Functional Analyses of Albumin Expression in a Series of Hepatocyte Cell Lines and in Primary Hepatocytes

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Abstract
A series of simian virus 40-immortalized hepatocyte cell lines which are heterogeneous with regard to expression of albumin protein and RNA were characterized for their ability to transcribe the albumin gene. Nascent chain extension assay showed that albumin RNA levels in these cells were determined predominantly at the transcription level. The albumin promoter and enhancer sequences were fused to the bacterial chloramphenicol acetyltransferase gene; the ability of the resulting expression constructs to drive chloramphenicol acetyltransferase expression after transfection into these hepatocyte cell lines was measured. The activity of the albumin promoter and enhancer constructs in primary hepatocytes was also measured. The albumin promoter was expressed differentially in these cells; however, no correlation was found between the transcriptional efficiency of the transfected albumin promoter and endogenous albumin transcription. The albumin enhancer was functional in some but not all albumin-positive cells. The minimal albumin enhancer was mapped to a 330-base pair fragment extending from −9.94 kilobases (kb) to −10.27 kb; three elements within this fragment recently shown to be necessary for enhancer function in a murine hepatocyte cell line were also essential for albumin enhancer function in the rat hepatocyte cell line CWSV1. A transcriptional silencer was identified which could suppress the expression of the homologous albumin promoter and the heterologous herpes simplex virus thymidine kinase promoter. Preliminary analysis localized the albumin silencer between −11 and −12 kb. Our results suggest that multiple regulatory sequences may act cooperatively to determine efficient tissue-specific expression of the albumin gene.

Introduction
With the long-term goal of understanding the basis of liver cell phenotypes, we developed a series of cell lines from normal adult rat hepatocytes transfected with SV40 DNA (1–4). These cell lines (designated by CWSV) exhibit considerable heterogeneity with respect to the expression of albumin and a variety of other liver-specific proteins. Furthermore, albumin gene expression can be regulated in these cell lines in response to hormone (dexamethasone), changes in cell density, and agents that trigger the acute-phase response. This "natural" variation in albumin gene expression among the CWSV cell lines, as well as their capacity to express albumin at liver-like levels and to regulate albumin expression, makes them valuable in elucidating the control mechanisms underlying albumin gene expression and its response to internal and external stimuli.

Serum albumin is a major protein synthesized almost exclusively by liver cells in all mammals, and its concentration increases from low levels early in fetal development to a high plateau level in the adult (5); down-regulation of albumin gene expression has been observed under some physiological states such as the acute-phase reaction (2, 6). Albumin gene expression appears to be regulated mainly at the level of transcription initiation (5, 7). Transient expression experiments in hepatoma cell cultures and in vitro transcription assays using the rat and mouse albumin promoters (8–11) have shown that 150 bp of DNA sequences immediately upstream from the cap site are able to direct hepatocyte-specific transcription of the albumin gene. Within this promoter sequence, which is highly conserved throughout mammalian evolution (12–14), are tightly packed at least six motifs that are binding sites for both liver-enriched (HNF-1, C/EBP, DBP, LAP) and general transcriptional factors (NF-1, NF-Y) (12, 13, 15–27). However, in contrast to its high activity in some hepatoma cell lines (10, 21), the albumin promoter by itself is only weakly expressed in most hepatoma cells (9, 28–30) and is not expressed when introduced into transgenic mice (31–33).

It was demonstrated using transgenic mice that a transcriptional enhancer located at −10 kb is essential for the expression of the albumin gene in vivo (33). The albumin enhancer is not functional in virtually all hepatoma cell lines tested except in two well-differentiated hepatocyte cell lines (9, 31, 34, 35). Initial mapping analysis using CWSV1, an SV40-immortalized rat hepatocyte cell line, indicated that the albumin enhancer consists of a 130-bp core enhancer element (−9.94 to −10.07 kb) which by itself can enhance the expression of the albumin

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5 The abbreviations used are: SV40, simian virus 40; kb, kilobase(s); bp, base pair(s); CAT, chloramphenicol acetyltransferase; HSV, herpes simplex virus; tk, thymidine kinase; PCR, polymerase chain reaction.
promoter in a cell type-specific fashion (34). However, a more recent report using an SV40-immortalized murine hepatocyte cell line showed that more than 300 bp of DNA sequence (mapping within the same general vicinity), which binds to at least three liver-specific transcriptional factors, is required for albumin enhancer function (35).

