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The Mouse Albumin Enhancer Contains a Negative Regulatory Element That Interacts with a Novel DNA-Binding Protein

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The far-upstream mouse albumin enhancer (−10.5 to −8.43 kilobases) has both positive and negative regulatory domains which contribute to the rate and tissue specificity of albumin gene transcription. (R. S. Herbst, N. Friedman, J. E. Darnell, Jr., and L. E. Babiss, Proc. Natl. Acad. Sci. USA 86:1553–1557). In this work, the negative regulatory region has been functionally localized to sequences −8.7 to −8.43 kilobases upstream of the albumin gene cap site. In the absence of the albumin-modulating region (in which there are binding sites for the transcription factor C/EBP), the negative region can suppress a neighboring positive-acting element, thereby interfering with albumin enhancer function. The negative region is also capable of negating the positive action of the heterologous transthyretin enhancer in an orientation-independent fashion. Within this negative-acting region we can detect two DNA-binding sites, both of which are recognized by a protein present in all cell types tested. This DNA-binding activity is not competed for by any of a series of known DNA-binding sites, and hence this new protein is a candidate for a role in suppressing the albumin gene in nonhepatic cells.

The initiation, maturation, and maintenance of the differentiated state in eucaryotic tissues result from the coordinate expression of a set of genes, many of which are regulated at the level of transcriptional initiation (for a review, see reference 28). An analysis of liver-specific gene regulation has led to the identification of two classes of required cellular transcription factors, some that are widely distributed and others that display a more limited cellular distribution (1, 4, 5, 8–11, 16–18, 26, 28). These factors interact with multiple DNA sequence-specific motifs present in the promoter and enhancer elements of hepatocyte-expressed genes so that a single gene (e.g., transthyretin [TTR]) can employ as many as five different proteins acting on as many as 10 independent sites (8). These factors may dictate liver specificity by either acting directly to positively stimulate transcription complex formation or, alternatively, by functioning to suppress the activity of negative-acting ubiquitous factors, thereby allowing expression in hepatocytes while preventing transcription in inappropriate cell types.

Transfection experiments with mutant recombinant genes have uncovered a number of DNA-binding sites that appear to have a negative effect on transcription. Such sites have been found in the retinol-binding protein, beta interferon, insulin, α-fetoprotein, c-*myc*, and immunoglobulin heavy chain genes among others (3, 7, 13, 15, 17, 19, 20, 28, 30, 31, 34). In some of these functionally proven cases of negative regulation, specific DNA sequences and their cognate DNA-binding proteins have been delineated. However, it is not yet clear how these proteins function to suppress gene expression.

Albumin is a prototypical tissue-specific gene product whose gene contains a highly liver-specific promoter segment (from −175 to −30 base pairs [bp]) regulated by the ubiquitous proteins NF1 and CAAT together with the liver-enriched C/EBP and HNF1 proteins (1, 4, 5, 26). In addition, the albumin gene contains an upstream enhancer element, consisting of both positive and negative regulatory domains, which contributes both to tissue specificity and to the maintenance of the high rate of albumin transcription in hepatocytes (18, 27, 33).

In the present study we have concentrated on the far-upstream enhancer of the mouse albumin gene (−10.5 to −8.43 kilobases [kb]) that was previously shown to be composed of three effective regions, an activator region (II), an inhibitory region (III), and a modulating region (I) that reverses the inhibitor (18) (see Fig. 7, top). In this work the functional negative-acting region has been more precisely mapped by deletion studies, and within a 267-nucleotide negative-acting region only two protein-binding sites were found, apparently for the same protein. Further analysis revealed that a region of the negative element which contained only one of these protein-binding sites (143 bp) could function to repress TTR enhancer activity. The negative-acting DNA element interfered with both the albumin enhancer and the heterologous TTR enhancer in HepG2 cells but was not able by itself to directly suppress promoter sequences. This candidate protein, which could function to specifically negate enhancer activity, does not appear to have been previously described based upon gel shift competition studies.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of rat CWSV1 cells were maintained in RPMI medium supplemented as previously described (18, 35). Human hepatoma (HepG2) cells were maintained as monolayer cultures and were grown in Ham F12 medium, supplemented with 5% fetal bovine serum, 1× essential amino acids, and 25 μg of garamycin per ml (22). Viral stocks were obtained by infection of monolayer cultures of 293 cells, and crude cellular lysates were obtained by repeated cycles of freezing and thawing. Titers of viral stocks were determined on 293 cells by fluorescent-focus assay with antiserum raised in rabbits against cesium chloride-banded purified virions (1, 14).

Construction of mouse albumin enhancer-containing expression plasmids. Plasmid constructs with differing

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amounts of mouse albumin enhancer sequence placed upstream of its promoter (at nucleotide −300) were constructed as previously described (18). Sequences were removed from the 3′ end by taking advantage of known restriction endonuclease sites (*Acc*I, −9.94 kb; *Bgl*II, −8.9 kb; *Pst*I, −8.7 kb), and the 5′ end was cleaved by digestion with *Hin*dIII. The DNA polymerase-repaired termini were subsequently ligated with *Eco*RI linkers; gel-isolated fragments were placed in position 5′ to the albumin promoter, and the orientation was determined by restriction endonuclease analysis. Heterologous constructs were designed by using the mouse TTR promoter. The TTR parent contains 202 bp of TTR upstream sequence (upstream of the RNA start site) along with the first two TTR exons, fused to the simian virus 40 (SV40) large T antigen 3′ end as previously described (9, 10). The TTR minimal enhancer sequences (a fragment taken from −1.96 to −1.86 kb upstream of the cap site) were fused immediately upstream of this promoter element (10). A unique *Xba*I site existing at the site of fusion between the promoter and enhancer elements (see Fig. 3, bottom) served as the site for insertion of the mouse albumin enhancer fragments, which were properly oriented by restriction endonuclease digestion.

Recombinant adenoviruses were isolated from plasmids which contained either the mouse albumin promoter (−300 bp upstream of the start site) alone or the albumin promoter with the mouse albumin enhancer sequences from −10.5 to −8.43 kb placed immediately upstream (1, 14). The method of in vitro overlap recombination, with human 293 cells, was used (1, 14).

Plasmid transfection, adenovirus infection, and RNase T_2 analysis of expression constructs. Mouse albumin promoter- and enhancer-containing plasmid DNA or the TTR heterologous constructs (50 μg), along with the SV40 enhancer-containing β-globin internal control (20 μg) (9, 18), were cotransfected into CWSV1 or HepG2 cells at 40% confluency by using the calcium phosphate-DNA coprecipitation method, as previously described (10, 18). All transfection experiments were performed at least four times. Cytoplasmic RNA was isolated 48 h posttransfection, and the accumulation of the albumin-adenovirus hybrid RNAs was scored by RNase T_2 protection against nuclease digestion of labeled antisence RNA probes, as shown in Fig. 1 and 2 and as previously described (9, 10, 18). The sizes of the protected fragments are as shown and described in the Results section.

HepG2 cells were infected with the appropriate recombinant adenovirus at 50 PFU per cell for 24 h in the presence of 1-β-D-arabinofuranosylcytosine (araC; 40 μg/ml) to prevent viral DNA replication. Following the infection, cytoplasmic RNA was harvested and analyzed by SP6 assays for albumin-virus fusion mRNAs and viral early region 2 mRNA as a control.

