Regulation of Albumin Gene Expression in Hepatoma Cells of Fetal Phenotype: Dominant Inhibition of HNF1 Function and Role of Ubiquitous Transcription Factors

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Two widely used hepatoma cell lines, mouse BW1J and human HepG2, express gene products characteristic of fetal hepatocytes, including serum albumin, whereas reporter genes driven by the albumin promoter are expressed at very low levels compared with highly differentiated hepatoma cells. We have investigated the low albumin promoter activity in BW1J cells to understand differences in liver gene regulation between fetal and adult cells. Addition of the albumin upstream enhancer, or any other fragment of the albumin gene, failed to modify expression of the transfected promoter in BW1J cells. Analysis of cis elements of the albumin promoter showed that, in contrast to highly differentiated H4II cells, in BW1J cells the activity largely depends on ubiquitous transcription factors. Both BW1J and HepG2 cells produce the liver-enriched transcription factor HNF1; dimerization and DNA binding properties are identical to those of liver HNF1, yet the protein fails to show the anticipated transcriptional stimulatory activity. A transfected HNF1 expression vector strongly trans-activates the albumin promoter in HepG2 but only weakly in BW1J cells, and in hybrids (BW1J $\times$ Fao), inefficient HNF1 function is dominant. We conclude that hepatoma cells of the fetal phenotype are deficient in the use of HNF1 to drive transcription of the albumin gene and that they harbor a dominant modulator of HNF1 function.

INTRODUCTION

In recent years, a number of liver-enriched transcription factors have been identified and cloned (for review, see De Simone and Cortese, 1991; Lai and Darnell, 1991; Tronche and Yaniv, 1992), most often using the criterion of binding to a cis-acting element of a liver-specific gene that was identified as functionally important from transfection tests in hepatoma cells. This strategy required focusing attention on those hepatoma lines where the promoters of liver-specific genes are efficiently expressed. However, in some cases, anomalous results have been obtained: promoters may be active in transfection tests in lines where the endogenous gene

is silent (Dierks *et al.*, 1981; Robins *et al.*, 1982) and, conversely, some hepatoma lines express the endogenous genes while failing to show transcription from the transfected promoter (see below). As the regulatory roles of the prominent liver transcription factors are initially defined, cell lines showing anomalous behavior tend to be neglected in favor of lines that appear to function similarly to hepatocytes. In a second phase of research, the exceptional lines may provide valuable models of "mutant" behavior, furnishing clues to unsuspected levels of regulation.

One of the most intensively investigated models of hepatocyte-specific transcription is the serum albumin gene. This gene is expressed selectively in hepatocytes,

where its transcription is first detected at the stage of liver bud formation in the mouse embryo (Schmid and Schulz, 1990; Cascio and Zaret, 1991). Thereafter, the gene is expressed at a low but significant level in the fetus (Tilghman and Belayew, 1982; Panduro *et al.*, 1987), whereas in the adult it is transcribed at a 1000-fold greater rate in liver than in other tissues (Powell *et al.*, 1984; Liu *et al.*, 1988). Upstream regulatory sequences of the albumin gene have been subjected to functional analysis by transfection into hepatoma cells (Ott *et al.*, 1984) and by *in vitro* transcription assays using liver nuclear extracts (Gorski *et al.*, 1986). Both of these approaches demonstrate that the 150 base pairs (bp) upstream of the transcription start site ensure a high rate of transcription in liver extracts and in H4II hepatoma cells (Gorski *et al.*, 1986; Heard *et al.*, 1987; Herbomel *et al.*, 1989). Moreover, a shorter promoter containing only the TATA box and the HNF1 binding site function efficiently in both systems (Lichtsteiner and Schibler, 1989; Maire *et al.*, 1989; Tronche *et al.*, 1989).

However, the albumin promoter is not sufficient to obtain liver-specific transcription in transgenic mice. In this context it is necessary to include an enhancer element 10 kilobase (kb) upstream of the promoter to obtain appropriate liver-specific expression (Pinkert *et al.*, 1987). It appears that the enhancer is necessary for activation of the albumin gene during development and to obtain optimal transcription of the gene in certain hepatocyte-derived cell lines *in vitro* (Zaret *et al.*, 1988; Herbst *et al.*, 1989).

The work described here concerns two hepatoma cell lines, mouse BW1J (Szpirer and Szpirer, 1975) (hereafter BW) and human HepG2 (Knowles *et al.*, 1980), that produce albumin messenger RNA yet fail to efficiently express reporter genes driven by the albumin promoter. The common feature of these hepatoma lines is their expression of fetal hepatic functions, such as α -feto-protein, as well as the expression of functions common to fetal and adult liver, such as albumin and other serum proteins. In addition, both cell lines fail to express the set of hepatocyte-specific metabolic enzymes that characterize the neonatal and adult liver (Cascio and Weiss, 1979; Knowles *et al.*, 1980). Hence, it can be postulated that the mechanism underlying the unexpected behavior of these two cell lines is relevant to the very different profiles of genes expressed in fetal versus adult hepatocytes. Because BW cells show a more strictly fetal phenotype than HepG2 (Kelly and Darlington, 1989), most experiments have been performed on the mouse cells.