The underlying mechanism for the differences observed when the activity of the albumin promoter and enhancer was examined in vivo in transgenic mice and in vitro in hepatoma cell lines is presently unclear. Because the hepatoma cells transcribe the albumin gene only at a very low level compared to liver (36, 37) and do not resemble normal hepatocytes phenotypically (38), it is difficult to assess the functional significance of the albumin-regulatory sequences in liver based on studies performed in hepatoma cells. Although it has been possible to measure the functional activity of the albumin enhancer in two well-differentiated cell lines, the discrepancy observed in mapping the albumin enhancer using the rat CWSV1 cell line and the murine H2.35 cell line has not been resolved. Neither of the two different mapping results has been independently confirmed. In this report, we have analyzed the activity of the albumin-regulatory sequences using our highly differentiated CWSV cell lines as well as primary hepatocytes in culture as model systems. First, we show that several CWSV cell lines transcribe the albumin gene at a liver-like level. These are the only hepatocyte cell lines that have this capacity. We demonstrate, in five albumin-positive cell types, four hepatocyte cell lines, and primary hepatocytes in culture, that there is no correlation between endogenous albumin transcription rate and albumin promoter activity and that there may be a correlation between the function of the albumin enhancer and the highly differentiated state of hepatocytes. We show for the first time that the albumin enhancer is functional when transfected into well-differentiated primary hepatocytes in culture; in fact, the enhancer is more active in primary hepatocytes than in any cell line. We demonstrate that the albumin enhancer is functional in a second SV40-immortalized hepatocyte cell line, CWSV2, in addition to CWSV1. Using CWSV1 cells, we map the albumin enhancer to a 330-bp fragment (−9.94 to −10.27 kb), and we demonstrate that at least three binding sites within this fragment are essential for enhancer function. This is in agreement with recently published findings using the murine H2.35 cell line (35). We also demonstrate for the first time that an albumin transcriptional silencer is present further upstream of the enhancer (−11 to −12 kb), and the silencer may be involved in ensuring hepatocyte-specific expression of albumin.

Results
Some SV40-immortalized Hepatocyte Cell Lines Transcribe the Albumin Gene at a Liver-like Level. We have shown in our previous characterizations of SV40-immortalized hepatocyte cell lines that the CWSV1, CWSV2, and CWSV14 cell lines secrete albumin and that the amount of albumin secreted by CWSV14 was less than that secreted by CWSV1 and CWSV2 (3). The CWSV8 cells beyond passage 12 lose the ability to secrete albumin (3). Northern blot hybridization revealed that the albumin mRNA levels for CWSV1, CWSV2, and CWSV14 are high, approaching the levels of albumin mRNA in liver (39).

In this study, we determined the ability of CWSV1, CWSV2, CWSV8, and CWSV14 to transcribe albumin RNA using isolated nuclei. Endogenous albumin transcription in CWSV1 and CWSV2 cells was equal to or greater than in nuclei isolated from liver (Table 1). In CWSV14 cells, albumin transcription approached, but did not equal, that found in liver. In CWSV8 cells, transcription of albumin RNA was essentially undetectable. We also measured the albumin transcription rate in a tumor cell line, NRAT-1, which produces high levels of albumin protein and mRNA (see “Materials and Methods” for further description; 40). The albumin transcription rate in NRAT-1 cells was about 3-fold higher than that in CWSV1 cells, which was consistent with high albumin mRNA levels in these cells.

Transcription of albumin in primary hepatocytes maintained in culture in a chemically defined medium supplemented with 2% dimethyloxal propan am 2%; it ranged from 11 to 37% of albumin transcription from liver nuclei (Table 1) and was lower than that in the CWSV1, CWSV2, and CWSV14 cell lines. The levels of albumin transcription in the CWSV cell lines and primary hepatocytes paralleled the levels of albumin steady-state RNA as measured by Northern blot analyses, indicating that endogenous albumin RNA levels are determined predominantly at the transcription level in these cells.

Activity of Albumin Promoter Differs in Different Hepatocyte Cell Lines and Primary Hepatocytes. The mouse albumin promoter sequence from −282 to +21 bp relative to the transcription start site was fused to the coding region of the bacterial CAT reporter gene (Fig. 1), and the recombinant plasmid plgCAT was transiently transfected into several different SV40-immortalized rat hepatocyte cell lines, CWSV1, CWSV2, and CWSV14 (see Table 1). In order to correct for the variation in transfection efficiency between individual experiments and be-

Table 1  Comparison of endogenous albumin transcription with the activities of transfected albumin promoter and enhancer in hepatocyte cell lines and primary hepatocytes

<table>
<thead>
<tr>
<th>Cell</th>
<th>Albumin mRNA*</th>
<th>Endogenous transcription (%)a</th>
<th>Relative promoter activityb</th>
<th>Enhancer activityc</th>
</tr>
</thead>
<tbody>
<tr>
<td>CWSV1</td>
<td>89 ± 6.1</td>
<td>145 ± 47</td>
<td>100</td>
<td>4.2 ± 1.47</td>
</tr>
<tr>
<td>CWSV2</td>
<td>62 ± 4.5</td>
<td>141 ± 30</td>
<td>119 ± 23</td>
<td>4.0 ± 0.72</td>
</tr>
<tr>
<td>CWSV14</td>
<td>38 ± 11</td>
<td>69 ± 25</td>
<td>387 ± 158</td>
<td>1.0 ± 0.35</td>
</tr>
<tr>
<td>NRAT-1</td>
<td>222</td>
<td>416 ± 271</td>
<td>120 ± 20</td>
<td>1.5 ± 0.00</td>
</tr>
<tr>
<td>CWSV8</td>
<td>1.3 ± 1.0</td>
<td>1.4 ± 1.9</td>
<td>4.9 ± 0.7</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>Primary hepatocytes</td>
<td>45</td>
<td>22 ± 11</td>
<td>0.6 ± 0.1</td>
<td>11.0 ± 1.93</td>
</tr>
</tbody>
</table>

*As determined by Northern blot analysis and expressed as percentage of liver albumin RNA level.

