of the reaction mix in 6% polyacrylamide gels using 0.5% TBE running buffer for 1.5 h. For supershift analysis, 2 μl anti-C/EBPα, C/EBPβ, or C/EBPδ (Santa Cruz Biotechnol.) were added to the reaction mix after 20 min, followed by incubation for an additional hour at room temperature prior to electrophoretic resolution as above. The gels were dried and autoradiographed at −70°C with X-OMAT film (Eastman Kodak Inc., Rochester, NY). The overall magnitude of transcription factor expression was graded semiquantitatively from absent (0) to maximally positive (4+) within the same class of transcription factor.

Statistical Analysis

Data were analyzed by the SigmaStat software and are expressed as mean ± SD (Jandel Scientific, San Rafael, CA). The significance of differences was tested where appropriate by Student's *t*-tests or chi-square tests, and $p < 0.05$ was considered significant.

RESULTS

HBV Enhancer/Promoter Activity in Various Cell Types

To document basal activity of HBV sequences, we first analyzed luciferase expression in our cells. The data were obtained in cultured cells transfected with either the HBV plasmid or the pGL2.basic plasmid (negative control), along with pSVβ-galactosidase plasmid to normalize for transfection efficiency. Primary mouse hepatocytes, Hepa1-6 cells, HBC-3 cells, WW6 ES cells, as well as NIH 3T3 cells, were studied initially. The data showed that HBV sequences were expressed well in cells of hepatic origin, with primary hepatocytes and Hepa1-6 cells being most supportive, although undifferentiated HBC-3 cells also supported HBV expression. Interestingly, HBV expression increased in HBC-3 cells following treatment with DMSO, which is known to differentiate HBC-3 cells along the hepatocyte lineage. In this situation, HBC-3 cells showed significant morphological change, as well as expression of albumin, glucose-6-phosphatase, etc., which was similar to detailed findings published previously (42). Also, the HBV sequences were active in undifferentiated ES cells, although the overall level of HBV expression was less pronounced than other permissive cells (Table 1). Overall, HBV expression was observed to range from 18 ± 8-fold greater levels above negative control pGL3.basic plasmid in ES cells to 265 ± 38-fold greater levels in primary hepatocytes ($p < 0.05$, *t*-test). In contrast, HBV activity was not supported by 3T3 fibroblasts and the plasmid showed no luciferase expression. The SV40 regulatory sequences were active in all cells following transfection with the pGL2.control plasmid, with mean increase of 2843 ± 1316-fold above negative controls, and this was much greater than the activity of HBV sequences ($p < 0.001$, *t*-test).

We further tested differences in cell type-specific regulation of our HBV sequences in response to α and β subunits of HNF-1, HNF-3, and C/EBP transcription factors, which are highly expressed in hepatocytes. Interestingly, HNF-1α and β overexpression did not regulate our HBV sequences in cells, which

TABLE 1
MAGNITUDE OF HBV EXPRESSION IN CELLS

	HBV Expression*	
Cell Type	Fold Increase Above Negative Controls	Relative Expression (Mean)
WW6 ES cells	18 ± 8	1
HBC-3 cells		
Undifferentiated	54 ± 13	3
After DMSO treatment	162 ± 16	9
Hepa1-6 cells	252 ± 30	14
Primary mouse hepatocytes	265 ± 38	15

*Luciferase activity was normalized with lacZ activity in transfected cells.

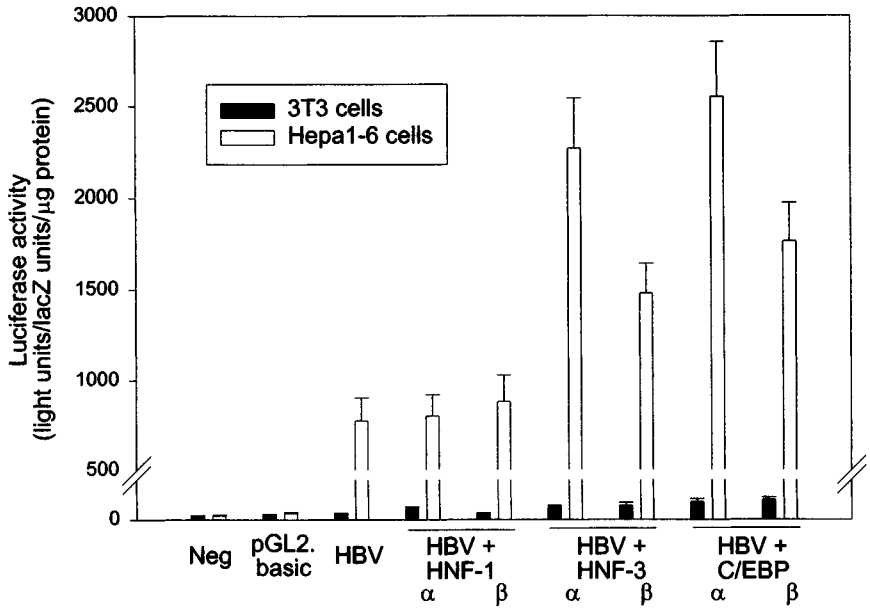

FIG. 1. Differential regulation of HBV sequences in Hepa1-6 hepatocytes and NIH 3T3 fibroblasts. Data show that HBV expression was downregulated in fibroblasts with no change in this situation following cotransfection of HNF-1, HNF-3, or C/EBP plasmids. In contrast, Hepa1-6 cells supported HBV expression. Moreover, the activity of HBV sequences increased in response to HNF-3 and C/EBP overexpression, although HNF-1 expression was ineffective. The data are representative of 3–6 independent experiments performed and include baseline luminiscence (neg), as well as transfection with the pGL2.basic negative control plasmid.

indicated loss of HNF-1 responsiveness, similar to previous findings in HuH-7 cells (35). In contrast, HNF-3 and C/EBP plasmids upregulated HBV expression in primary mouse hepatocytes, Hepa1-6 cells, as well as HBC-3 cells. However, HBV sequences remained inactive in 3T3 fibroblasts, despite cotransfection of the HBV plasmid with these upregulatory transcription factors (Fig. 1).