Experiments were designed to understand why the transfected albumin promoter exhibits much weaker activity in BW cells than in highly differentiated hepatoma cells. The albumin promoter was first tested in BW cells for its ability to be stimulated by the upstream albumin enhancer (Pinkert *et al.*, 1987), as well as for possible cryptic regulatory elements residing within the

transcribed region of the gene. We next examined BW cells for transcriptional activation of albumin promoter segments by hepatocyte-enriched and ubiquitous transcription factors and found striking differences compared with similar tests of well-differentiated hepatoma cells. Among the hepatocyte-enriched transcription factors, the homeodomain protein HNF1 is the most important for the function of the albumin promoter (Herbomel *et al.*, 1989; Lichtsteiner and Schibler, 1989; Tronche *et al.*, 1989). Both BW and HepG2 cells produce HNF1. However, in contrast to H4II cells, the factor is not sufficient to drive transcription from a minimal promoter. Cotransfection of an HNF1 expression vector into BW cells only partially rescues the promoter activity, suggesting that function of the protein is compromised in the cells. Finally, genetic evidence indicates the existence in BW cells of a dominant factor that blocks HNF1 function and that could account for the anomalous behavior of the cells. Taken together, these results suggest the existence of a novel mechanism by which fetal-like hepatoma cells fail to express a fully differentiated phenotype.

MATERIALS AND METHODS

Cell Culture and Transfection

BW1J (Szpirer and Szpirer, 1975), HepG2 (Knowles *et al.*, 1980), and FGC4 cells (Angrand *et al.*, 1990) were maintained either in modified Ham's F12 medium (Coon and Weiss, 1969) or in a 1:1 mixture of F12 and Dulbecco's modified Eagle's medium (DMEM), with either 5 or 10% fetal calf serum at 37°C. C33 cells (Rey-Campos *et al.*, 1991) were grown in DMEM with 5% fetal calf serum. Cells were transfected by the calcium phosphate procedure as described previously (Heard *et al.*, 1987; Zaret *et al.*, 1988); the amounts of DNA used for each experiment are indicated in Figure legends and the Table. One to 2 d after transfection, cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) and β -galactosidase activity (Heard *et al.*, 1987; Herbomel *et al.*, 1989), or RNA was prepared and specific transcripts from the plasmids were quantitated by the primer extension method (Zaret *et al.*, 1988).

Plasmid Constructions

All of the albumin promoter-containing test plasmids have been described elsewhere (Heard *et al.*, 1987; Zaret *et al.*, 1988; Herbomel *et al.*, 1989; Tronche *et al.*, 1989; Frain *et al.*, 1990). Mouse albumin gene fragments for the enhancer tests were isolated from bacteriophage clones λ alb6 and λ alb8 (Kioussis *et al.*, 1981) and inserted at position -787 of the mouse albumin promoter in the plasmid pAT2 (Zaret *et al.*, 1988). For constructing the mouse albumin promoter oligo-scan (OS) plasmid series, double-stranded oligonucleotides were prepared that spanned the following sequences with respect to the transcription start site (as defined in Gorski *et al.*, 1986; Liu *et al.*, 1988): OS1, -13/-32; OS2, -25/-39; OS3, -33/-52; OS4, -46/-65; OS5, -57/-76; OS6, -69/-87; OS7, -79/-97; OS8, -91/-110; OS9, -104/-120; OS10, -114/-132; OS11, -126/-145; OS12, -138/-157. The oligonucleotides contained additional 5' extensions of three nucleotides that allowed them to form head-to-tail multimers. After annealing and ligation, 6 $\times$ multimers were isolated by gel electrophoresis and inserted at position -109 of the thymidine kinase promoter (McKnight, 1980), which was fused to the neo gene in the plasmid pTN1. The structures of all pTN1-OS plasmids were confirmed by DNA sequencing. Plasmids were prepared as described using two

cesium chloride gradients. For the rat albumin promoter plasmids and the oligoscan OS4 plasmid, *dam*⁻ bacteria were used to avoid *dam* methylation of the GATC sequence in the HNF1 binding site (Lichsteiner and Schibler, 1989; Tronche *et al.*, 1989).

HNF1 Analysis

Northern blot analysis of HNF1 mRNA (Cereghini *et al.*, 1990), preparation of nuclear extracts, and electrophoretic mobility shift assays (Cereghini *et al.*, 1988) were as previously described.