As determined by nascent chain extension and expressed relative to that in liver, which was set to 100%.

CAT activity from plgCAT in each cell type was expressed relative to that in CWSV1 cells, which was set to 100. It is important to note that promoter activity has been calculated so that the promoter strength in each cell type is being compared to CWSV1.

The values given represent fold increase in normalized CAT activity from p-α-γ-α1bCAT over that from plgCAT in each cell type. To determine enhancer activity, the promoter activity for each cell type was arbitrarily set to 1.
between different plasmids and cell lines, we chose to quantitate the plasmid DNA level in the transfected cells so that we could directly compare the reporter gene expressions relative to the gene copy number (see "Materials and Methods" for details). As judged by the CAT enzyme activity, the albumin promoter was actively transcribed in CWSV1, CWSV2, and CWSV14 cells. The transfected mouse albumin promoter showed similar activity in driving CAT expression in both CWSV1 and CWSV2 cells; however, it was more than 3 times as active in CWSV14 as in CWSV1 and CWSV2 cells. When transfected into the tumor cell line NRAT-1, the mouse albumin promoter demonstrated about the same level of activity as was seen in CWSV1 and CWSV2 cells. In the albumin-negative cell line CWSV8, the mouse albumin promoter was more than 20-fold less active than in CWSV1 cells, although it retained residual activity in that pSalB consistently gave about 3-fold higher activity than the promoterless CAT construct pOCAT.

The rat and mouse albumin promoters are 95% homologous over 180 nucleotides upstream of the initiation site (12, 13). To determine whether there was a species-specific effect on promoter activity, the rat albumin promoter sequence from −180 and +16 bp relative to the transcription start site was fused upstream to the CAT coding region to make pRSalB. When tested in CWSV1 cells, pRSalB gave 98.5% of the CAT expression of pSalB. This finding was in agreement with previous studies using hepatoma cells, showing that the rat and mouse albumin promoters behave similarly (11), and it supports the concept that albumin promoter function is not species specific (29, 30).

The mouse albumin promoter was also tested in primary rat hepatocyte cultures, which transcribe the endogenous albumin gene at approximately 22% of the level of that in rat liver (Table 1). The transfected mouse albumin promoter showed extremely low activity: 170-fold lower than in CWSV1 cells and 8-fold lower than in CWSV8 cells. This very low CAT expression was 4-fold higher than the activity from p0CAT in these cells, indicating that the albumin promoter exhibits residual activity in primary rat hepatocytes. Data are shown for the primary hepatocytes cultured in vitro for 15 days; similar results were obtained for primary hepatocytes cultured for 2 days (data not shown).

Mapping of the Mouse Albumin Enhancer in CWSV1 Cells. The mouse albumin enhancer sequences from −10.5 to −8.7 kb and various deletion mutants of the enhancer region were inserted upstream of the pSalB construct (Fig. 1) and were tested for enhancer activity in CWSV1 cells (Fig. 2). In agreement with previous findings (33, 34), the mouse albumin enhancer sequences from −10.5 to −8.7 kb (p−8.7 SalBcat) increased the CAT expression about 4-fold as compared with the enhancerless pSalB construct; this increase was seen in the albumin-expressing CWSV1 cell line and not in the albumin-negative CWSV8 cells.

Similar results were obtained with p−8.9 SalBcat, which lacks 200 bp at the 3' end of the enhancer domain. Consistent with this result, when the 200-bp region from −8.7 to −8.9 kb was fused upstream of the mouse albumin promoter and transfected into the CWSV1 cells, no enhancement of the albumin promoter was observed. These findings demonstrated that deletion of the 200-bp fragment from −8.7 to −8.9 kb, which has been shown to contain the binding sites for the ubiquitous transcription factor NF-1 and the liver-specific factor HNF-3 and was implicated in suppressing albumin enhancer activity (34), had no effect on the strength of the mouse albumin enhancer.

Further deletion from −8.7 to −9.94 kb of the mouse albumin enhancer had little deleterious effect on enhancer activity; the construct p−9.94 SalBcat enhanced the expression of the albumin promoter to about the same extent as the construct p−8.7 SalBcat in CWSV1 cells. However, when a 90-bp fragment containing sequences from −9.94 to −10.03 kb was tested, no enhancer activity was detected in spite of the fact that this same fragment has been previously postulated to be the albumin core enhancer element (34).