The results concerning regulation of HBV sequences with various transcription factors are shown in Table 2. In primary hepatocytes, as well as Hepa1-6 cells, HBV activity was significantly upregulated in response to both α and β HNF-3 and C/EBP subunits. The findings in HBC-3 cells were interesting because responses to transcription factors were different after differentiation induction with DMSO. In undifferentiated HBC-3 cells, cotransfection with HNF-3 and C/EBPβ plasmids was less effective in increasing HBV expression, compared with HNF-3 and C/EBPα plasmids. In contrast, following differentiation of HBC-3 cells, HNF-3β and C/EBPβ transcription factors assumed greater activity in upregulating HBV, whereas HNF-3α became less effective. These findings were compatible with the coordinate regulation of transcription factor activity in relationship with cell differentiation states.

In WW6 ES cells, our HBV sequences showed activity in the absence of cotransfection with transcription factor plasmids with $6 \pm 3 \times 10^4$ luciferase units/lacZ activity/μg protein, which was significantly dif-

TABLE 2
CHANGE IN HBV EXPRESSION FOLLOWING COTRANSFECTION OF CELLS WITH TRANSCRIPTION FACTOR PLASMIDS

	HBV Enhancer I/preS1 Promoter Activity*					
Cell Type	HNF-1α	HNF-1β	HNF-3α	HNF-3β	C/EBPα	C/EBPβ
Primary mouse hepatocytes	0.95 ± 0.1	1.0 ± 0.2	2.7 ± 0.3†	2.5 ± 0.2†	3.2 ± 0.5†	2.7 ± 0.5†
Hepa1-6 cells	1.0 ± 0.2	1.1 ± 0.2	2.9 ± 0.4†	1.9 ± 0.2†	3.3 ± 0.4†	2.3 ± 0.3†
HBC-3 cells						
Undifferentiated	0.99 ± 0.3	1.0 ± 0.3	4.9 ± 0.8†	1.2 ± 0.4	2.0 ± 0.3†	1.7 ± 0.2†
After DMSO treatment	0.97 ± 0.2	1.0 ± 0.2	1.5 ± 0.1†	1.9 ± 0.5†	2.4 ± 0.5†	2.9 ± 0.4†

*Percent luciferase activity against control cells without transfection of transcription factor plasmids.
†$p < 0.05$.

ferent from background luminescence, as well as following transfection of pGL2.basic plasmid ($p < 0.001$). However, in response to transcription factor plasmids, HBV activity was regulated in a fashion that was different from other cells. In this situation, HBV expression remained unchanged, similar to upon transfection of HNF-3β plasmid in undifferentiated HBC-3 cells or HNF-3α plasmid in differentiated HBC-3 cells. These findings suggested that HBV transcription in ES cells was controlled at the episomal level, in view of this location of the HBV and transcription factor plasmids in our transient transfection assays. Moreover, an absence of regulation of our HBV sequences by transfected transcription factors in ES cells was compatible with the existence of negative controls.

Development and Analysis of Fusion Cells

Following completion of our electrofusion protocol, a number of clones were selected for further study on the basis of cell morphology (Fig. 2A–C). We found cell clones that exhibited different morphology in comparison with parental Hepa1-6 and NIH 3T3 cells. This morphology of Zeocin and G418-resistant fusion cells varied from fibroblastoid with elongated and spindly cytoplasmic projections, as well as hepatocytic with large nuclei and complex cytoplasm along with spindly cell projections. We randomly chose to evaluate several clones with the latter morphology that exhibited luciferase activity. Flow cytometry showed significantly increased DNA content in these cell clones compared with the parental cells, which was compatible with the presence of additional genetic material (Fig. 2D). Karyotyping of a clone designated Hepa1/3T3 showed extra chromosomes in fusion cells compared with parental cells, along with significantly increased modal chromosome number. We further determined whether Hepa1/3T3 fusion cells expressed hepatic genes, because our major interest was in analyzing cells of this particular phenotype. For this purpose, albumin expression was analyzed by Western blotting of total cell lysates (Fig. 2E). The cells expressed albumin, which allowed us to proceed with further studies using fusion cells exhibiting this desirable phenotype.