RESULTS

There are many albumin-producing hepatoma cell lines available, but they do not all express reporter functions driven by the albumin promoter. In fact, functional analysis of the albumin promoter has been limited to lines where the vectors are expressed at high levels, ranging from 20 to 100% of the activity obtained with the SV40 early region promoter and enhancer. These lines include H4II rat hepatoma cells and its derivatives (Ott *et al.*, 1984), Hep3B cells (Frain *et al.*, 1990), and mouse and rat hepatocyte lines immortalized by SV40 transformation (Zaret *et al.*, 1988; Herbst *et al.*, 1989). Two widely used albumin-producing hepatoma lines fail to efficiently express different versions of the albumin promoter: mouse BW cells (Izban and Papacostantinou, 1989) and the human hepatoma line HepG2. Three different promoter fragments of the rat albumin gene, extending to -253 with respect to the transcription start site, were tested in vectors containing the CAT reporter gene (Table 1). The two longer constructs show high activity in H4II cells; the -68/+4 fragment, which contains only the TATA box and the binding site for HNF1, shows reduced but still significant activity in H4II. However, none of the fragment showed comparable activity in BW1J or HepG2 cells (Table 1).

Independent assays confirmed the weak activity of the albumin promoter in these cells. To verify that low activity of the albumin promoter is not due to a species difference (rat promoter in mouse cells), we tested a mouse promoter fragment extending to -787 bp, this time using a primer extension assay and the tk gene as a reporter. Transcription from the albumin promoter in BW cells (Figure 1B, lane -) and HepG2 cells was <2% of that recorded with the RSV-LTR linked to the same tk gene (Table 1). Similar constructs containing the human albumin promoter (Frain *et al.*, 1990) also showed very low activity (see Figure 7 below).

To investigate the basis for the failure of BW cells to express the albumin promoter-containing vectors described above, we tested the possibility that additional upstream sequences, including the upstream albumin enhancer, were necessary for high level expression. The entire upstream region from -11 kb was fragmented and inserted in front of the albumin promoter construct extending to -787 bp (Figure 1A). None of the fragments displayed even a twofold stimulatory effect on the weak activity of the promoter (Figure 1B, lanes 1-

Table 1. Expression of rat albumin-CAT and mouse albumin-tk plasmids in various hepatoma cell lines

	H4II	BW1J	HepG2
Rat albumin*			
-253/+16	90	5	15
-151/+16	70 (40-100)	4 (1-10)	7 (2-12)
-68/+4	20 (15-40)	<1	4 (2-6)
Mouse albumin†			
-787/+8	ND‡	1.7 (1.2-2.0)	0.25 (0.09-0.78)

* Values are normalized for that of the internal control cotransfected plasmid, and expressed as percentages of the activity obtained with the SV40 promoter-enhancer. Cells were transfected with 2-2.7 μ g of test plasmid and 1.5-1.8 μ g of RSV-lacZ.

† Values are normalized as above except that they are compared with RSV-tk. Cells were transfected with 6 μ g of test plasmid and 15-75 ng of the RSV-tk plasmid pRT1.

‡ ND, not done.

4), despite the fact that the same sequence bearing the -10-kb enhancer (fragment 1 in Figure 1) functioned efficiently in other hepatocyte-derived cell lines (Zaret *et al.*, 1988; Herbst *et al.*, 1989). Finally, we tested the possibility that an additional enhancer resided downstream of the albumin transcription start site. Various albumin DNA fragments up to 20 kb downstream of the start site, which spanned known DNaseI hypersensitive sites in liver chromatin (Liu *et al.*, 1988) (vertical arrows in Figure 1), were linked to the -787-bp albumin promoter. None of the fragments stimulated the activity of the albumin promoter in transfected BW cells (Figure 1B, lanes 5-10). One interpretation of these results is that the upstream albumin enhancer as well as potential downstream elements are intrinsically inactive in the cells. Alternatively, the albumin promoter could be unable to respond to distal regulatory elements in BW cells. However, this latter possibility is unlikely because the addition of the SV40 enhancer led to a very strong activation of the promoter in BW cells (Figure 1C, lane 2).