Cell and tissue extracts. Tissue extracts were prepared as previously described, except that they were subjected to ammonium sulfate precipitation (0.3 g/ml) followed by dialysis against BC100 buffer (1, 12, 18). Cultured cell extracts (CWSV1, HepG2, FaO, and HeLa) were made by the method of Dignam et al. (12) and concentrated by ammonium sulfate precipitation.

DNA-protein-binding assays. In vitro exonuclease III (ExoIII) stop reactions were preformed as described by Kovesdi et al. (23). For all reactions, 1 μg of uniquely end-labeled DNA probe was combined with 30 μg of nuclear extract and 4 μg of poly (dI-dC) which was included as a nonspecific competitor of DNA-binding proteins. Following ExoIII digestion (100 U per reaction; Boehringer Mannheim Biochemicals), the products were analyzed on 8% denaturing polyacrylamide gels.

Gel retardation analysis was performed as previously described (8, 18). End-labeled fragment was incubated for 25 min at room temperature with 1 to 10 μg of nuclear protein in 40 mM KCl–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–1 mM $MgCl_2$–0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–0.5 mM dithiothreitol–320 μg of poly (dI–dC) per ml–40 μg of pGEM1 DNA per ml–4% Ficoll in a final volume of 20 μl. Protein-DNA complexes were separated from free probe on polyacrylamide gels (the percentage was varied depending on the size of the probe) in 50 mM TBE. (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA).

DNase I footprinting studies were performed with liver nuclear extract prepared as above, which had been fractionated on a heparin-agarose column by using a KCl salt gradient, and HeLa extract, which had been fractionated on a P11 column by using KCl steps (0.25 M KCl for liver and 0.4 M step for HeLa, respectively). The fractions were dialyzed against BC100 buffer, and a gel shift reaction was used to assay for albumin negative factor (ANF) activity. Subsequently, 1 ng of ^{32}P-labeled probe was incubated at 0°C for 60 min with 5 to 30 μg of extract, 5 μg of poly(dI-dC), and 4% polyethylene glycol in a buffer containing 10 mM HEPES (pH 7.9), 7 mM $MgCl_2$ and 0.75 mM dithiothreitol. DNase I (10 to 50 ng) was added for 3 to 4 min, and the reaction was terminated by the addition of proteinase K (200 μg/ml) and sodium dodecyl sulfate (0.5%). The DNA was phenol extracted and ethanol precipitated, and the labeled fragments were analyzed on an 8% denaturing acrylamide gel. G + A sequence reactions were performed by the procedure of Maxam and Gilbert (29).

RESULTS

The negative regulatory region resides between −8.7 and −8.43 kb of the mouse albumin enhancer. In our previous study, we identified a negative-acting DNA sequence element in the mouse albumin enhancer (region III [18]). To better characterize this negative acting region, we sequenced the most promoter-proximal portion of the enhancer (previously termed −8.7 to −8.5 kb) and found it to be 267 bp in length. To continue using the general coordinates previously reported, we have labeled the most promoter-proximal *Pst*I site −8.43 kb (Fig. 1, bottom) while leaving the other sequence reference numbers unchanged.

To specifically localize the DNA sequences which conferred the negative effect on the mouse albumin enhancer, we performed transient plasmid transfection assays with rat CWSV1 cells (18, 35). A plasmid containing the albumin promoter-proximal sequences from −10.5 to −8.43 kb relative to the transcriptional start site was joined to a DNA fragment that included the albumin gene sequences from −300 to +22 bp fused to a reporter gene (adenovirus E1B; for expression in transient transfection [Fig. 1, bottom]). Expression from this construct was subsequently compared with the enhancerless control plasmid (containing only −300 bp of albumin sequence). In agreement with our previously reported result, a fivefold stimulation in albumin promoter function was found when the entire albumin enhancer was included (Fig. 1A; −10.5 versus −0.3). The relative mRNA accumulation from each albumin construct was scored by using a 5′-end RNase T_2 assay, protecting a band of 205 nucleotides, as described previously (Fig. 1, bottom) (18).

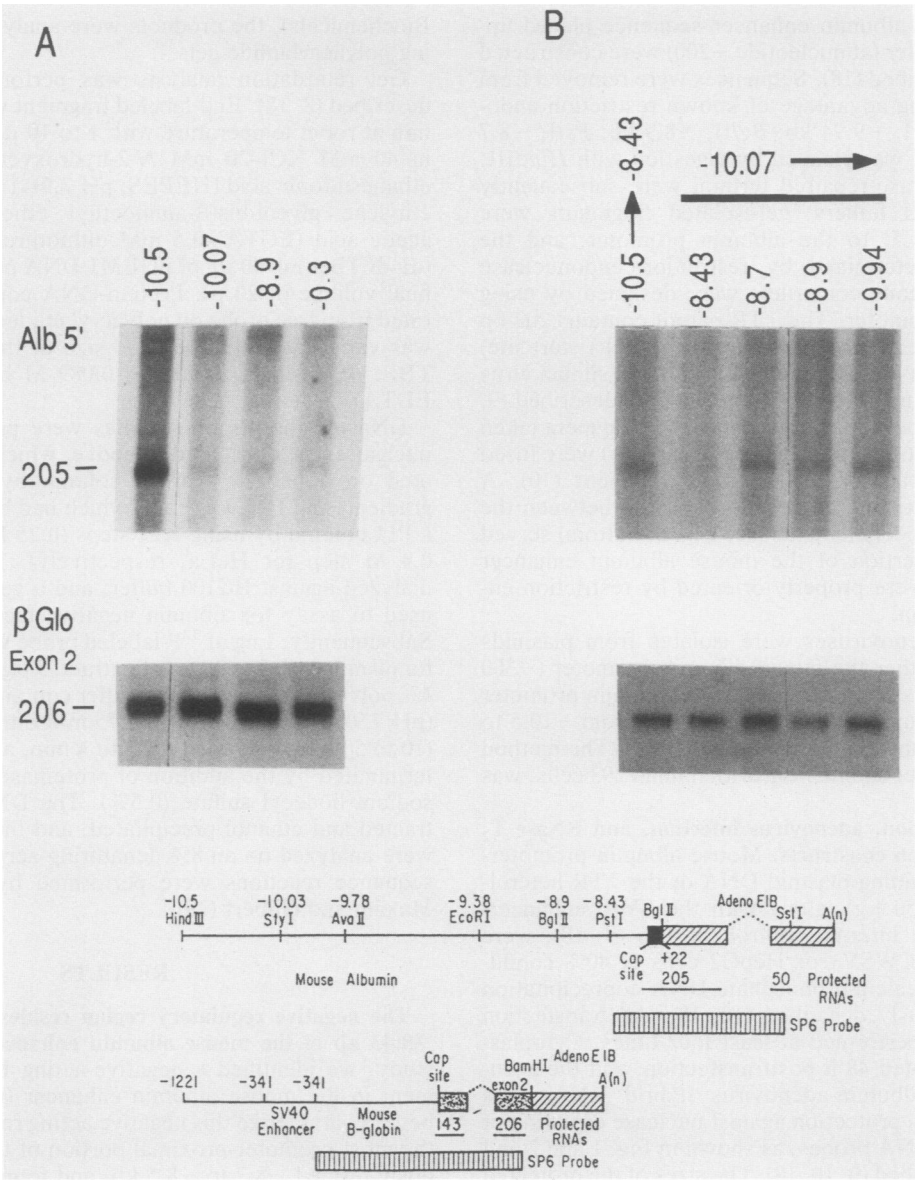

FIG. 1. Functional analysis of the mouse albumin enhancer element in CWSV1 cells. (A) The parent construct (bottom) and deletions (marked by their 5′ boundary in kilobases) were cotransfected into CWSV1 cells with a control plasmid (bottom; an SV40 enhancer containing β-globin promoter) to standardize for variations in transfection efficiency (see Methods and Materials). Assays for expression were carried out by protection of labeled SP6 probes. The lane marked −0.3 indicates the enhancerless albumin promoter plasmid containing −300 bp of promoter sequence. (B) The parent construct is assayed (−10.5 to −8.43 kb), along with the additional consturct, as shown, which maintains a 5′ enhancer boundary of −10.07 kb while having variable 3′-end positions as indicated above each lane. Abbreviations: Alb, albumin; βGlo, β-globin.