Regulation of Endogenous Transcription Factor Activity in Cells

We documented patterns of transcription factor activity in various cells with gel shift analysis of nuclear extracts using oligonucleotide probes. In primary mouse hepatocytes, large amounts of C/EBP, as well as HNF-3, DNA binding activities were present, whereas AP1 and SP1 transcription factors were expressed at a relatively low level (Fig. 3A–E). In undifferentiated HBC-3 cells, SP1 and AP1 transcription factors were expressed much more abundantly than HNF-3 and C/EBP, presumably indicating their greater proliferative activity or other differences. In contrast, following induction of differentiation with DMSO, HBC-3 cells expressed greater amounts of HNF-3 and C/EBP, whereas AP1 and SP1 levels declined in this situation. In ES cells, AP1 transcription factor was particularly abundant, while SP1 transcription factor was not detected by the gel shift assay. In addition, some HNF-3 activity was present in ES cells, whereas C/EBP activity was not detected in these cells. In NIH 3T3 fibroblasts, both AP1 and SP1 transcription factors were expressed abundantly but expression of HNF-3 and C/EBP was not detected. Finally, C/EBP, HNF-1 and HNF-3 binding activity was detected in fused Hepa1/3T3 cells, which was compatible with our observation of albumin expression in these cells. The overall pattern of transcription factor activity is summarized in Table 3.

HBV Expression in Fused Hepa1/3T3 Cells

To document regulation of HBV expression in fusion cells, we performed transient transfection assays with the lacZ reporter plasmid (Fig. 4). When cells were transfected with the HBV plasmid alone, HBV enhancer and promoter showed activity, which was similar to the parental Hepa1-6 cells but dissimilar from NIH 3T3 cells. The findings were compatible with the maintenance of albumin expression, as well as the presence of endogenous C/EBP, HNF-1, and HNF-3 expression as shown above. However, when transcription factors were cotransfected with the HBV plasmid in fusion cells, we found that HBV expression was downregulated. There was no perturbation in cell morphology or viability in this situation. Nonetheless, HBV expression was attenuated following cotransfection of cells with HNF-1α plus β plasmids, HNF-3α plus β plasmids, as well as C/EBPα and C/EBPβ plasmids.

DISCUSSION

These findings indicate that HBV sequences were active in pluripotential ES cells, as well as HBC-3 progenitor cells, which can differentiate along both hepatocyte and biliary lineages (42). Differentiation of HBC-3 cells along the hepatocyte lineage was associated with increased expression of transfected HBV sequences. In contrast, fibroblasts showed significant differences in transcription factor activity with extinction of HBV sequences, despite the provision of suitable transcription factors in *trans*. This

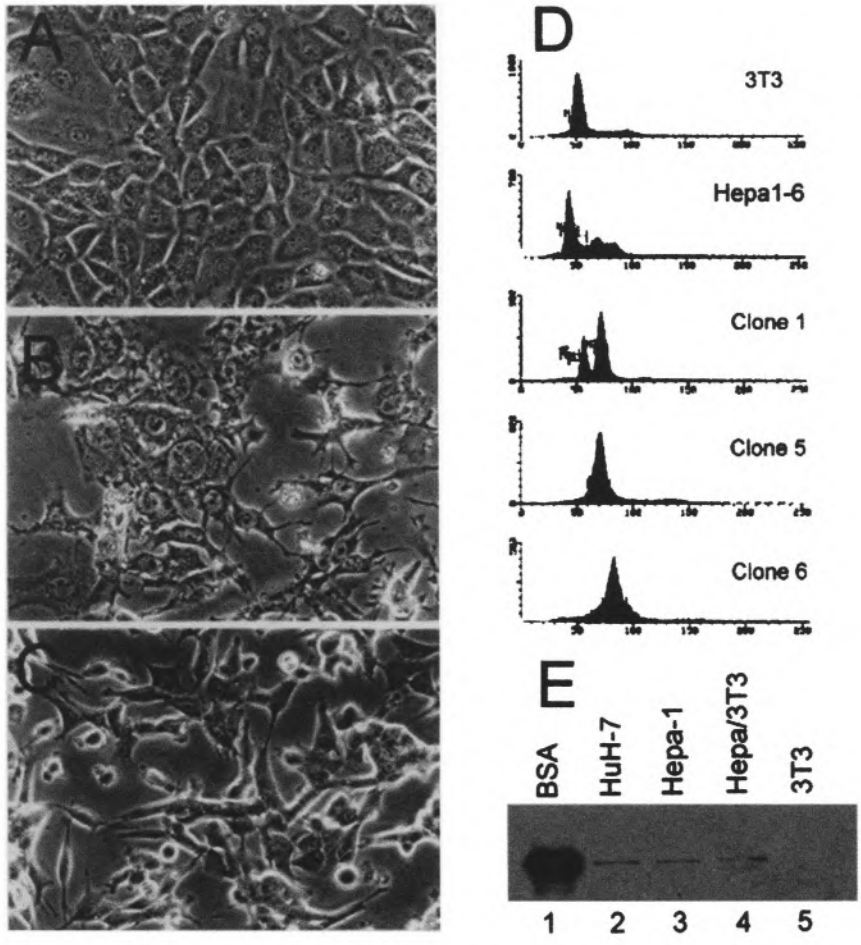

FIG. 2. Analysis of fused Hepa1/3T3 cells. Cultured parental cells are shown under phase contrast microscopy (×200), with NIH 3T3 fibroblasts in (A) and Hepa1-6 cells in (B). (C) Fused Hepa1/3T3 cells with a different morphology compared with parental cells. (D) Flow cytometric analysis of nuclear DNA content in normally cultured cells following staining with propidium iodide. The panels show parental 3T3 fibroblasts and Hepa1-6 cells (top two panels), as well as three individual clones of fused cells (bottom three panels). The DNA content of fused cells was greater than that of either parental cell. (E) Western blot analysis with an albumin antibody, which cross-reacts with several animal species. Lane 1 contains molecular weight marker with bovine serum albumin. Lanes 2–5 contain total lysates from HuH-7 cells, parental Hepa1-6 cells, fused Hepa1/3T3 cells, and parental 3T3 fibroblasts, respectively. Albumin was detected in hepatocyte-derived cells, as well as fused cells, whereas NIH 3T3 cells were negative.

suggested the possibility of episomally mediated transcriptional controls in fibroblasts.