Extensive analysis of sequences critical for expression of the rat albumin promoter in H4II hepatoma cells revealed that binding sites for HNF1 and NF-Y contribute, albeit at different levels, to expression (see Tronche *et al.*, 1990 and references therein). To analyze the contribution of the different promoter elements to the low level of expression in BW and HepG2 cells, two different approaches were used. First, selected internal mutations and 5' deletions of the rat promoter previously described (Herbomel *et al.*, 1989) were tested by transfection (Figure 2). For this analysis, significant values can be recovered only from very efficient transfections because the reference -151/+16 CAT plasmid gives only low activity. For comparison, the values reported for H4II cells are included. Although the effect of mutation in the DEI and CAAT elements is slight in H4II cells, these

Figure 1. Lack of enhancer activity of albumin gene fragments in BW1J cells. (A) Restriction fragments of the albumin gene (numbered 1–10) were inserted upstream of the albumin promoter in the vector pAT2, and 15 μ g were transfected into BW1J cells with 1.5 μ g of the internal control vector pSVneo. Schematic drawing at the top shows the positions of the fragments relative to the transcribed portion (wavy line) of the albumin gene (double lines); dark blocks between the double lines indicate exon positions. Arrows above the double lines indicate the positions of DNaseI hypersensitive sites in liver chromatin (Liu *et al.*, 1988). (B) Primer extension analysis of transfected cell RNAs. Extension products are indicated to the right of the figure. Lane M contains a sequencing ladder as size markers, lane s is a primer extension analysis of sham-transfected cells; lane – is of cells that were transfected with the pAT2 vector without an insert; lanes 6 and 7 contain the same fragment in the different orientations shown in the schematic diagram; and lane SV is of cells that were transfected with a vector containing the SV40 promoter, lacking the enhancer, driving tk. The primer extension product from the SV-tk transcript is of a larger size than that from the transcript of pAT2. Densitometry scanning of the albumin promoter signals, relative to the internal control, showed that none of the albumin gene fragments significantly stimulated transcription. (C) Activation of the albumin promoter by the SV40 enhancer. Primer extension analysis of RNAs from cells transfected with 15 μ g of the plasmid pAN2, which contains an albumin promoter fragment from –787 to +8 driving the neo gene (lane 1) and pANS2 containing the SV40 enhancer (lane 2) inserted downstream of the neo gene. These transfections included 0.7 μ g of a plasmid with the SV40 enhancer and promoter driving neo as an internal control. A similar induction of transcription was observed when the SV40 enhancer was placed upstream of the albumin promoter.

mutations decrease activity by 5- to 10-fold in BW. This implies for BW cells a more important role of the DEI and CAAT elements. HepG2 cells show an intermediate response to these mutations. The role of the proximal element (PE) was first examined by transfection of the minimal promoter –68/+4 that contains only PE and the TATA element. This minimal promoter is totally devoid of activity in BW, demonstrating a deficiency in the activity of HNF1, the factor that binds to PE. By contrast, the same –68/+4 construct was highly active in H4II cells. However, modifications

that reduce the binding of HNF1 to PE render the full-length promoter dependent on upstream elements in H4II cells (Tronche *et al.*, 1989). Effectively, in BW cells, a similar situation prevails: mutation of the PE site (LS –67/–55) destroys activity, but the PE element alone permits no activity in BW, implying an absolute requirement for cooperation with upstream elements (see DISCUSSION). These observations serve to highlight a critical difference in the activity of HNF1 between H4II and BW cells: in the former cells an intact HNF1 site is necessary and sufficient

Figure 2. Effect of internal mutations in the albumin promoter. Details of the constructions, including the sequences mutated and substituted, are given in Tronche *et al.* (1989). Here, black boxes indicate the binding sites (top line) and the sequences mutated (lower lines). The results shown by BW1J and HepG2 are from one representative experiment. The values for H4II are taken from Tronche *et al.* (1989). Nil, a significantly positive value was not obtained. The dashes indicate that the transfection was not performed. The values obtained with the -151/+16 promoter are set at 100 to permit comparison with the mutant promoters. For BW1J cells, activity of the -151/+16 construct was 4% of SV-CAT, and for HepG2 it was 12%.

for promoter activity, whereas in the latter it is necessary but not sufficient.

The second approach, "oligo-scan" depicted in Figure 3, involved a scan of the mouse promoter for sequences that might repress or augment a reporter gene. The mouse promoter sequence is nearly identical to that for the rat in this region (Heard *et al.*, 1987). Homopolymeric promoter segments were inserted upstream of the thymidine kinase promoter; together, the 12 segments tested spanned from positions -13 to -157 of the mouse promoter. The ability of the multimers to stimulate activity of the thymidine kinase promoter, when normalized to an internal transfection control, is depicted in Figure 3B and quantitated in C. The only oligo-scan construction that shows an inhibitory effect is OS9, corresponding to the DE11 element, which is an NF1 binding site (Cereghini *et al.*, 1987). The inhibition is only slight and entirely compatible with the fact that mutation of this element leads to slight increases in reporter gene activity (Figure 2, mutant LS -121/-114). Consequently, the weak activity of the albumin promoter in BW cells cannot be explained by a strong repressor acting on one of the promoter elements.