The SV40–β-globin internal control used to monitor transfection efficiency is shown (9, 18). A 206-nucleotide protected band represents the second exon of the β-globin gene (Fig. 1, bottom). Because of the similar sizes of the RNA protected by these probes, each was assayed separately.

Figure 1A depicts a 5′ deletion analysis of the parent albumin enhancer construct described above, which has the 3′ enhancer boundary set at −8.43 kb. When sequences between −10.5 and −10.07 kb (which includes region I) were removed, a complete loss of enhancer function was observed (Fig. 1A, −10.07 kb), owing to the absence of the enhancer-modulating sequences (region I [see Fig. 7, top]) (18). A DNA fragment which contains the negative element exclusively (sequences from −8.9 to −8.43 kb) did not suppress the basal level of albumin promoter function (Fig. 1A, compare lanes marked −8.9 and −0.3). Therefore, the negative element would appear to exert its effect exclusively by negating the activity of the enhancer.

To more precisely define the functional boundaries of the negative element, a plasmid containing mouse albumin regulatory domains II (the positive-acting domain) and III (the negative-acting domain, −10.07 to −8.43 kb) was used to make deletions from the 3′ boundary by using three different restriction sites: *Pst*I (−8.7 kb), *Bgl*II (−8.9 kb), and *Acc*I (−9.94 kb). Plasmids with these deletions (maintaining a set 5′ end at −10.07 kb) were again assayed in CWSV1 cells

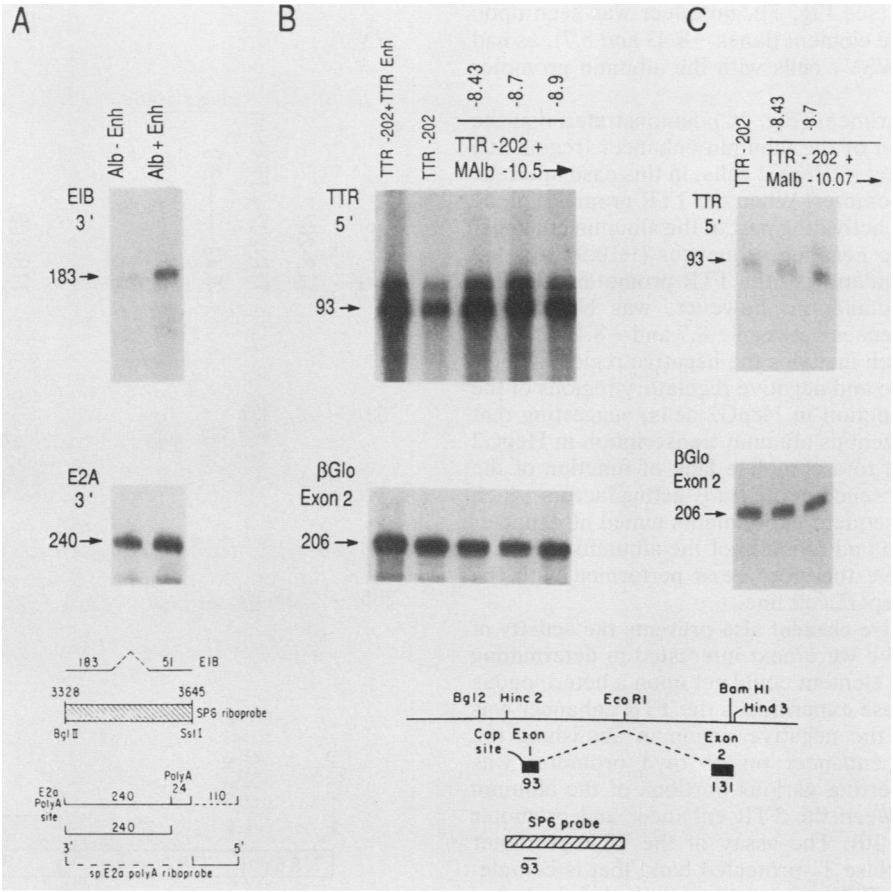

FIG. 2. The mouse albumin enhancer is capable of functioning in HepG2 cells and of activating a heterologous promoter. (A) Analysis of viral infections (50 PFU per cell for 24 h in the presence of araC) by using recombinant adenoviruses containing the albumin −300 promoter (Alb − Enh) or a virus also contianing the −10.5 to −8.43 kb albumin enhancer sequence (Alb + Enh) in HepG2 cells. Expression of RNA was assayed after 24 h by using RNase T_2 assays and probes as depicted at the foot of the figure to score for either the albumin signal (E1B) or the viral E2A gene as a control for viral infectivity. (B) The mouse albumin enhancer sequences (−10.5 to −8.43, −8.7, or −8.9 kb) were placed at the −202 position of the mouse TTR promoter (9, 10), transfected, and assayed by protection of labeled SP6 probes. The SV40–β-globin (βGlo) construct served as a control. (C) The mouse albumin enhancer-negative region is demonstrated to function in human hepatoma cells (HepG2 cells) by using the heterologous TTR promoter. The albumin enhancer-TTR heterologous constructs contain enhancer sequence as depicted by the coordinates above each lane and were transfected into human HepG2 cells, along with the SV40 enhancer containing β-globin plasmid as an internal control.

(Fig. 1B). The plasmid construct with a 3′ coordinate of −8.43 kb (containing the entire region from −8.9 to −8.43 kb) did not show any enhancer function, owing to the presence of the negative element (without the modulatory element, region I, the negative element is dominant [18]). In contrast, when 270 bp was deleted to the site at −8.7 kb, full enhancer function was restored, thus locating the functional negative sequences of region III between −8.7 and −8.43 kb. As observed previously (18), further 3′ deletion of DNA sequences to −9.94 kb resulted in an identical enhancement, indicating that no additional negative regulatory elements exist.

The mouse albumin enhancer can function in HepG2 cells. Although we have used CWSV1 cells to analyze the regulation of the albumin enhancer, most studies of this kind have used rodent and human hepatoma cell lines (including FaO and HepG2). One problem with hepatoma cells, however, is that they transcribe their endogenous albumin genes at only about 10% of the level found in rat liver (1, 6, 14), suggesting that the albumin enhancer may not be contributing to the rate of albumin transcription in these cells. We were interested in addressing this question, but had previously found that the mouse albumin promoter region gave very weak signals upon transient transfection into HepG2 cells (1, 14). We therefore elected to test the enhancer in HepG2 cells by using a recombinant adenovirus construct containing the albumin upstream enhancer (−10.5 to −8.43 kb) fused to the albumin promoter and an adenovirus reporter gene (the E1B gene [14]). In addition, we used constructs for transfection that had the albumin enhancer coupled to the promoter for the mouse TTR gene, which is known to be active and susceptible to stimulation by its own enhancer in HepG2 cells (8–10).