While this study was in progress, Liu et al. (35) reported that at least three elements within 330 bp of the mouse albumin enhancer sequences from −9.94 to −10.27 kb were necessary for enhancer function in a murine hepatocyte cell line. Three liver-specific (enriched) transcriptional factors, C/EBP or a C/EBP-related factor, HNF-3, and an unidentified factor called eH-TF, bind to the eE, eG, and eH sites within this 330-bp fragment. The eE and eG sites were outside the 130-bp core enhancer

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Fig. 1. Construction of albumin promoter/enhancer-containing plasmids. A, partial restriction map of the upstream region from −8.7 to −12 kb of the mouse albumin gene (coordinates per Refs. 33 and 34). Restriction sites: A, AccI; B, EcoRV; C, BglII; H, HindIII; P, PstI; T, StyI; V, EcoRV. B, CAT plasmids constructed in pUC8 vector contained SV40 early gene polyadenylation signal downstream from the CAT coding region. The mouse albumin promoter (−282 to +21 bp), the rat albumin promoter (−180 to +16 bp), the HV-Ik promoter, and the mouse albumin gene upstream sequences are marked.
sequences identified by Herbst et al. (34). We inserted the 330-bp sequence from −9.94 to −10.27 kb and its derivatives with the eE or eG site mutated (35) upstream to the albumin promoter in palbCAT. The 5' portion of this 330-bp fragment, spanning from −10.03 to −10.27 kb which contained the eE and eG sites but not an eH site, was also cloned into palbCAT. The intact 330-bp enhancer fragment increased the expression of the albumin promoter to the same extent as the 1.8-kb region from −8.7 to −10.5 kb did in CWSV1 cells. However, mutation of either the eE (pmtAlbCAT) or eG (pmGalbCAT) site or deletion of the eH site (p−10.27 AlbCAT) abolished enhancer function (Fig. 3).

To test whether the albumin enhancer was able to increase transcription from a heterologous promoter, the sequence from −8.9 to −10.5 kb was fused to the HSV-tk promoter in a vector directing CAT expression. There were no differences in the CAT activity of CWSV1 cells transfected with p−8.9 to −10.5 tkCAT and an enhancerless ptkCAT control plasmid, indicating that the albumin enhancer does not function on the heterologous HSV-tk promoter (data not shown).
than palbCAT in the hepatocytes. Data are shown for primary hepatocytes cultured in vitro for 15 days; similar results were obtained for primary hepatocytes cultured for 2 days (data not shown).

An Albumin Silencer Is Present from −11 to −12 kb Upstream of the Mouse Albumin Gene. In the original transgenic mouse experiments, the mouse albumin enhancer was identified as a 3.5-kb region between −8.5 and −12 kb upstream to the mouse albumin gene transcription start site (33). Having determined that enhancer activity mapped downstream of −10.5 kb, we next wanted to test the functional activity of sequences upstream of −10.5 kb. Two constructs containing either the sequences from −10.5 to −11 kb or from −11 to −12 kb ligated to the albumin promoter construct (palbCAT) were transfected into the hepatocyte cell lines (Fig. 5). The sequence from −11 to −12 kb, when inserted upstream of the albumin promoter in a reversed orientation, decreased the expression of the albumin promoter 4-fold in CWSV1 cells. Moreover, this region also decreased the expression of the albumin promoter in the albumin-negative cell line CWSV8 by 4-fold so that p−11 to −12 AlbCAT was no more active than the promoterless pOCAT (background activity) in CWSV8 cells. The region from −10.5 to −11 kb had no enhancing or suppressing activity. When the sequences from −11 to −12 kb were inserted upstream of the heterologous HSV-tk promoter, they also suppressed the expression of the HSV-tk promoter, although to a lesser extent (2-fold reduction).

The negative element in its normal orientation also decreased the activity of the mouse albumin promoter and enhancer. Construct p−11 to −12 AlbCAT, which contained both the enhancer and the negative sequences in their normal orientation, increased the expression of the albumin promoter by only 2-fold in CWSV1 cells as opposed to the 4.2-fold increase when the same cells were transfected by the construct p−11 to −12 AlbCAT, which did not contain the negative sequences. In CWSV8 cells in which the albumin enhancer is not active, the expression of p−11 to −12 AlbCAT was 5-fold lower than palbCAT.

Direct Determination of CAT RNA in Transfected Cells. To determine directly the amount of albumin promoter-specific transcription initiation, we analyzed total RNA from transfected CWSV1 and CWSV8 cells by RNAse T1 analysis. The protected fragments corresponding to albumin promoter-specific initiations were 266 bp (Fig. 6). In addition, nonspecific initiations of CAT RNA from prokaryotic vector sequences (read-through transcriptions) were present in both the promoterless pOCAT and the albumin promoter-containing constructs. The protected bands from autoradiograms were quantitated by densitometric scanning and normalized as described above by using the plasmid DNA level in the transfected cells. The overall results were in good agreement with CAT assays. In CWSV1 cells, the albumin enhancer-containing construct p−8.7 to −10.5 AlbCAT showed approximately 10-fold higher activity than the basic construct palbCAT, and the albumin silencer-containing construct of p−11 to −12 AlbCAT decreased the expression of the albumin promoter by 4-fold. In agreement with the extremely low level of activity of the albumin promoter in CWSV8 cells as measured by CAT assays, CAT RNA

![Diagram](image-url)
initiated from the albumin promoter was not detectable in either palbCAT- or p.11 AlbCAT-transfected CWSV8 cells even when 160 µg total RNA/reaction were used (data not shown).

Discussion

The availability of a series of characterized SV40-immortalized hepatocyte cell lines that exhibit heterogeneity with regard to albumin gene expression made it possible to begin to evaluate in vitro the function of cis-acting sequences controlling albumin expression. In this report, we determined that the albumin transcription in several of the cell lines approaches or equals the levels in liver. This is in contrast to other hepatocyte and hepatoma cell lines, which transcribe the albumin gene at less than 5% of liver (36, 37). Variations in the albumin transcription levels in the cell lines paralleled the changes observed in the steady-state albumin mRNA level, indicating that albumin gene expression in these cells, as in liver, is determined predominantly at the transcriptional level.