Our HBV sequences were active in hepatocytes, in agreement with the expression of endogenous transcription factors, such as HNF-3 and C/EBPs. Similarly, increased expression of these transcription factors in HBC-3 cells following DMSO treatment was associated with greater activity of HBV sequences. On the other hand, absence of liver-specific transcription factors in fibroblasts was associated with downregulation of HBV sequences that was not overcome after expression of HNF-3 and C/EBP transcription factor plasmids. These findings suggest multiple possibilities for future investigations, including neutralization of transcription factors by repressors, inaccessible DNA binding sites, and absence of additional subunits necessary for transcription factor activity. Although ES cells expressed little by way of HNF-3 and C/EBP hepatic transcription factors, we found expression of HNF-1 and 4, as well as copious expression of the AP1 transcription factor, the latter at levels similar to fibroblasts. However, unlike fibroblasts, ES cells supported HBV expression. Nonetheless, transfection of HNF-3 and C/EBP plasmids was not associated with further increases in HBV expression in ES cells. Also, the overall magnitude of HBV expression in ES cells was limited. These findings indicated that, unlike fibroblasts, ES cells and HBC-3 progenitor cells lacked repressors under basal conditions that could block HBV expression. However, the ability of ES cells to express HBV sequences at higher levels presumably required additional cellular factors, which were not reconstituted by our supply of transcription factors. We found that transfection of

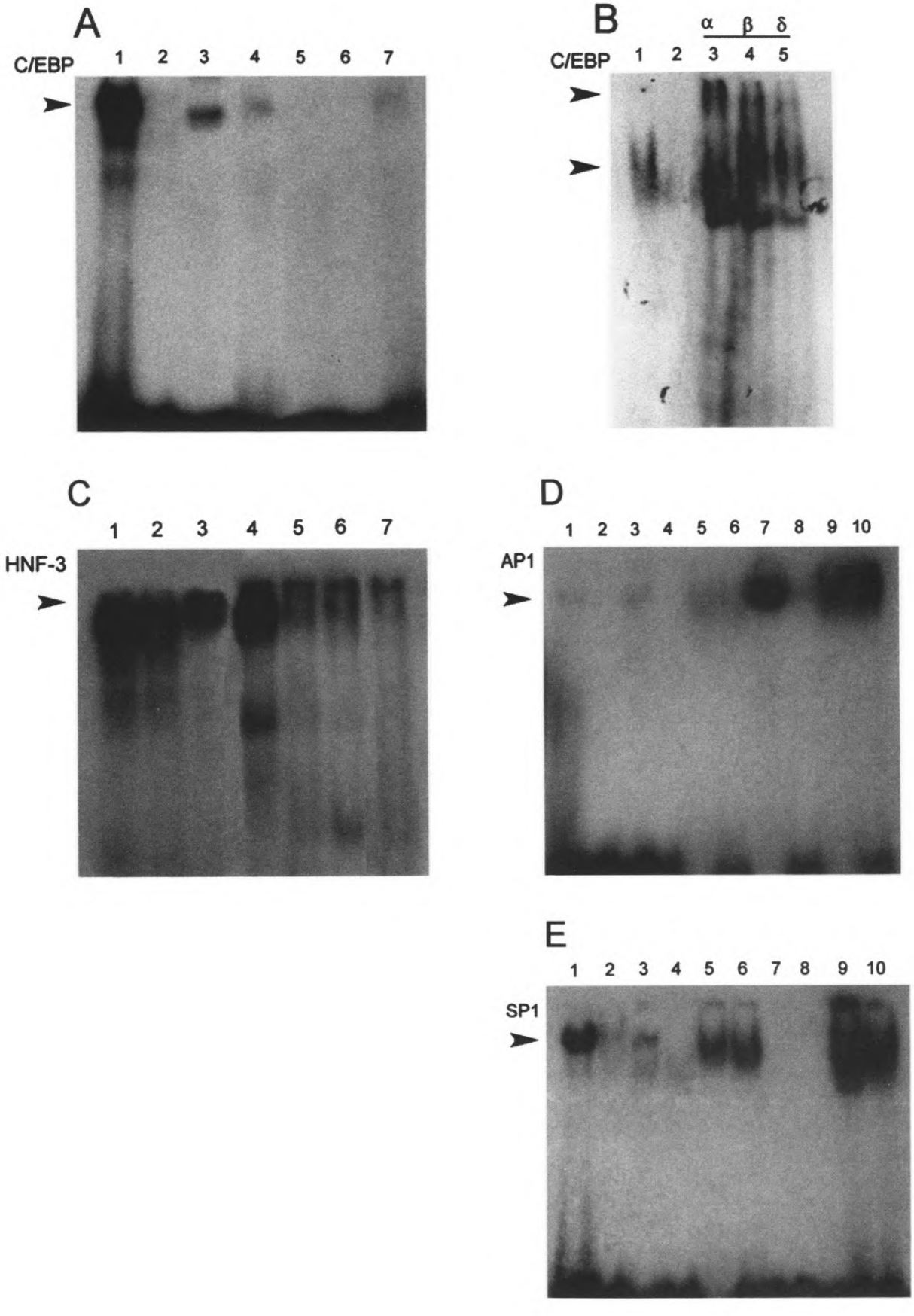
A
C/EBP
1 2 3 4 5 6 7
B
α β δ
C/EBP
1 2 3 4 5
C
1 2 3 4 5 6 7
HNF-3
D
1 2 3 4 5 6 7 8 9 10
AP1
E
1 2 3 4 5 6 7 8 9 10
SP1