Infection by the adenovirus albumin construct and transfection of plasmids with the albumin enhancer and TTR promoter both showed that HepG2 cells could support albumin enhancer function to stimulate transcription about fivefold just as CWSV1 cells do (Fig. 2A and B). These experiments also indicated that the albumin enhancer was capable of stimulating a heterologous promoter, a canonical attribute of enhancer function (Fig. 2B) (24, 28). The enhancement generated by the albumin enhancer was slightly smaller than that by the TTR enhancer itself (Fig. 2B). Since albumin enhancer region I was present (including both

C/EBP-binding sites [see Fig. 7]), no effect was seen upon removing the negative element (lanes −8.43 and 8.7), as had been observed in CWSV1 cells with the albumin promoter (18).

An additional experiment (Fig. 2C) demonstrated that the negative-acting region of the albumin enhancer (region III) also produced an effect in HepG2 cells, in this case upon the heterologous TTR promoter. When the TTR promoter alone was coupled with the activating part of the albumin enhancer in the absence of the negating sequences (−10.07 to −8.7 kb), there was a stimulation of the TTR promoter (Fig. 2C, lane −8.7). This stimulation, however, was blocked by inclusion of the sequences between −8.7 and −8.43 kb (Fig. 2C, lane −8.43), which includes the negative region. Therefore, both the positive and negative regulatory regions of the albumin enhancer function in HepG2 cells, suggesting that the low rate of endogenous albumin transcription in HepG2 cells may not be due to a complete lack of function of the enhancer or a total absence of the *trans*-acting factors which interact with it. Subsequent experiments aimed at delineating the exact location and function of the albumin enhancer negative element have therefore been performed with the more widely used HepG2 cell line.

The albumin negative element also prevents the activity of the TTR enhancer. We were next interested in determining whether the negative element could act upon a heterologous enhancer, and for these experiments the TTR enhancer was used. The effect of the negative region in abolishing the action of the TTR enhancer on its own promoter was demonstrated by inserting various portions of the albumin enhancer region between the TTR enhancer and promoter (Fig. 3, bottom) (9, 10). The assay of the TTR promoter generates a 93-bp RNase T_2-protected band that is complementary to the first TTR exon, although a slightly larger band is also observed (nominally 95 and 96 bases) which is thought to represent either an upstream start site or an RNase T_2 artifact. In any event, the two bands are usually about equal. The approximately fivefold stimulation of the TTR enhancer on its own promoter (Fig. 3A, leftmost two lanes) was blocked by the presence of a 470-bp fragment which included the albumin negative region (Fig. 3A, lane −8.43 to −8.9). This negative effect was mapped more precisely by dividing the negative element into two roughly equal-sized portions (−8.9 to −8.7 kb and −8.7 to −8.43 kb). The negative effect was contained entirely within the region from −8.7 to −8.43 kb, which could exert its action when present in either orientation (indicated by + or − in Fig. 3B). Similar experiments were performed by placing the same negative-region fragments between the SV40 enhancer–β-globin promoter construct described previously and assaying for expression in both HepG2 and HeLa cells (data not shown). In neither case was a negative effect observed; perhaps the SV40 enhancer is too potent for suppression and there exists a limited group of enhancers that can be suppressed by this negative element.

Two protein-binding sites reside between −8.7 and −8.43 kb. We next turned to experiments that could locate protein-binding sites within the 273-bp portion of the albumin enhancer containing the negative element. It had previously been found that this region gave a complex gel shift pattern, suggesting that several proteins might be interacting with these sequences (18). To simplify our analysis, we bisected the DNA fragment at a unique *Ase*I restriction site (−8,651 bp). End-labeled DNA fragments from −8,700 to −8,562 bp and −8,561 to −8,430 bp were prepared and mixed with nuclear extracts from either rat liver or HeLa cells (an undifferentiated human cell line). Assays for binding were carried out by gel retardation shifts with these labeled DNA fragments. Both fragments bound protein from both extracts, and one particular complex (Fig. 4A, arrow) was specifically prevented from forming by inclusion of an excess of unlabeled homologous oligonucleotide. The two portions of the negative region (−8,700 to −8,562 bp and −8,561 to −8,430 bp) showed cross-competition with each other, indicating a binding site in each half for the same (or a similar) DNA-binding protein (Fig. 4B). Interestingly, although the specific activities of the two probes were the same, probe 1 gave a stronger gel shift band and was better able to compete for itself than an equal molar amount of probe 2, suggesting that probe 1 might bind this protein with a higher affinity than probe 2. The specificity of this complex

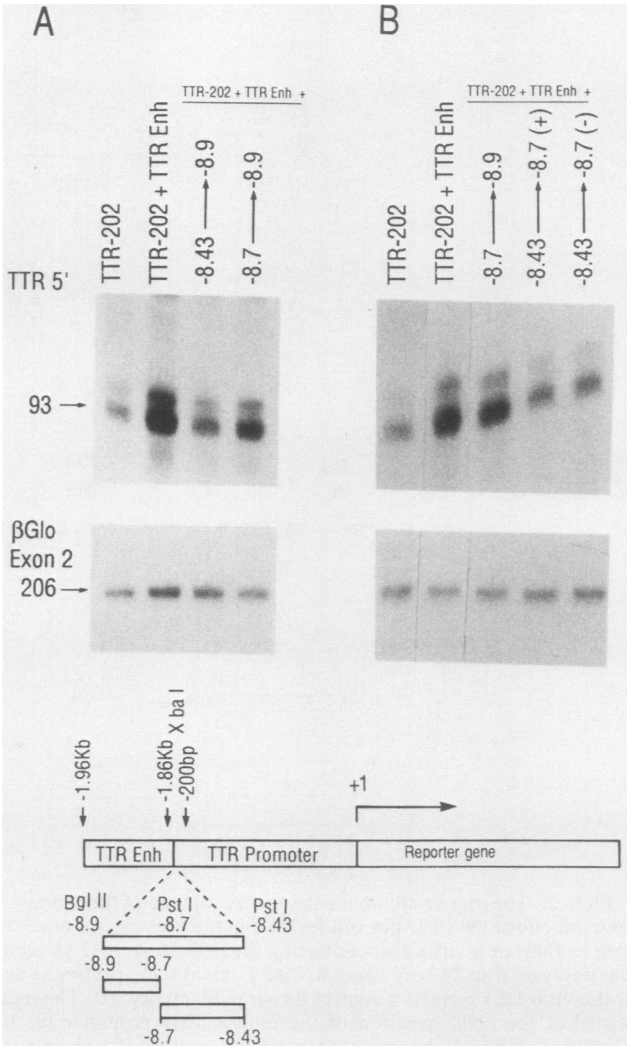

FIG. 3. The mouse albumin enhancer-negative region, when placed between the TTR enhancer and promoter, can negate the TTR enhancer effect. Constructs were prepared as described in Materials and Methods and are diagrammed at the bottom of the figure. These constructs were transfected into human hepatoma cells (50 μg) along with the SV40–β-globin (βGlo) plasmid (20 μg) as an internal control. The TTR −202 promoter containing plasmid and the TTR promoter-TTR minimal enhancer (TTR Enh) construct are included as controls in both panels A and B, which represent separate experiments.