Using the CWSV cell lines, we began to analyze the functional significance of the various albumin-regulatory sequences in determining albumin gene expression. With respect to the albumin promoter, we confirmed that the activity of the albumin promoter is cell specific in that its activity in the albumin-negative CWSV8 cells was more than 20-fold lower than that in the albumin-positive CWSV1, CWSV2, CWSV14, and NR4T-1 cells. However, we also demonstrated that the expression level of the promoter varied among the albumin-positive cells, but in general, there was no correlation between the expression level of the transfected mouse albumin promoter and the endogenous albumin gene expression in the cell types used in this study. CAT activity driven by the promoter in CWSV14 cells was more than 3-fold that in CWSV1 even though endogenous albumin transcription was slightly lower in CWSV14 cells than in CWSV1 cells. In primary rat hepatocytes in culture, the activity of the promoter was more than 100-fold less than in CWSV1 cells, and yet the level of endogenous albumin transcription was only 6-fold less than in CWSV1 cells. The extremely low activity of the albumin promoter in the primary hepatocyte culture is consistent with the fact that it showed no activity in the transgenic mice (31–33). It is not clear why the albumin promoter has low activity in the liver and in the primary hepatocyte culture but is relatively active in immortalized or transformed hepatocyte cell lines. It is possible that the promoter by itself is suppressed in the postmitotic, terminally differentiated hepatocytes, as suggested by Gorski et al. (8). Our results also suggest that the albumin promoter may be activated during the process of hepatocyte immortalization/transformation. Indeed, preliminary results from this laboratory suggest that some of the transcriptional factors important for the promoter function are more abundant in the CWSV cell lines than in normal liver and, in addition, are more abundant in CWSV14 than in CWSV1.

The function of the mouse albumin enhancer is cell specific in that it was active only in albumin-positive cells and not in albumin-negative cells. In addition, the enhan-
transcriptional factors for the albumin enhancer are still present in CWSV1 and CWSV2 cells and may have been lost or rendered inactive in other CWSV cells and most hepatoma cells.

Even though the CWSV14 cells transcribe the endogenous rat albumin gene at a level about 70% that of liver, the albumin enhancer was not functional in these cells. CWSV14 cells also differ from the CWSV1 and CWSV2 cells in that they (a) express higher levels of c-Ha-ras RNA at low passage; (b) are more tumorigenic at low passage; (c) lose albumin expression when passaged in culture; and (d) begin to express the oncofetoprotein α-fetoprotein at late passage (3, 4, 39). Similarly, the albumin enhancer was not functional in the tumor cell line NR4T-1 even though the endogenous albumin transcription rate is very high in these cells. These results suggest that there may be a correlation between the function of
the albumin enhancer and the differentiation state of the hepatocytes. That is, the enhancer functions poorly or not at all in CWSV14 cells and NR4T-1 cells (cells that express oncoproteins, e.g., Ha-ras, and are tumorigenic), many hepatoma cells, and fetal liver (31). In contrast, the enhancer is functional in adult liver and in well-differentiated primary hepatocytes and CWSV1 and CWSV2 cells. In agreement with this hypothesis, it has been reported that the activity of the albumin enhancer, but not the promoter, can be induced by various conditions that promote hepatocyte differentiation in vitro in the murine hepatocyte cell line H2.35 (35, 37). It was demonstrated in H2.35 cells that extracellular matrix plays an important role in maintaining the differentiation state of the hepatocytes and the albumin enhancer function. Since the CWSV1 and CWSV2 cells are cultured on a plastic surface, either these cells can produce the necessary extracellular matrix components or they possess other mechanisms that maintain their differentiation state and albumin enhancer function.

The albumin enhancer did not increase the expression of a heterologous promoter, the HSV-tk promoter, in our transient assay. Similarly, the albumin enhancer did not enhance the expression of the heterologous human growth hormone promoter in the transgenic study (33). However, the albumin enhancer was shown previously to enhance the expression of the heterologous β-globin and transthyretin promoters in CWSV1 cells (34, 41). Since the albumin enhancer can cooperate with some, but not all, heterologous promoters, factors bound to the albumin enhancer may require interaction with factors bound to promoters to exert an effect.