A positive effect on transcription was observed for three of the oligomerized segments, which span sequences just upstream of the TATA box (OS3); the CCAAT element (OS7), which binds NF-Y (Raymondjean *et al.*, 1988); and a portion of the DEIII element (OS11) (Herbomel *et al.*, 1989). Of these sequences, the OS3 and OS7 segments are bound by transcription factors found in numerous cell types (Raymondjean *et al.*, 1988; Maire *et al.*, 1989). Strikingly, the segments that bind the liver-enriched transcription factors HNF1 (OS4) and C/EBP (OS8) failed to show either a stimulation or inhibition of transcription. FGC4, a well-characterized and transfectable subline of H4II (Angrand *et al.*, 1992) was transfected as a positive control. Figure 4 shows that the OS4 segment markedly stimulated the thymidine kinase promoter in FGC4 cells, whereas the OS7 segment had no effect. The studies of

the multimerized HNF1 site also demonstrated that the inactivity of HNF1 in BW1J cells is not specific for the albumin promoter, because the multimers efficiently stimulated the heterologous tk promoter in FGC4 cells. Thus, the pTN1-OS4 construct is competent to respond to HNF1. The absence of an effect of the oligomerized HNF1 site in BW cells is in agreement with the fact that the -68/+4 promoter, which contains only the TATA box and PE, fails to show activity. These results underscore the necessity of cooperation between PE and the upstream elements of the albumin promoter.

Given the importance of HNF1, BW and HepG2 cells were examined for the expression of this transcription factor. Poly A⁺ RNA from the cells was used to prepare Northern blots hybridized to an HNF1 cDNA probe (Cereghini *et al.*, 1990). Figure 5A shows that both BW and HepG2 cells produce HNF1 mRNA of the size expected for the lines' species of origin. Note that this RNA is particularly abundant in HepG2 cells. Mendel *et al.* (1991b) have recently reported on the existence of DCoH, a protein involved in stabilization of HNF1 dimers. Figure 5B shows that both BW and HepG2 cells produce the corresponding mRNA.

Next, HNF1 protein was examined for its DNA binding properties, using a competition assay with a series of oligonucleotides containing mutations at bases known to be critical for binding (Cereghini *et al.*, 1988). Figure 6A shows the results: binding and competition patterns for HNF1 from HepG2 and BW cells were identical to those for rat liver, showing that changes in DNA binding cannot explain the deficiency of function in these cells. The amounts of HNF1 binding activities were within a two- to threefold range of that observed in rat or mouse liver nuclear extracts.

Proteins binding to the HNF1 site include not only HNF1 but also vHNF1 (LF-B3 or HNF1β) (De Simone *et al.*, 1991; Mendel *et al.*, 1991a; Rey-Campos *et al.*, 1991), which can also function as an activator via its binding sites. HNF1 and vHNF1 have been shown to heterodimerize. The binding of these proteins and their

Figure 3. Oligo-scan analysis of the mouse albumin promoter in BW1J cells. (A) Strategy for preparing oligo-scan (OS) constructs. The double line at the top indicates the albumin promoter sequence; double line segments below the line indicate the regions spanned by 15–20 bp double-stranded oligonucleotides (OS1–OS12). The OS segments were inserted as sixfold head-to-tail multimers in front of the thymidine kinase promoter, which was fused to the neo gene. A schematic view of the tk promoter and prominent factor binding sites (McKnight, 1980) is shown. (B) Fifteen-microgram amounts of the pTN1-OS plasmids were transfected into BW1J cells with 0.4 μ g of RSV-tk plasmid pRT1, and 2 d later transcripts from the plasmids were quantitated by primer extension. The respective extension products are indicated at the right side of the autoradiograph. Lane m, sequencing marker lane; lane s, from sham-transfected cells; lane c, cells transfected with the pTN1 vector only; lane –, cells transfected with the pTN1 vector without an insert. (C) Quantitation of the effects of the albumin segments on the tk promoter. The pTN1-OS and pTN1 signals were quantitated by laser densitometry scanning and normalized to the internal control in each lane; the ratios of normalized values for each OS multimer to that of the basal promoter are shown. Stippled bars indicate OS elements that reproducibly affected tk promoter activity. OS4 and OS8, which had no effect, represent binding sites for HNF1 and C/EBP, respectively. Note that OS3 corresponds to a region of the mouse promoter that clearly binds C/EBP, whereas the corresponding region of the rat promoter shows no binding (Cereghini *et al.*, 1987; Lichtsteiner *et al.*, 1987).