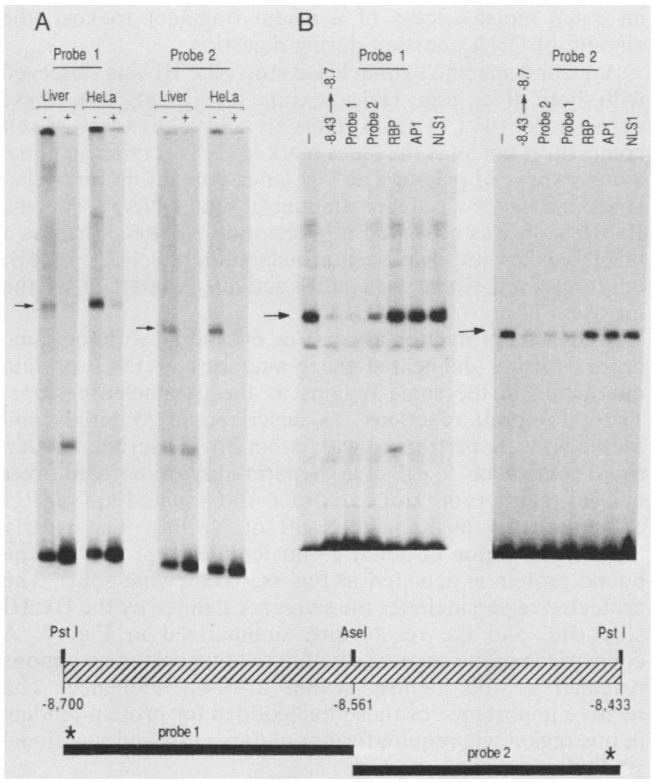

FIG. 4. Definition of protein-binding sites within the mouse albumin negative region, −8.7 to −8.43 kb, by gel shift assay. Two roughly equal-sized probes were prepared by restriction endonuclease digestion at the unique *Ase*I site as shown. These two end-labeled probes were then used in separate gel shift assays (probe 1 and probe 2). (A) The probes were 3′ end labeled and incubated with liver or HeLa cell nuclear extract in the absence (−) or presence (+) of homologous unlabeled DNA (50-fold molar excess), and the products were analyzed by gel retardation on an 5.5% native acrylamide gel. (B) The same two probes were used (as marked), except that other competing fragments and oligonucleotides (also in 50-fold excess) were present. These include the enitre frament from −8.7 to −8.43 kb (+), unlabeled probes 1 and 2, and DNA fragment from the retinol-binding protein (RBP) gene promoter containing the functional negative-acting region (a gift of R. Cortese [7, 13]). Oligonucleotides representing binding sites for the factors NLS1 and AP-1 were also included as competitors (18, 24).

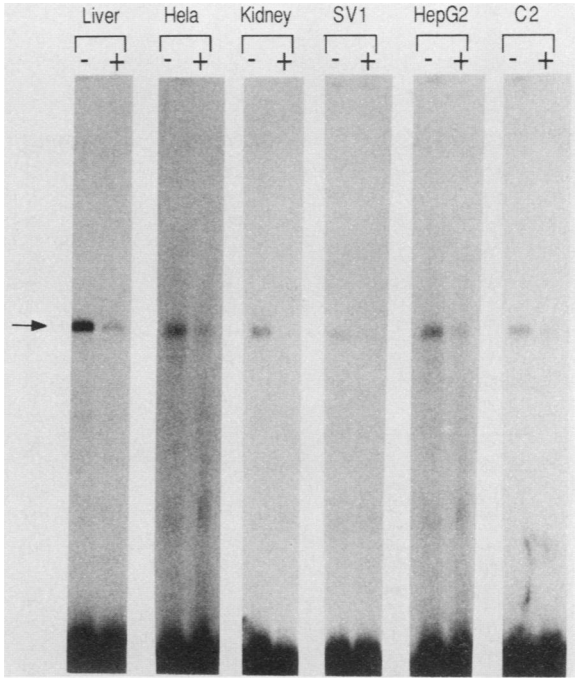

FIG. 5. The negative-region DNA-protein binding activity is ubiquitous. Probe 2 (Fig. 4) was end labeled and subjected to a gel shift assay with equal protein amounts of nuclear extracts prepared from the cell types identified above each lane, in the presence (+) or absence (−) of specific self competitor. The arrow depicts the specific ANF complex.

was tested further by competition during complex formation with excess molar amounts of several other oligonucleotides. No other oligonucleotide tested reduced complex formation (Fig. 4B). These unsuccessful competitors included the previously described negative elements in the retinol-binding protein gene and a group of other positive-acting factors (shown in Fig. 4 are sites for AP-1 and NLS1, the latter representing the protein that binds to the activating region of the albumin enhancer; not shown, but also with no effect, were oligonucleotides representing SP-1-, HNF1-, HNF3-, HNF4-, C/EBP-, and Oct1-binding sites) (8, 11, 24).

The cellular distribution of the protein that bound to the negative-acting region was determined next. The same total amounts of protein from nuclear extracts from six different cell sources were used in a gel shift reaction with negative region probe 2 (−8,561 to −8,433 bp) (Fig. 5). Each extract used was subjected to a gel shift reaction by using a known AP-1-binding site to ensure its integrity (data not shown), and all contained approximately equal amounts of AP-1. All the cell lines or tissues were able to react with the negative region of the albumin enhancer, and when normalized to the AP-1 content, the concentration varied by no more than threefold. Our results therefore illustrate that the factor recognizing the negative region of the albumin enhancer is widely distributed in various cell and tissue types which do (liver, CWSV1, and HepG2) or do not (HeLa, kidney, and C2) transcribe albumin.

To more precisely define the binding site for the proteins that produce the gel retardation described above and test for any other possible protein-binding sites, we carried out two types of experiments, both of which used end-labeled DNA probes. Each of the fragments (Fig. 6, probe 1 or probe 2) was uniquely 5′ end labeled (Fig. 6, bottom) and subjected to separate analyses. Note that only the results for one DNA strand (the bottom strand for probe 1 and the top strand for probe 2) are shown in Fig. 6; the data representing the results from both strands are summarized in Fig. 7.