The core enhancer sequence of the mouse albumin enhancer was initially mapped to a 130-bp fragment extending from −9.94 to −10.03 kb by deletion analysis using the CWSV1 cells as recipients for transient transfections (34). Within this 130-bp fragment, the 90-bp sequence from −9.94 to −10.03 kb was shown to bind a non-liver-specific factor (NLS1) in a gel-shift assay. Since no other protein-binding sites were found by gel-shift analysis within the 130-bp core enhancer, it was postulated that either NLS1 underwent liver-specific modifications or there was a liver-specific factor that cooperated with NLS1, since the albumin core enhancer functioned only in the hepatocyte cell line. However, Liu et al. (35) reported recently that a 330-bp fragment from −9.94 to −10.27 kb was necessary for the albumin enhancer to function in a murine hepatocyte cell line, H2.35. Within the 330-bp fragment were at least three binding sites which bound three liver-specific (enriched) factors. Mutations of any one of these sites abolished enhancer activity even though two of these sites were outside the 130-bp core enhancer sequences identified by Herbst et al. (34). Because two different hepatocyte cell lines, the murine H2.35 and the rat CWSV1 used in these two different studies, the discrepancy between these two studies in mapping the mouse albumin enhancer might have been due to species specificity. In this study, we have shown that (a) the 90-bp fragment from −9.94 to −10.03 kb had no enhancer activity in either the albumin-positive cell line CWSV1 or the albumin-negative cell line CWSV8, suggesting that NLS1 is not sufficient for enhancer activity, and (b) mutation or deletion of any one of the three protein-binding sites identified by Liu et al. (35) abolished enhancer function, whereas the wild-type 330-bp fragment from −9.94 to −10.27 kb containing all three sites was fully functional. Therefore, our mapping result obtained using the rat CWSV1 cell line is in complete agreement with that of Liu et al. (35) using the murine H2.35 cell line, indicating that the albumin enhancer, like the albumin promoter, is highly conserved between these two rodent species.

The sequence from −11 to −12 kb (the putative albumin silencer) was shown to decrease the expression of homologous and heterologous promoters in both albumin-expressing and albumin-negative cells. This far upstream silencer-like region may play an important role in determining the cell-specific expression of the albumin gene by suppressing inappropriate expression in nonhepatocytes. This region reduced the residual activity of the albumin promoter in the albumin-negative CWSV8 cells to background levels. Previous reports show that tissue specificity of the albumin promoter is not tightly controlled; the albumin promoter is expressed in fibroblast cell lines, in albumin-negative hepatoma cells (10, 11, 28, 37), and in nuclear extracts from the brain and spleen (8, 27). The albumin promoter in the absence of the far upstream enhancer region was also expressed occasionally in the kidney in transgenic mice, but this expression was abolished when the sequences from −8.5 to −12 kb were present (31, 33). Previous work from several groups has suggested that negative-acting DNA regions may be involved in suppressing hepatocyte-specific gene expression in non-liver tissues (42–48). Negative regulation has also been implicated in determining cell type specificity of other tissue-specific genes, such as the immunoglobulin heavy chain (49) and lysozyme genes (50). In addition, since the albumin silencer was able to decrease albumin promoter activity in the albumin-expressing cells, it could also potentially play a role in mediating down-regulation of albumin expression that occurs under altered physiological conditions, such as the acute-phase response.

Our results clearly indicate the importance of the combination of different cis-regulating sequences (the promoter, enhancer, and silencer) in determining the tissue-specific and high-level expression of the albumin gene in CWSV cell lines and primary hepatocytes. Moreover, different regulatory sequences may be responsible for directing albumin transcription, depending upon the hepatocyte differentiation state. Furthermore, in addition to the promoter, enhancer, and silencer sequences identified so far, additional cis-acting elements may play a role in albumin expression. In NR4T-1 cells which are transformed, the activity of the albumin enhancer is lost in spite of the high transcriptional rate of the endogenous albumin gene in these cells. Although it is possible that mechanisms such as changes in chromatin structure or DN methylation may be responsible for maintaining the high transcription rate of albumin genes, it is also possible that other, as yet unidentified, regulatory sequences may be involved in controlling albumin transcription in these cells. One possible candidate is the group of α-fetoprotein enhancers located at the intergenic region between the mouse albumin and α-fetoprotein gene and downstream from the albumin gene which was shown to significantly increase the expression of the albumin promoter in transgenic mice (31).
Materials and Methods

Plasmid Construction. The plasmid ptkCAT (Fig. 1) contains a fragment spanning the promoter (−105 to +56 bp) of the herpes simplex virus tk gene cloned 5' to the CAT gene coding region and the SV40 splice and polyadenylation signals (51). p\(^{−11} to −12\)kCAT contains, in addition, a 1.0-kb fragment from an EcoRV site at −12 kb to a HindIII site at −11 kb of the mouse albumin gene (33). p\(^{−8.9 to −10.3}kCAT\) sequence contains a fragment generated from a HindIII site at −10.5 kb to a BglII site at −8.9 kb. Both fragments were inserted 5' to the tk promoter.