Figure 4. Oligo-scan analysis in differentiated FGC4 hepatoma cells. (A) pTB1-OS plasmids were transfected into FGC4 cells and resulting transcripts were quantitated as described in Figure 3B. (B) Quantitation of primer extension signals as in Figure 3C.

heterodimers to the probe corresponding to the albumin proximal element (HNF1 binding site) was examined at high resolution in the electromobility shift assay shown in Figure 6B. Extracts from dedifferentiated H5 rat hepatoma cells, which lack HNF1, provide a positive control for the migration of vHNF1 homodimers (Rey-Campos *et al.*, 1991). These studies revealed HNF1 and vHNF1 homodimers and HNF1/vHNF1 heterodimers in both HepG2 and BW cells, although BW had slightly less of all binding species. In addition, no novel binding activity, which might act to displace HNF1 and vHNF1, was detected in either of the lines (Figure 6, A and B).

These results demonstrate that HNF1, shown from earlier results to be necessary for expression of the al-

Figure 5. Presence of HNF1 mRNA and DCoH mRNA in hepatoma cell lines. Three micrograms of polyA⁺ RNA from the indicated cell lines and from rat and mouse liver were fractionated on agarose-formaldehyde gels, transferred to a membrane, and hybridized with rat HNF1 and GAPDH probes (A) and then with a DCoH probe (B) as described (Cereghini *et al.*, 1990). The positions of specific mRNAs are indicated at the right of the autoradiograph and the HNF1 and GAPDH mRNA sizes in kilobases are indicated on the left. The two HNF1 mRNA species of 3.6 and 3.2 kb, observed in rat-derived tissues or cell lines, are due to the use of different polyadenylation sites (Chouard *et al.*, 1990); human and mouse tissues and cells contain a unique HNF1 mRNA species of 3.6 kb, respectively. H4II rat cell mRNA serves as a positive control for HNF1 and DCoH mRNA in hepatoma cells.

Figure 6. Presence of HNF1 DNA binding activity in HepG2, BW1J, and FOB10 (Fao × BW1J hybrid clone) cells. Electrophoretic mobility shift assays of nuclear protein from the indicated sources. (A) Binding reactions contained 10 µg of nuclear protein, 0.2 ng of 32P-labeled PE56 oligonucleotide as probe, and, when indicated, 20 ng of wild-type PE56 or mutant oligonucleotides that either compete weakly (mut3, left, mut4; right) or not at all (DS34) (Cereghini *et al.*, 1988). (B) Six and 10 µg of the indicated nuclear extracts were analyzed on a gel that was run for a longer time to resolve the binding species. The positions of HNF1 and vHNF1 homodimers and HNF1:vHNF1 heterodimers are indicated. The H5 extract serves as a control for the migration of vHNF1 homodimers. The slowest migrating complex observed with high-resolution gel shift assays in both BW and HepG2 nuclear extracts is not a peculiarity of these cell lines. It has been observed previously in fully differentiated hepatoma cells, notably H4II, and appears to be composed of HNF1 as revealed by ultraviolet cross-linking experiments (see Rey-Campos *et al.*, 1991, for a discussion).

bumin proximal promoter, is present in HepG2 and BW cells and shows identical binding properties compared with rat liver and H4II cells. Since BW cells contain slightly less of the binding activity than liver (Figure 5) and results obtained from transfection of linker scan mutants in BW parallel those obtained in H4II when a binding site showing slightly reduced affinity for HNF1 is used, it could be postulated that HNF1 is limiting. To

test this hypothesis, an HNF1 expression vector was cotransfected with the albumin promoter.

A series of cotransfections was carried out with different amounts of the HNF1 expression plasmid (Rey-Campos *et al.*, 1991), using as recipient cells HepG2 and BW and as a positive control C33. C33 is a line derived from a cervical carcinoma and has been used, in cotransfection tests with the albumin promoter, to demonstrate the trans-activation potential of both HNF1 and vHNF1 (Rey-Campos *et al.*, 1991). As shown in Figure 7, both C33 and HepG2 cells show a strong stimulation, even with low ratios of the HNF1 expression vector relative to the albumin-CAT construct (upper curve). The stimulation observed in HepG2 cells reaches a maximum close to 10-fold with either the -151/+16 or -68/+4 fragments of the rat promoter. The degree of sensitivity and responsiveness of HepG2 cells is that expected of cells that are limiting for HNF1 protein; in fact, the dose-response curve exactly parallels the curve obtained with HNF1-deficient C33 cells.

BW cells gave different results. Here, trans-activation with exogenous HNF1 was significantly weaker, especially considering the ratio of HNF1 expression vector relative to the test construct necessary to obtain the effect (Figure 7, lower curve). With a variety of albumin promoter fragments of both rat and human origin, the maximal trans-activation is generally limited to two- to fourfold (the range extending from 1 to 6), even at high ratios of HNF1 expression vector to albumin test constructs. The trans-activation obtained remained inferior to twofold even when an expression vector for DCoH (unpublished data), the protein shown to be implicated in stabilization of HNF1 dimers (Mendel *et al.*, 1991b), was included. BW cells are thus only poorly responsive to exogenous HNF1, implying a more complex situation than a simple deficiency of active protein. These observations are compatible with the idea that BW cells possess an activity that interferes with HNF1 function or that they lack an activity necessary to render HNF1 competent. This "activity" could be a modification of the protein or a cofactor.