For each ExoIII analysis, 1 ng of labeled probe was incubated with 30 μg of nuclear protein (either liver, HeLa, or kidney) for 30 min and subsequently subjected to ExoIII digestion. First, ExoIII digestion of the labeled probe in the presence of nuclear proteins showed one major block to digestion in each probe; each exonuclease stop was specifically inhibited by the presence of excess unlabeled probe (Fig. 6A and B). The starred bands indicate specific, self-competable exonuclease stop sites representing points of DNA-protein interaction. With probe 1 (Fig. 6A), which contains the somewhat stronger binding site based on gel retardation analysis, a predominant exonuclease stop was seen at nucleotide −8,598 with crude liver, HeLa, and kidney nuclear extracts (Fig. 6A). Samples in lanes marked (+) contained a 30-fold molar excess of cold unlabeled self competitor, whereas samples in lanes marked (−) contained

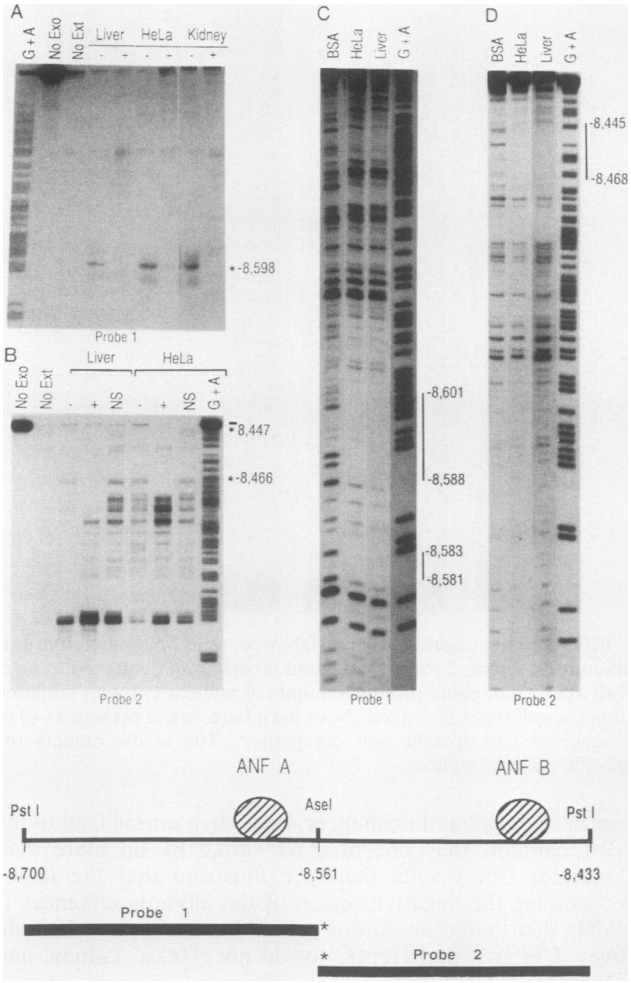

FIG. 6. ExoIII stop and DNase I protection experiments to delineate the boundaries of factor binding. The two probes were 5′ end labeled at the position shown and subjected to the assays as described in Materials and Methods. (A and B) ExoIII assays for probes 1 and 2, respectively. (C and D) DNase I footprints for the same two probes. Note that in panel A the lanes labeled (+) contain homologous self competitor (30-fold) while the (−) lanes contain an equal molar amount of pGem1. In panel B, however, the (−) lanes contain no unlabeled nonspecific competitor. This control is instead now included in the lanes labeled NS, containing an equal molar amount of pGem1. The lanes labeled "No Ext" received no protein extract, and the lanes labeled "No Exo" received neither protein extract nor ExoIII. The ExoIII stop fragments are indicated by asterisks and by their positions relative to the albumin transcription start site. A G + A sequence ladder is included. The DNase I protection assay (C and D) used the same probes incubated with either bovine serum albumin (BSA; as a control), P11-purified HeLa extract, or a heparin-agarose-purified liver protein fraction (see Materials and Methods). These complexes were treated with DNase I, and the products were analyzed by 8% denaturing polyacrylamide gel electrophoresis as shown. Regions of protection from DNase I digestion are denoted by the bar, with the coordinates as shown. See Fig. 7 for a summary of the binding sites and sequence comparison. For this and subsequent figures, we have used ANF (albumin-negative factor) to denote these proteins that interact with the negative element.

an equal molar excess of a pGem fragment to keep the amount of DNA constant during digestion.

A second specific exonuclease stop (site B) was observed with both liver and HeLa extracts (Fig. 6B). In these experiments the (+) again refers to a 30-fold excess of self competitor, whereas the lanes marked (NS) contain an equal molar excess of pGem. The (−) lanes contain no unlabeled DNA and hence a reduced amount of total DNA. A specific ExoIII stop was observed at nucleotide −8,466, whereas a much weaker stop was seen at nucleotide −8,447 (Fig. 6B, only observed when the ExoIII activity is reduced by the inclusion of nonspecific DNA).

A DNase I protection assay (Fig. 6C and D) with the same probes further delineated the boundaries of DNA-protein interaction in the same regions as the exonuclease stops. These footprint reactions (as described in Materials and Methods) were performed with either bovine serum albumin as a control or 5 μg of a heparin-agarose-purified liver nuclear extract or HeLa extract fractionated on a P11 column (a 0.4 M KCl step; gift of X. Fu). The clearly footprinted region of about 23 nucleotides representing the bound protein is depicted in Fig. 6C (somewhat split). The protected region includes the sequence defined by the ExoIII stop site, and the results are summarized in Fig. 7. A comparison of the sequence within the two protected regions revealed a 7-bp identity within a 10-bp sequence. The relative importance of these nucleotides for protein binding in this region will require further mutagenesis and functional studies.

The more upstream protein-binding site alone can exert a negative effect. Having identified these binding sites for a candidate negative-acting protein (labeled ANF [albumin negative factor] in Fig. 6), a final experiment compared the effectiveness of the two binding sites in suppressing TTR gene enhancer activity. Transient transfections were performed with similar plasmid constructs as shown in Fig. 3 with fragments containing the two sites now independently inserted between the TTR elements (all constructs shown are in the + orientation). Following cotransfection into HepG2 cells (50 μg of TTR DNA) using an SV40 enhancer-driven β-globin plasmid (20 μg) as a control, both TTR and β-globin gene expression were assayed in the same reaction (diagrams of the β-globin and TTR assays are shown at the foot of Fig. 1 and 2, respectively). The 93-nucleotide TTR signal (labeled in Fig. 8) can be evaluated after correcting for variations in transfection efficiency and/or RNA extraction by using the β-globin signal which here consists of a summation of the 143 and 206 bands, representing the first and second exons, respectively (labeled in Fig. 8).

The first 144-nucleotide fragment (−8.56 to −8.7 kb) had about the same negative activity as the larger fragment previously described (−8.43 to −8.7 kb) whereas the smaller fragment (−8.43 to −8.56 kb) had at best a very slight negative effect (Fig. 8). Recall that Fig. 4 suggested that the affinity of the first site (−8.56 to −8.7 kb; probe 1) was stronger than that of the second smaller segment (−8.56 to −8.43 kb; probe 2). Thus, the apparent stronger binding site possessed the greater negative effect in transfection assays, suggesting the possibility that the entire functional negative effect in region III was due to this 144-nucleotide fragment −8.56 to −8.7 kb.