palbCAT was constructed by replacing the tk promoter in ptkCAT with a 303-bp BamHI fragment containing sequences from −282 to +21 bp of the mouse albumin promoter. p\(^{−11} to 1\)albCAT contains the same albumin gene upstream fragment as p\(^{−11} to −12\)kCAT. p\(^{−8.7 to −10.5}albCAT\) contains a segment spanning a HindIII site at −10.5 kb to a BamHI site at −8.7 kb. p\(^{−10.5 to −11.5}\)albCAT contains a 0.5-kb HindIII albumin gene upstream fragment from −11 to −10.5 kb. To generate p\(^{−8.7 to −10.5}\)albCAT, p\(^{−8.7 to −10.5}\)albCAT was cut with BamHI and BglII, and the small 200-bp fragment from −8.7 to −8.9 kb upstream of the mouse albumin gene was removed. The remaining sequences were religated by virtue of the cohesive ends generated by the BamHI and BglII digestions. The same 200-bp sequences that were deleted in p\(^{−8.9 to −10.3}kCAT\) were inserted into the BamHI and HindIII sites upstream to the albumin promoter in palbCAT after filling-in of the HindIII end in palbCAT and the BglII end in the inserted fragment and subsequent ligation; the resulting plasmid was designated p\(^{−8.7 to −10.5}albCAT\). To make p\(^{−8.9 to −10.5}albCAT\), the albumin upstream sequences from −9.94 to −10.5 kb relative to the transcription start site were cut out from p\(^{−8.7 to −10.5}\)albCAT by HindIII and Accl digestion and were inserted into the HindIII and Sall sites upstream to the albumin promoter in palbCAT after partial filling-in of the Accl end (GAS' → GAS) in the inserted fragment and the GCT/Sall end (5' TCGA → 5' TCGA) in palbCAT and subsequent ligation.

To make p\(^{−8.7 to −10.5}\)albCAT, the mouse albumin upstream sequences from −9.94 to −10.3 kb were cut out from p\(^{−8.9 to −10.5}albCAT\) by BamHI and StyI digestions and were inserted into the BamHI and Sall sites upstream to the albumin promoter in palbCAT after filling-in of the StyI and Sall ends in the inserted fragment and palbCAT, respectively. To make p\(^{−8.7 to −12}\)albCAT, the albumin upstream sequences from −8.7 to −12 kb were inserted into the BamHI and Sall sites upstream to the albumin promoter in palbCAT. To make pRahlCAT, the rat albumin promoter sequences from −180 to +16 bp relative to the transcription start site were cut out from a plasmid containing the rat albumin promoter (a gift from Dr. James Darnell) by BamHI and HindIII digestion and were inserted into palbCAT after the mouse albumin promoter was deleted from it by XhoI and BamHI digestion and the XhoI end in the vector was filled-in. To construct the promoterless p0CAT, the mouse albumin promoter was cut out by XhoI and Sall from palbCAT, and the resulting promoterless plasmid was religated.

p\(^{−8.9 to −10.27}albCAT\), pmEalbCAT, pmGalbCAT, and p\(^{−10.01 to −10.27}\)albCAT were generated by PCR cloning as follows. Two oligonucleotides, oligo 1 gcg AACGCTT GCGACATAGCAAGCAGCAA and oligo 2 gcg GTCGAC TCGTAGACAAATGGCCCTTTC, were used to amplify the albumin enhancer sequences between −9.94 and −10.27 kb from pAT2-337/669, pAT2-M1 (same as pAT2-337/669 except that the eE site was mutated), pAT2-M2 (eE site mutated), and pAT2-M3 (eE site mutated) (35). The HindIII site in oligo 1 and SalI site in oligo 2 that were introduced to facilitate subsequent cloning are underlined. The GCC spacer sequence in the oligonucleotides introduced to facilitate restriction digestion is shown in lower case letters. PCR amplification was performed with Taq DNA polymerase (Perkin Elmer) in a Perkin Elmer thermocycler, as suggested by the manufacturer. The PCR products were digested with HindIII and SalI, and the digests were gel purified. The PCR amplified DNA fragments were then cloned into the HindIII and SalI sites in palbCAT. p\(^{−9.94 to −10.27}albCAT\) and pmE and pmGalbCAT contained the same enhancer sequences as pAT2-337/669, pAT2-M1, and pAT2-M2, respectively. The PCR product amplified from pAT2-M3 contained an internal SalI site at −10.03 kb of the enhancer sequences. Subsequently, the enhancer sequences between −9.94 and −10.03 kb were deleted following SalI digestion and subsequent cloning into palbCAT to make p\(^{−10.03 to −10.27}albCAT\).

Cell Cultures. Rat CWSV1, CWSV2, CWSV8, and CWSV14 cells were cultured in RPMI medium supplemented as previously described (3, 4). Adult rat primary hepatocyte cultures were prepared from 180- to 200-g Fischer male F344 rats (52–54) and maintained as described (1, 55). Primary rat hepatocytes were plated at a density of 10\(^5\) cells/60-mm plastic cell culture dish that was coated with rat tail collagen and fed RPMI medium (4) supplemented with 2% dimethyl sulfoxide and 25 ng/ml epidermal growth factor. Hepatocytes were gel purified with the fresh medium every 2 days. NRAT-1 is a tumor cell line that was derived from a tumor generated by injection of NR4 cells into newborn syngeneic rats. NR4 cells were derived by transformation of CWSV1 cells with the activated c-Ha-ras oncogene (40). All cell lines used for transfection were low-passage cells that were passaged in vitro less than 25 times.

Nascent Chain Extension Assay. Nuclei were extracted from liver, hepatocyte cell lines, and primary hepatocytes in culture, and nascent chain extension reactions were performed as described (36, 55). Denatured plasmid DNA obtained from an albumin genomic clone [AL4 (56)], K-ras DNA [pKSm (57)], and control vector DNA were spotted onto nitrocellulose. Extension products were counted by a liquid scintillation method, and equal numbers of counts were hybridized to the immobilized DNA. Individual dots were cut from filters, dried, placed in liquid scintillation fluid, and counted. Transcription rates of the K-ras gene were similar in all of the CWSV cell lines tested, indicating that the cell lines did not differ in the basic transcriptional machinery.