TABLE 3
ANALYSIS OF TRANSCRIPTION FACTOR EXPRESSION WITH GEL-SHIFTS USING CELLULAR NUCLEAR EXTRACTS

	Transcription Factors					
Cell Type	HNF-1	HNF-3	HNF-4	C/EBP	AP1	SP-1
Primary hepatocytes	+++	+++	++	++++	+	++
HBC-3 cells						
Undifferentiated	++	++	+/–	+	++	+++
DMSO treated	++	+++	+	++	+	+
WW6 ES cells	++	+/–	+	–	++++	–
NIH 3T3 cells	+	+/–	–	–	+++	++++
Hepa1/3T3 fusion cells	+	+	ND	+	ND	ND

ND = not done.

HNF-1 transcription factor plasmid did not upregulate HBV expression in our cells, which was similar to our previous findings in HuH-7 cells (35).

A common feature of fusion cells concerns the loss of chromosomes from one or the other fusion partner (5,13). We utilized electrofusion because of superior probabilities of preserving differentiated function in fused cells (38). Presence of albumin, C/EBP, HNF-1, and HNF-3, along with expression of transfected HBV sequences, was in agreement with the predominance of the Hepa1 phenotype in our fusion cells. On occasion, cell type-specific genes escape extinction in fusion cell systems (5,6,43). Nonetheless, it was noteworthy that our HBV sequences were downregulated following transfection of transcription factors in Hepa1/3T3 cells. We interpreted this to indicate episomal negative control mechanisms, presumably reflecting perturbed balance among transcriptional controls, with negative regulators overwhelming the positive ones.

In previous studies, genetic loci mediating gene repression have been analyzed in intertypic hybrid cells, similar to the ones reported here, but which generally fail to express tissue-specific gene products of either parent (5,13,27,28). Whereas gene extinction is observed regularly in hybrid cells containing one set of chromosomes from either parent, hybrid cells with two sets of chromosomes from liver cells may retain hepatic gene expression (36). Hepatoma–fibroblast hybrid cells have been investigated extensively, including for expression of multiple liver-specific mRNAs (12). Among these, the majority of liver-specific mRNAs (14 of 16) failed to accumulate in karyotypically complete hybrid cell clones, with steady-state mRNA levels declining by up to 1000-fold. Interestingly, all 14 liver-specific mRNAs were reexpressed in hybrid cells following the deletion of fibroblast chromosomes. These data indicate that expression of tissue-specific genes is regulated in intertypic hybrid cells and suggest that repression of heterologous genetic elements is a frequent control mechanism. Similarly, microcell hybrids formed by the transfer of single fibroblast chromosomes into liver cells exhibit extinction of liver-specific genes, such as albumin and tyrosine aminotransferase (TAT) (11). Two genetic loci, designated tissue-specific extinguisher (tse) 1 and tse2, which are localized on mouse chromosome 11 and 1, respectively, have been shown to mediate gene repression in hepatoma–fibroblast hybrid cells (13,28). Tse1 mediates extinc-

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FIG. 3. Typical gel shifts showing abundance of cellular transcription factors. (A) C/EBP binding (arrowhead) is shown in cells as indicated with unbound probe at the bottom of the gel. Lane 1: primary mouse hepatocytes with abundant C/EBP activity; lane 2: mouse hepatocytes incubated with 70-fold excess of unlabeled competitor showing loss of gel shift; lane 3: HBC-3 cells following differentiation with DMSO; lane 4: undifferentiated HBC-3 cells showing less C/EBP than after cell differentiation; lane 5: WW6 ES cells showing absence of C/EBP activity; lane 6: NIH 3T3 cells showing absence of C/EBP activity; lane 7: Hepa1/3T3 fusion cells showing weak C/EBP activity. (B) C/EBP supershifts following incubation of nuclear extracts from primary hepatocytes with C/EBP antibodies. Lane 1: gel shift without incubation with antibodies (lower arrowhead); lane 2: similar to lane 1 except for the presence of cold competitor in 70-fold excess; lanes 3–5: supershifts of the C/EBP band (upper arrowhead) following incubation with antibodies against C/EBP isoforms indicated. (C) HNF-3 gel shifts. Lanes 1 and 2: primary mouse hepatocytes; lane 3: undifferentiated HBC-3 cells; lane 4: differentiated HBC-3 cells after incubation with DMSO; lane 5: WW6 ES cells; lane 6: NIH 3T3 cells; and lane 7: fusion Hepa1/3T3 cells. (D) AP1 gel shifts and (E) SP1 gel shifts with the order of samples in both (D) and (E) as follows. Lane 1: primary mouse hepatocytes; lane 2: primary hepatocytes with unlabeled competitor in 30-fold excess; lane 3: HBC-3 cells following differentiation with DMSO; lane 4: differentiated HBC-3 cells with unlabeled competitor; lane 5: undifferentiated HBC-3 cells; lane 6: undifferentiated HBC-3 cells with unlabeled competitor in 30-fold excess; lanes 7 and 8: WW6 ES cells; lanes 9 and 10: NIH 3T3 cells.