DISCUSSION

The experiments described in this paper show that the albumin enhancer contains a functionally acting negative

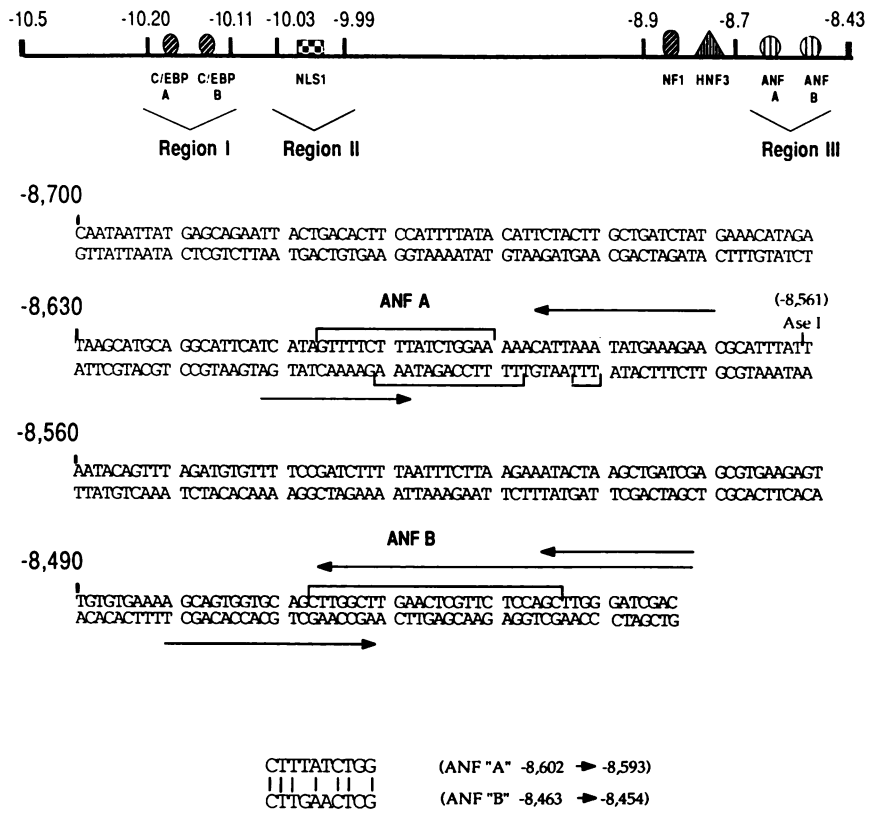

FIG. 7. Summary of the DNA-protein interactions occurring on the mouse albumin enhancer along with the DNA sequence of the negative region (−8.7 to −8.43 kb). Our previous study identified three general regions of functional importance (identified as I, II, and III), and the proteins that interact with them are shown schematically (18). Region I has two sites for the protein C/EBP. Region II has a factor binding between −10.03 and −9.94 kb, which we have named NLS1 (non-liver specific). Region III contains the negative-acting sequences which we describe in this report. The sequence from the negative region (III) is shown −8.7 to −8.43 kb, with the exonuclease stop sites from both strands represented by the arrows. The DNase I-protected regions are represented by the brackets. No footprint has been obtained for ANF site B lower strand, which represents the weaker binding site. A comparison of the ANF A and B binding sites is shown at bottom, indicating a 7-of-10 sequence match between the two protected regions.

sequence between −8.43 and −8.7 kb upstream of the albumin gene RNA start site. A negative action is also evident when the element is placed between the enhancer and promoter of the mouse TTR gene; however, the negative element had no direct effect on the mouse albumin promoter by itself, suggesting that the mechanisms of action of this element was to prevent the action of distant positive acting sequences. Two site-specific binding sites with similar mobilities in gel retardation assays were located within the negative-acting region. Each of these sites could interfere with complex formation by the other, suggesting that one protein factor recognized both sites. The stronger binding site as part of a 144-nucleotide sequence could act as an enhancer suppressor on its own. No other protein interactions could be found within the 267 nucleotides of the negative region by either gel retardation or exonuclease assays, and no oligonucleotide representing the binding site of any of the large collection of known proteins interrupted the formation of the DNA-protein complexes in the negative-acting region. Thus, the protein that binds this site is a candidate for a unique negative-acting protein. Further studies, including mutagenesis of the negative-acting site and a demonstration of the effect of the negative element in other cell types (e.g., HeLa cells), are necessary to determine the possible role for this protein in regulating albumin-specific gene expression.

The transgenic animal experiments of Pinkert et al. (33) that first demonstrated the positive role of the albumin enhancer suggested that removal of a large section of upstream DNA, which we now know includes this negative region, allowed expression of the albumin gene in inappropriate tissues. Thus, it might be that the negative element described here operates in nonhepatic cell types as a general suppressor of neighboring enhancer function. This negative action is overcome in hepatocytes by the cluster of positive-acting sites nearby, including the two previously described C/EBP sites or the site for the widely distributed NLS1 positive-acting protein that binds in region II and might be correctly modified to stimulate transcription in several non-liver, differentiated tissues (18). We presume that in the experimental constructs in which the negative element divided the TTR elements, the negative element is able to prevent the action of other site enhancer DNA-binding proteins.

In many ways the albumin negative element resembles the yeast silencers in their ability to function in an orientation- and distance-independent fashion to block transcription (2). Like the yeast silencers, our element is not promoter specific and works through a *cis*-acting sequence known to bind cellular factors. As the SIR proteins and binding sites from yeasts and this negative-acting factor are better characterized, a comparison will certainly be warranted.

From the work of several groups there were strong suggestions and precedent that negative-acting DNA regions

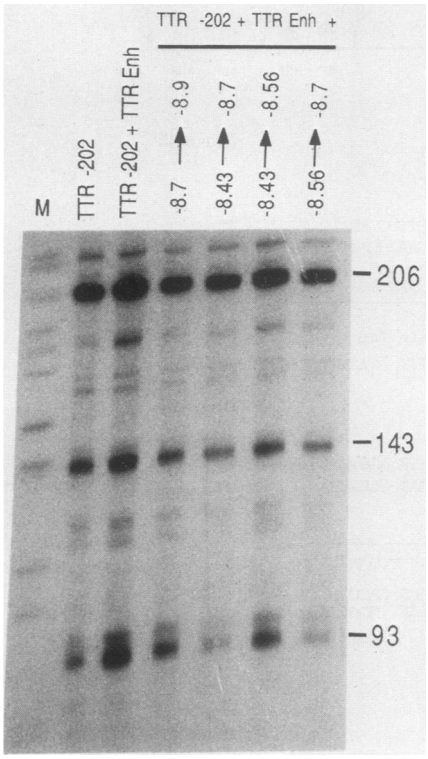

FIG. 8. The mouse albumin enhancer negative region sequences were placed between the TTR enhancer and promoter, and in this assay, both probes (for the TTR or the β-globin control) were employed simultaneously. Again the TTR promoter (TTR −202) and TTR promoter-enhancer controls are shown (TTR −202 + TTR Enh). The coordinates of the inserted albumin sequences are as marked, and are all in the (+) orientation. The 93-nuceotide band represents the specific TTR signal, whereas the 143- 206-bp bands represent exons 1 and 2 of the β-globin gene, respectively.

exist to suppress hepatocyte-specific gene expression in nonliver tissue and that without them, inappropriate expression may result (7, 13, 21, 30, 32, 33). In most of these cases, however, the nature of the proteins has not yet been established. Negative-acting proteins have recently been implicated in the regulation of c-*myc* and immunoglobulin heavy-chain promoter and enhancer, respectively, which play critical roles in determining cell type specificity (17, 19, 20, 34).

The ability of the albumin enhancer to function in hepatoma cells deserves special note. The albumin gene is liver specific and reaches its maximal level of expression several weeks following birth in the rodent (3). Furthermore, the function of the enhancer in the partially differentiated HepG2 cell (a fetal cell type in which the endogenous gene is expressed at a much reduced rate compared with adult liver [1, 14]) is surprising and demonstrates that the decreased albumin transcriptional level observed in this cell type is not due to a complete lack of enhancer function. Possibly the relatively low levels of several different transcription factors are responsible for the decreased transcription of albumin (and, for that matter, several other genes encoding serum protein) in hepatoma cells.