To examine whether the defect in HNF1 function is dominant, we turned to a hybrid clone, FOB10 (H4II derived Fao cells × BW) isolated previously and found to show expression of both rat and mouse albumin (Cassio *et al.*, 1980). Cells of this hybrid were transfected with the -151/+16 rat albumin-CAT plasmid. As described above for the BW parent, expression was <5% of that obtained with the SV40 enhancer-promoter construct (Figure 8). Because FOB10 hybrid cells contain HNF1 (Figure 6B), we conclude that the defect in expression of the albumin promoter and function of HNF1 in BW cells is epistatic.

When the hybrid cells were cotransfected with the albumin promoter construct -151/+16 and an HNF1 expression vector, trans-activation in the range of that obtained in BW was observed (Figure 7). These findings

Figure 7. Trans-activation of different versions of the albumin promoter with a cotransfected HNF1 expression vector (Rey-Campos *et al.*, 1991). Different amounts of expression vector were cotransfected with a constant amount of the designated albumin promoter test plasmids, and CAT and β -galactosidase levels were quantitated as described (Heard *et al.*, 1987). The fold activation by HNF1 is plotted against the ratio of HNF1 expression to test plasmid, normalized to β -galactosidase. In the legends, the symbols are indicated as well as the cell line used, and the 5' extremity of the promoter fragment, including two of human (H) origin that span $-486/+1$ and $-221/+1$. Cells were transfected with 2–2.7 μ g of test plasmid in 6-cm dishes or 10 μ g plasmid in 10-cm dishes and included the indicated amounts of RSV-HNF1 plasmid (the RSV-LTR concentration being held constant by inclusion of the appropriate amount of RSV-luc). RSV-lacZ was included as specified in Table 1. Note that a single line has been drawn for BW1J cells, even though a variety of promoters were used; the dispersion of the individual points more likely represents experimental variation than differences in the behavior of the promoters.

show that even though the rat hepatoma Fao parental cells are known to possess transcriptionally competent HNF1, a function of the hybrid cells leads to its inactivation.

DISCUSSION

Here, we have investigated the unexpected behavior of two hepatoma lines, human HepG2 and mouse BW cells, that express the endogenous albumin gene and show only weak transcription from a transfected albumin promoter. Our strategy has consisted of a search for sequences capable of stimulating transcription from the albumin promoter. Subsequently, we evaluated the importance of previously characterized binding sites present in the albumin promoter and searched for sequences involved in negative regulation. Finally, HepG2 and BW cells have been characterized for expression of HNF1, a factor known to be critical for transcription from the albumin promoter.

A scan of fragments of the entire albumin locus for regulatory activity revealed no evidence for a site of either "enhancement" or "repression" in BW cells that concerns either a fragment previously identified as a potential regulatory region, such as DNaseI hypersensitive sites or others that had not been studied. Furthermore, the upstream albumin enhancer was unable to stimulate the promoter in BW cells (Figure 1B) and HepG2 cells (DiPersio *et al.*, 1991). Failure to respond to the albumin enhancer is not due to an inability of the promoter to respond to activating sequences: the SV40 enhancer added upstream or downstream causes

a dramatic stimulation in promoter activity in BW and HepG2 as well as all cell lines that have been tested (Figure 1C) Rollier and Tronche, unpublished data). It can be concluded that the inactivity of the albumin enhancer, and presumably its binding factors (Liu *et al.*, 1991; DiPersio *et al.*, 1991), is one level at which the cells lack a regulatory function.

We have shown previously for H4II cells that the strength and tissue-specific character of the rat albumin promoter depend on the HNF1 binding site and, only marginally, on the NF-Y site (Tronche *et al.*, 1991). The situation is very different in BW cells. Although the HNF1 binding site is required, it is not sufficient to drive transcription: cooperation of upstream sites binding NF-Y and C/EBP family members is now necessary. We recover here, in a natural context, the artificial situation previously observed for H4II cells where an HNF1 site of slightly reduced binding efficiency owing to bacterial methylation similarly required cooperation of upstream elements (Tronche *et al.*, 1989). The ability of HNF1 site multimers to stimulate the tk promoter in FGC4 cells, but not in BW1J, is inherent to the factor and not specific to its interactions with other proteins that bind to the albumin promoter.