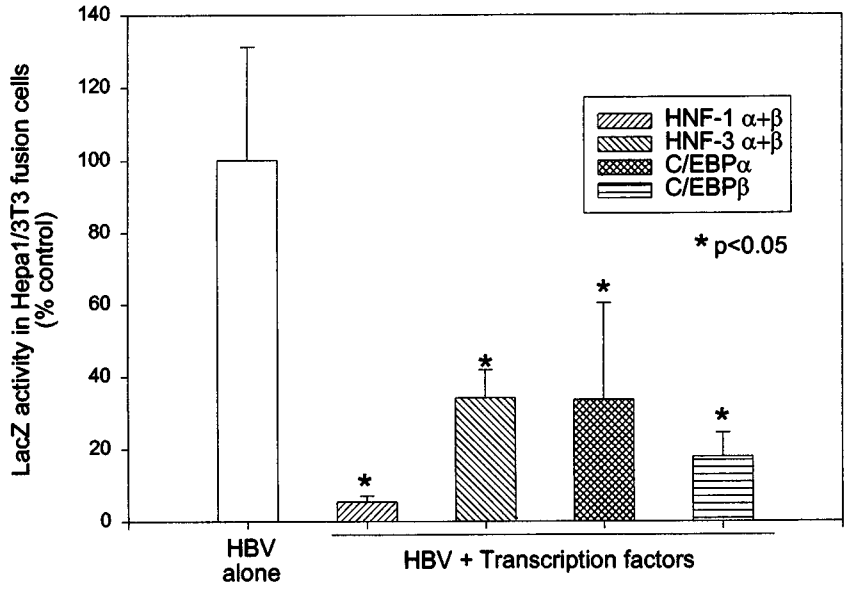

FIG. 4. Regulation of HBV activity in fusion Hepa1/3T3 cells. The data are from transfection experiments performed with HBV plasmid alone and with various transcription factor plasmids. Although the HBV plasmid showed activity in these cells, its activity was significantly attenuated after transfection with HNF-1, HNF-3, as well as C/EBP plasmids.

tion of TAT and several other liver genes and tse2 represses albumin and alcohol dehydrogenase genes (28). Also, it has been demonstrated that another locus on mouse chromosome 3 can extinguish albumin synthesis but not alcohol dehydrogenase gene (32). Our fusion cells were capable of albumin synthesis, suggesting absence of gene repression mediated by tse loci. In most systems analyzed so far, genetic extinction has been correlated with the absence of inactivity of critical cell type-specific transcription factors. In hepatoma-fibroblast fusion cells, decreased production of HNF-1, HNF-3, and HNF-4 is associated with extinction of liver genes, such as albumin and α-1 antitrypsin (10,26). However, in one study, expression of HNF-1, HNF-3α and β, and HNF-4 mRNAs was unaffected in hybrid cells containing human fibroblast chromosome 2, which is the counterpart of mouse chromosome 1 (20). These studies defined a genetic locus on human chromosome 2, which extinguished albumin gene expression in *trans*, and this was interpreted to indicate that tse2-mediated extinction is independent of HNF-1 and -4, and HNF-3 expression. Moreover, overexpression of HNF-4 in hepatoma cells has recently been shown to be associated with reexpression of hepatic genes, as well as morphological and other changes (44). Although our findings are in agreement with the close relationships among transcription factor abundance and cellular gene expression, our data also indicate perturbation of the activity of transfected transcription factors by unidentified negative regulators.

Our results indicate that isolation and further characterization of specific negative transcriptional regulators of HBV could be helpful in developing novel therapeutic strategies to block HBV expression. Also, our systems will be helpful in the analysis of progenitor cells derived from various organs. As further efforts are undertaken to isolate progenitor cells, such as the recent success in isolating pluripotential human ES cells (45), convenient methods will be required to analyze cell differentiation along various lineages. Use of HBV sequences, which demonstrate cell type specificity, as well as regulation by specific transcription factors, will be an excellent system for such analysis in respect with the hepatocyte lineage. On the other hand, persistence of HBV expression in progenitor cells, as shown by our studies in ES cells and HBC-3 cells, will be of interest in mechanisms concerning viral clearance and oncogenesis. Our studies showing the activity of SV40 promoter and enhancer in all cells tested, including ES cells, provide further substantiation of this mechanism concerning viral persistence. Similarly, human papillomavirus, another oncogenic DNA virus, is regulated in a cell type-dependent manner (41). The ability of HBV to reside in progenitor cell compartments might account for difficulties in eradicating viral infection in chronic carriers, despite the intrinsically cytopathic nature of HBV infection, as well as potent immune responses against virally infected hepatocytes (22). Whether clonal hepatocellular carcinomas arise from activated progenitor cells could be another possibility.