Northern Blot Analyses. Total RNA was isolated from liver, cell lines, and primary hepatocytes as previously described (3, 55, 58). For Northern blot hybridization, equal amounts of formamidine-denatured total cellular RNA were electrophoretically separated on 1.4% agarose gels. RNA was transferred to nitrocellulose filters, and the filters were baked. Prehybridization, hybridization, and
autoradiography were carried out as previously described (55).

**Transient Transfection, CAT Assay, and Quantitation of Plasmid DNA from Transfected Cells.** Plasmid DNA transfections were performed by the calcium phosphate precipitation method (59). CWSV cell lines and the NN4T-1 tumor cell line, grown to 50% confluency in 100-mm plastic dishes, were transfected with 15 μg DNA/dish. For the primary rat hepatocytes, the cells were transfected with 5 μg DNA/dish at day 2 or day 15 in culture. The amount of plasmid DNA used was predetermined to be within the linear range of DNA uptake
and CAT expression. The precipitates were removed 4 to 5 h after transfection, and the cells were refed with fresh media. For CWSV6 cells or as indicated for other cells, the cells were shocked with 15% glycerol for 2 min before being refed with fresh medium. The cells were harvested 48 h after transfection, and cells harvested from several plates were pooled. A portion of the pooled transfected cells were frozen and thawed three times for protein extraction. Protein concentrations were determined using the Bio-Rad (Richmond, CA) protein assay. CAT assays were performed as described (60) using appropriate amounts of protein extracts and 0.2 μCi [14C]-chloramphenicol (50 to 60 mCi/mmol; NEN, Boston, MA). The reaction products were separated by thin-layer chromatography, and the radioactive spots from the thin-layer plates were cut out and counted in a liquid scintillation counter to quantitate the conversion rate of chloramphenicol to the acetylated products. Initial experiments were performed using varying amounts of protein extracts and incubation time to ensure that all CAT assays were performed within the linear range of the assay.

The remainder of the pooled harvested cells were lysed by the method of Hirt (61) to extract plasmid DNA. The lysed cell extracts were digested with proteinase K, and the Hirt supernatants were extracted with phenol-chloroform and precipitated with ethanol. Portions of each Hirt DNA sample from equivalent numbers of cells were digested with restriction enzymes, electrophoretically separated on agarose gels, and blotted to nitrocellulose filters. Southern hybridizations were performed as described (62) using as probe a nick-translated 1-kb fragment containing the CAT coding sequence. The plasmid DNA bands from autoradiograms were quantitated by densitometric scanning.

CAT activity from each transfection was normalized to the amount of protein used and the plasmid DNA level in the transfected cells. In agreement with previous reports (63), we found this approach to be accurate and highly reproducible throughout our experiments. For example, when plasmid p°bCAT was transfected into CWSV14 cells in two independent experiments, a 3-fold (2.4% versus 6.5% CAT conversion) difference in CAT expression was noticed. Similarly, in NN4T-1 cells, when construct palbCAT was transfected and the DNA uptake was deliberately increased by glycerol shock, a 10-fold (from 0.3% to 3.5%) increase in CAT expression as a result of glycerol shock was observed. In each case, the variation in CAT expression directly reflected the difference in uptake of the plasmid DNAs. When the CAT activity was corrected for variation in levels of DNA uptake, the resulting normalized CAT activities became essentially the same. In some cells, the promoterless p0CAT had residual CAT activity, and this background activity was subtracted from the albumin promoter/enhancer-containing constructs before comparing CAT activity between different constructs or different cell lines. Each transfection was repeated multiple times, and the average values and standard deviations were reported.

**RNase T1 Analysis.** Total cellular RNA was extracted by the guanidine-phenol extraction method (58). To make the template construct pSPtalbCAT for riboprobe preparation, the EcoRI-BamH1 fragment from palbCAT, which contains the mouse albumin promoter sequences from 282 to +21 bp and the S' portions of the CAT gene (245 bp), was cloned into the multiple cloning site in pSP18 (Boehringer Mannheim, Indianapolis, IN). The T7 albCAT riboprobe was synthesized from pSPtalbCAT by using [a-32P]CTP (3000 Ci/mmol; NEN) and T7 polymerase as specified by the supplier (Boehringer Mannheim). An excess of labeled probe (about 2 × 106 cpm/reaction) was hybridized with 80 μg of RNA at 37°C overnight in 30 μl of hybridization buffer (40 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.4-0.4 M NaCl-1 mM EDTA-80% formamide). RNase T1 digestion was performed by adding to each reaction 350 μl of 4 μg/ml RNase T1 (Bethesda Research Laboratories, Gaithersburg, MD) in RNase digestion buffer (0.01 M Tris, pH 7.5-0.005 M EDTA-0.3 M NaCl) and incubating at 37°C for 1 h (64). The protected fragments were analyzed on a 4% polyacrylamide-urea sequencing gel with end-labeled Haelll-digested pBR322 as a molecular weight standard.

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**References**