From this discussion we draw support for the picture of combinations of transcription factors interacting to produce the final picture of regulated gene action that is the hallmark of differentiated cells. At the moment we know too little to suggest specific mechanisms of action of negative-acting proteins. However, the first step in solving such interactions is the clear delineation of negative-acting sequences on DNA and identification of the cognate proteins for these sites.

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LITERATURE CITED

1. **Babiss, L. E., R. S. Herbst, A. L. Bennett, and J. E. Darnell, Jr.** 1987. Factors that interact with the rat albumin promoter are present both in hepatocytes and other cell types. Genes Dev. **1**:256–267.
2. **Brand, A. H., L. Breeden, J. Abraham, J. Sternglanz, and K. Nasmyth.** 1985. Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell **41**:41–48.
3. **Camper, S. A., and S. M Tilghman.** 1989. Postnatal repression of the α-fetoprotein gene is enhancer independent. Genes Dev. **3**:537–546.
4. **Cereghini, S., M. Blumenfeld, and M. Yaniv.** 1988. A liver-specific factor essential for albumin transcription differs between differentiated and dedifferentiated rat hepatoma cells. Genes Dev. **2**:957–974.
5. **Cereghini, S., M. Raymondjean, A. G. Carranca, P. Herbomel, and M. Yaniv.** 1987. Factors involved in control of tissue-specific expression of albumin gene. Cell **50**:627–638.
6. **Clayton, D. F., M. Weiss, and J. E. Darnell, Jr.** 1985. Liver-specific RNA metabolism in hepatoma cells: variation in transcription rates and mRNA levels. Mol. Cell. Biol. **5**:2623–2632.
7. **Colantuoni, V., A. Pirozzi, C. Blance, and R. Cortese.** 1987. Negative control of liver-specific gene expression: cloned human retinol-binding protein gene is repressed in HeLa cells. EMBO J. **6**:631–636.
8. **Costa, R. H., D. R. Grayson, and J. E. Darnell, Jr.** 1989. Multiple hepatocyte-specific nuclear factors function in the regulation of the transthyretin and α1-antitrypsin genes. Mol. Cell. Biol. **9**:1415–1425.
9. **Costa, R. H., E. Lai, and J. E. Darnell, Jr.** 1986. Transcriptional control of the mouse prealbumin (transthyretin) gene: both promoter sequences and a distinct enhancer are cell specific. Mol. Cell. Biol. **6**:4697–4708.
10. **Costa, R. H., E. Lai, D. R. Grayson, and J. E. Darnell, Jr.** 1988. The cell-specific enhancer of the mouse transthyretin (prealbumin) gene binds a common factor at one site and a liver-specific factor(s) at two other sites. Mol. Cell. Biol. **8**:81–90.
11. **Courtois, G., S. Baumhueter, and G. R. Crabtree.** 1988. Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. Proc. Natl. Acad. Sci. USA **85**:7937–7941.
12. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
13. **D'Onofrio, C., V. Colantuoni, and R. Cortese.** 1985. Structure and cell-specific expression of a cloned human retinol binding protein gene: the 5'-flanking region contains hepatoma specific transcriptional signals. EMBO J. **4**:1981–1989.
14. **Friedman, J. M., L. E. Babiss, D. F. Clayton, and J. E. Darnell, Jr.** 1986. Cellular promoters incorporated into the adenovirus genome: cell specificity of albumin and immunoglobulin expression. Mol. Cell. Biol. **6**:3791–3797.
15. **Goodbourn, S., H. Burstein, and T. Maniatis.** 1986. The human β-interferon gene enhancer is under negative control. Cell **45**:601–610.
16. **Grayson, D. R., R. Costa, K. G. Xanthopoulos, and J. E.**

Darnell, Jr. 1988. One factor recognizes the liver-specific enhancers in α_1-antitrypsin and transthyretin genes. Science **239:** 786–788.

17. **Hay, N., M. Takimoto, and J. M. Bishop.** 1989. A FOS protein is present in a complex that binds a negative regulator of MYC. Genes Dev. **3:**293–303.
18. **Herbst, R. S., N. Friedman, J. E. Darnell, Jr., and L. E. Babiss.** 1989. Positive and negative regulatory elements in the mouse albumin enhancer. Proc. Natl. Acad. Sci. USA **86:**1553–1557.
19. **Imler, J. L., C. Lemaire, C. Wasylyk, and B. Wasylyk.** 1987. Negative regulation contributes to tissue specificity of the immunoglobulin heavy-chain enhancer. Mol. Cell. Biol. **7:**2558–2567.
20. **Kakkis, E., K. J. Riggs, W. Gillespie, and K. Calame.** 1989. A transcriptional repressor of c-myc. Nature (London) **339:**718–721.
21. **Killary, A., and R. E. K. Fournier.** 1984. A genetic analysis of extinction: trans-dominant loci regulate expression of liver-specific traits in hepatoma hybrid cells. Cell **38:**523–534.
22. **Knowles, B., C. C. Howe, and D. P Arden.** 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science **209:**497–499.
23. **Kovesdi, I., R. Reichel, and J. R. Nevins.** 1986. Identification of a cellular transcription factor involved in E1A trans-activation. Cell **45:**219–228.
24. **Jones, N. C., P. W. Rigby, and E. B. Ziff.** 1988. Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. **2:**267–281.
25. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2:**786–800.
26. **Lichsteiner, S., J. Wuarin, and U. Schibler.** 1987. The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. Cell **51:**963–973.
27. **Liu, J.-K., Y. Bergman, and K. S. Zaret.** 1988. The mouse albumin promoter and a distal upstream sites are simultaneously DNase I hypersensitive in liver chromatin and bind similar liver-abundant factors *in vitro*. Genes Dev. **2:**528–541.
28. **Maniatis, T., S. Goodbourne, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236:** 1237–1244.
29. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
30. **Muglia, L., and L. B. Rothman-Denes.** 1986. Cell type-specific negative regulatory element in the control region of the rat α-fetoprotein gene. Proc. Natl. Acad. Sci. USA **83:**7653–7657.
31. **Nir, U., M. D. Walker, and W. J. Rutter.** 1986. Regulation of rat insulin 1 gene expression: evidence for negative regulation in nonpancreatic cells. Proc. Natl. Acad. Sci. USA **83:**3180–3184.
32. **Petit, C., J. Levilliers, M. O. Ott, and M. C. Weiss.** 1986. Tissue-specific expression of the rat albumin gene: genetic control of its extinction in microcell hybrids. Proc. Natl. Acad. Sci. USA **83:**2561–2565.
33. **Pinkert, C. A., D. M. Ornitz, R. L. Brinster, and R. D. Palmiter.** 1987. An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. Genes Dev. **1:**268–276.
34. **Scheuermann, R., and U. Chen.** 1989. A developmental-specific factor binds to suppressor sites flanking the immunoglobulin heavy-chain enhancer. Genes Dev. **3:**1255–1266.
35. **Woodworth, C. D., and H. C. Isom.** 1987. Regulation of albumin gene expression in a series of rat hepatocyte cell lines immortalized by simian virus 40 and maintained in chemically defined medium. Mol. Cell. Biol. **7:**3740–3748.