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REFERENCES

1. Akira, S.; Isshiki, H.; Sugita, T.; Tanabe, O.; Kinoshita, S.; Nishio, Y.; Nakajima, T.; Hirano, T.; Kishimoto, T. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. 9:1897–1906; 1990.
2. Ang, S-L.; Rossan, J. HNF-3β is essential for node and notochord formation in mouse development. Cell 78: 561–574; 1994.
3. Aragona E.; Burk, R. D.; Ott, M.; Shafritz, D. A.; Gupta, S. Cell type-specific mechanisms regulate hepatitis B virus transgene expression in liver and other organs. J. Pathol. 180:441–449; 1996.
4. Bannister, A. J.; Kouzarides, T. The CBP co-activator is a histone acetyl-transferase. Nature 384:641–643; 1996.
5. Baron, M. H.; Maniatis, T. Rapid reprogramming of globin gene expression in transient heterokaryons. Cell 46:591–602; 1986.
6. Blau, H. M.; Pavlath, G. K.; Hardeman, E. C.; Chiu, C. P.; Silberstein, L.; Webster, S. G.; Miller, S. C.; Webster, C. Plasticity of the differentiated state. Science 230:758–766; 1985.
7. Boshart, M.; Nitsch, D.; Schuetz, G. Extinction of gene expression in somatic cell hybrids—a reflection of important regulatory mechanisms? Trends Genet. 9:240–245; 1993.
8. Cereghini, S. Liver-enriched transcription factors and hepatocyte differentiation. FASEB J. 10:267–282; 1996.
9. Cereghini, S.; Ott, M. O.; Power, S.; Maury, M. Expression patterns of vHNF1 and HNF1 homeoproteins in early postimplantation embryos suggest distinct and sequential developmental roles. Development 116:783–797; 1992.
10. Cereghini, S.; Yaniv, M.; Cortese, R. Hepatocyte dedifferentiation and extinction is accompanied by a block in the synthesis of mRNA coding for the transcription factor HNF1/LBP1. EMBO J. 9:2257–2263; 1990.
11. Cerosaletti, K. M.; Fournier, R. E. Extinction of albumin gene expression in a panel of human chromosome 2 microcell hybrids. Genomics 31:348–358; 1996.
12. Chin, A. C.; Fournier, R. E. K. A genetic analysis of extinction: Transregulation of 16 liver-specific genes in hepatoma-fibroblast hybrid cells. Proc. Natl. Acad. Sci. USA 84:1614–1618; 1987.
13. Chin, A. C.; Fournier, R. E. K. Tse-2: A trans-dominant extinguisher of albumingene expression in hepatoma hybrid cells. Mol. Cell. Biol. 9:3736–3743; 1989.
14. Dabeva, M.; Shafritz, D. A. Activation, proliferation, and differentiation of progenitor cells into hepatocytes in the D-galactosamine model of liver regeneration. Am. J. Pathol. 143:1606–1620; 1993.
15. Darlington, G. J.; Bernhard, H. P.; Miller, R. A.; Ruddle, F. H. Expression of liver phenotypes in cultured mouse hepatoma cells. J. Natl. Cancer Inst. 64:809–819; 1980.
16. De-Medina, T.; Faktor, O.; Shaul, Y. The S promoter of hepatitis B virus is regulated by positive and negative elements. Mol. Cell. Biol. 8:2449–2455; 1988.
17. Elfassi, E. Broad specificity of the hepatitis B virus enhancer. Virology 160:259–262; 1987.
18. Garcia, A. D.; Ostapchuk, P.; Hearing, P. Functional interaction of nuclear factors EF-C, HNF-4, and RXR alpha with hepatitis B virus enhancer I. J. Virol. 67: 3940–3950; 1993.
19. Gourdeau, H.; Fournier, R. E. K. Genetic analysis of mammalian cell differentiation. Annu. Rev. Cell. Biol. 6:69–94; 1990.
20. Griffo, G.; Hamon-Benais, C.; Angrand, P. O.; Fox, M.; West, L.; Lecoq, O.; Povey, S.; Cassio, D.; Weiss, M. HNF-4 and HNF-1 as well as a panel of hepatic functions are extinguished and reexpressed in parallel in chromosomally reduced rat hepatoma-human fibroblast hybrids. J. Cell. Biol. 121:887–898; 1993.
21. Gupta, S.; Ott, M. Viral hepatitis and liver disease. In: Jameson, J. L., ed. Principles of molecular medicine. Clifton, NJ: Humana Press Inc.; 1998:387–400.
22. Gupta, S.; Shafritz, D. A. Viral mechanisms in hepatic oncogenesis. In: Arias, I. M.; Boyer, J.; Fausto, N.; Jacoby, E. R.; Schachter, D.; Shafritz, D. A., eds. The liver: Biology and pathobiology. New York: Raven Press Limited; 1994:1429–1453.
23. Gupta, S.; Vemuru, R. P.; Lee, C. D.; Yerneni, P.; Aragona, E.; Burk, R. D. Hepatocytes exhibit superior transgene expression after transplantation into liver and spleen compared with peritoneal cavity or dorsal fat pad: Implications for hepatic gene therapy. Hum. Gene Ther. 5:959–967; 1994.
24. Honigwachs, J.; Faktor, O.; Dikstein, R.; Shaul, Y.; Laub, O. Liver-specific expression of hepatitis B virus is determined by the combined action of the core gene promoter and the enhancer. J. Virol. 63:919–924; 1989.
25. Ioffe, E.; Liu, Y.; Bhaumik, M.; Poirier, F.; Factor, S. M.; Stanley, P. WW6: An embryonic stem cell line with an inert genetic marker that can be traced in chimeras. Proc. Natl. Acad. Sci. USA 92:7357–7361; 1995.
26. Junker, S.; Pedersen, S.; Schreiber, E.; Matthias, P. Extinction of an immunoglobulin kappa promoter in cell hybrids is mediated by the octamer motif and corre-