The oligo-scan analysis of the mouse albumin promoter confirmed the activity of NF-Y in BW and revealed two further possibly active regions: one located just upstream of the TATA element, OS3 (and recognized by various unidentified proteins) (Maire *et al.*, 1989), and another, OS11, corresponding to a portion of the rat DEIII site of the promoter (Tronche *et al.*, 1990) that has been shown to bind various liver-en-

Figure 8. CAT chromatogram. Fao, hybrid FOB10, and BW1J were transfected with the SV-CAT and rat albumin (-151/+16)-CAT plasmids (10-cm dishes, 10 μ g plasmid) and assayed as described.

riched factors (Cereghini *et al.*, 1987; Lichtsteiner *et al.*, 1987; Zaret *et al.*, 1990). However, in the context of H4II cells or in liver nuclear extracts, where all regulatory elements of the albumin promoter are functional, the DEIII site does not play a prominent role.

The principal conclusion to be drawn from these studies is that the transcription of the endogenous albumin gene in BW1J cells may not be a consequence of the activity of previously characterized liver-enriched transcription factors and cis elements to which they bind. We suggest that weak additive effects of the ubiquitous factors are sufficient to ensure transcription, although we cannot eliminate the possibility that very far upstream or downstream cis elements are involved.

We have focused on HNF1 because this factor is known to be critical for transcription from the albumin promoter and is present in both HepG2 and BW cells. Both cell lines express the normal size of the HNF1 and DCoH mRNA. Gel mobility shift assays reveal HNF1 binding activity which is 1) nuclear, because the extracts are prepared from purified nuclei; 2) fairly proportional to the amount of mRNA, by comparison with lines such as H4II that express functional HNF1; and 3) composed of HNF1 and vHNF1 homo- and heterodimers in the proportions expected. These analyses fail to reveal the existence of novel species that could act to compete, dissociate, or destabilize homo- and heterodimers of HNF1 and vHNF1 (e.g., Mendel *et al.*, 1991b; Ron and Hebener, 1992).

It has been reported that hybrids showing extinction of HNF1-dependent functions lack the binding activity (Baumhueter *et al.*, 1988; Cereghini *et al.*, 1988) and show absence of transcription of the HNF1 gene (Cereghini *et al.*, 1990). Our findings differ in that the absence of transcriptional activation by HNF1 in BW cells is not due to a loss of the protein or its DNA binding capacity. We conclude that the regulation of HNF1 can be controlled both at levels of synthesis and function.

In HepG2 cells, cotransfection of promoter constructs and an HNF1 expression vector results in trans-activation, showing a dose-response similar to that observed with HNF1-deficient C33 cells. These observations imply either that endogenous HNF1 in HepG2 cells lacks

transcription activation potential in the context of the albumin promoter or that a negative factor is involved that can be titrated readily by relatively small increases in HNF1. BW cells also produce HNF1, but trans-activation of the albumin promoter by an HNF1 expression vector is relatively inefficient. In addition, the phenotype is fully epistatic in hybrids: the albumin promoter is expressed only at low levels, and trans-activation by exogenous HNF1 is inefficient. These results imply that BW contributes a factor, acting in trans, that inhibits the activity of HNF1. Although by the criterion of the trans-activation test BW and HepG2 appear to constitute different phenotypic classes, the difference could be quantitative rather than qualitative.

Two classes of hypotheses, including cofactor molecules and posttranslational modifications, should be considered in the light of the results presented. BW and HepG2 cells could lack a coactivator (see Berger *et al.*, 1992) that mediates an interaction between HNF1 and the transcription complex. A posttranslational modification could activate or inactivate the protein: both types of effects could be at least partially reversed in the trans-activation test where an excess of substrate is provided. The ensemble of results presented indicate that BW cells may contain a factor that inhibits a function required specifically for transcriptional activation. This is similar in principle to the "lack of activation" phenotype imposed by the TSE1 tissue specific extinguisher, which encodes a regulatory subunit of protein kinase A (Boschart *et al.*, 1991; Jones *et al.*, 1991). Overexpression of TSE1 causes a failure to express the TAT gene, apparently by inhibiting the basal activity of protein kinase A and, consequently, the transcription factor CREB. Because TSE1 does not affect albumin expression (Chin and Fournier, 1989), TSE1 and the inhibitor of HNF1 activity that we have described are likely to be distinct.

During the transition from fetus to neonate, changes in the hormonal and cellular environment alter the pattern of genes expressed in hepatocytes. To the extent that BW cells mimic the gene expression pattern of fetal hepatocytes, we suggest that the functional state of transcription factors that we have discovered for BW cells might also characterize the fetal state. By this model, ubiquitous factors binding to the albumin promoter would play a more prominent role in fetal hepatocytes than the host of liver-enriched factors that bind the promoter and enhancer in adult liver. Further study will be needed to discern if hormonal and other intercellular regulatory signals that induce the fetal to neonatal transition in vivo could shift the balance of transcription factor activities in BW cells. Experiments to address this prediction are in progress.

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