lates with suppression of Oct-2 expression. Cell 61: 467–474; 1990.
27. Kaneko, K. J.; DePamphilis, M. L. Regulation of gene expression at the beginning of mammalian development and the TEAD family of transcription factors. Dev. Genet. 22:43–55; 1998.
28. Killary, A. M.; Fournier, R. E. K. A genetic analysis of extinction: Trans-dominant loci regulate expression of liver-specific traits in hepatoma hybrid cells. Cell 38:523–534; 1984.
29. Kuo, C. F.; Santopoulos, K. G.; Darnell, J. E., Jr. Fetal and adult localization of C/EBP: Evidence for combinatorial action of transcription factors and cell-specific gene expression. Development 109:473–481; 1990.
30. Kuo, C. J.; Conley, P. B.; Chen, L.; Sladik, F. N.; Darnell, J. E., Jr.; Crabtree, G. R. Transcriptional heirarchy involved in mammalian cell-type specification. Nature 355:457–461; 1992.
31. Lai, E.; Prezioso, V. R.; Tao, W. F.; Chen, W. S.; Darnell, J. E., Jr. Hepatocyte nuclear factor 3 α belongs to a gene family in mammals that is homologous to the *Drosophila* homeotic gene forkhead. Genes Dev. 5: 416–427; 1991.
32. Petit, C.; Levilliers, J.; Ott, M. O.; Weiss, M. C. Tissue-specific expression of the rat albumin gene: Genetic control of its extinction in microcell hybrids. Proc. Natl. Acad. Sci. USA 83:2561–2565; 1986.
33. Ori, A.; Shaul, Y. Hepatitis B virus enhancer binds and is activated by the hepatocyte nuclear factor 3. Virology 207:98–106; 1995.
34. Ott, M.; Gupta, S. Regulation of gene expression in hepatocytes. In: Mito M.; Sawa, M., eds. Hepatocyte transplantation. Basel: Karger Landes Systems; 1997: 234–254.
35. Ott, M.; Thyagarajan, S. P.; Gupta, S. *Phyllanthus amarus* suppresses hepatitis B virus by interrupting interactions between HBV enhancer I and cellular transcription factors. Eur. J. Clin. Invest. 27:908–915; 1997.
36. Peterson, J. A.; Weiss, M. C. Expression of differentiated functions in hepatoma cell hybrids: Induction of mouse albumin production in rat hepatoma-mouse fibroblast hybrids. Proc. Natl. Acad. Sci. USA 69:571–575; 1972.
37. Pontogiolo, M.; Barra, J.; Hadchouel, M.; Doyen, A.; Kress, Ch.; Poggi, B. J.; Babinet, C.; Yaniv, M. Hepatocyte nuclear factor 1 (HNF-1) inactivation results in hepatic dysfunction, phenylketonuria and renal Fanconi syndrome. Cell 84:575–585; 1996.
38. Radomska, J. S.; Eckhardt, L. A. Mammalian cell fusion in an electroporation device. J. Immunol. Methods 188:209–217; 1995.
39. Rana, B.; Mischolon, D.; Xie, Y.; Bucher, N. L.; Farmer, S. R. Cell–extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes. Reciprocal expression of C/EBP alpha and immediate early growth-response transcription factors. Mol. Cell. Biol. 14:5858–5869; 1994.
40. Raney, A. K.; Easton, A. J.; McLachlan, A. Characterization of the minimal elements of the hepatitis B virus large surface antigen promoter. J. Gen. Virol. 75:2671–2679; 1994.
41. Rapp, B.; Pawallek, A.; Kraetzer, F.; Schaefer, M.; May, C.; Purdie, K.; Grassmann, K.; Iftner, T. Cell-type-specific separate regulation of the E6 and E7 promoters of human papillomavirus type 6a by the viral transcription factor E2. J. Virol. 71:6956–6966; 1997.
42. Rogler, L. E. Selective bipotential differentiation of mouse embryonic hepatoblasts in vitro. Am. J. Pathol. 150:591–602; 1997.
43. Shurman, L.; Laskov, R.; Bergman, Y. Direct and indirect mechanisms of repression participate in suppression of T-cell-specific gene expression in T × L-cell hybrids. Gene Expr. 5:285–300; 1996.
44. Spath, G. F.; Weiss, M. C. Hepatocyte nuclear factor 4 provokes expression of epithelial marker genes, acting as a morphogen in dedifferentiated hepatoma cells. J. Cell Biol. 140:935–946; 1998.
45. Thomson, J. A.; Itskovitz-Eldor, J.; Shapiro, S. S.; Waknitz, M. A.; Swiergiel, J. J.; Marshall, V. S.; Jones, J. J. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147; 1998.
46. Tian, J-M.; Schibler, U. Tissue-specific expression of the gene encoding hepatocyte nuclear factor 1 may involve hepatocyte nuclear factor 4. Genes Dev. 5:2225–2234; 1991.
47. Turker, M. S.; Bestor, T. H. Formation of methylation patterns in the mammalian genome. Mutat. Res. 386: 119–130; 1997.
48. Wang, N-D.; Finegold, M. J.; Bradley, A.; Ou, C. N.; Abdelsayed, S. V.; Wilde, M. D.; Taylor, L. R.; Wilson, D. R.; Darlington, G. J. Impaired energy homeostasis in C/EBPα knockout mice. Science 269:1108–1112; 1995.