An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice

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Transgenic mice were used to locate the *cis*-acting DNA elements that are important for efficient, tissue-specific expression of the mouse albumin gene in the adult. Chimeric genes with up to 12 kb of mouse albumin 5′-flanking region fused to a human growth hormone (hGH) reporter gene were tested. Remarkably, a region located 8.5–10.4 kb upstream of the albumin promoter was essential for high-level expression in adult liver and the region in between −8.5 and −0.3 kb was dispensable. The far-upstream region behaved like an enhancer in that its position and orientation relative to the albumin promoter were not critical; however, it did not function well with a heterologous promoter. Two of four DNase hypersensitive sites found in the 5′-flanking region of the albumin gene map to the far-upstream and promoter regions; the others may reflect regions involved in developmental or environmental control of this gene.

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The albumin and α-fetoprotein genes are evolutionarily related and separated by 14 kb on mouse chromosome 5 (Ingram et al. 1981). Furthermore, they are developmentally regulated in a similar fashion in that they are induced coordinately in fetal liver, yolk sac, and gastrointestinal tract (Krumlauf et al. 1985a). However, albumin expression is maintained in the adult liver whereas α-fetoprotein expression declines to undetectable levels shortly after birth; both genes are repressed in nonhepatic tissues of the adult. They are regulated primarily at the transcriptional level and their mRNAs are among the most abundant polymerase II transcripts in the liver and code for the most abundant serum proteins of fetus and of adult (Tilghman and Belayew 1982).

Progress has been made in dissecting the *cis*-acting DNA elements involved in the developmental and tissue-specific control of α-fetoprotein gene expression by assaying various constructs in transgenic mice (Krumlauf et al. 1985a,b; Hammer et al. 1987) or after transfection into tissue culture cells (Godbout et al. 1986; Muglia and Rothman-Denes 1986; Widen and Papaconstantinou 1986). When tested in transgenic mice, a construct with 7 kb of 5′-flanking region associated with an α-fetoprotein minigene allowed proper tissue-specific expression at levels exceeding those of the endogenous genes in some cases (Hammer et al. 1987), suggesting that all of the elements necessary for proper expression lie within that construct. Further dissection revealed that the α-fetoprotein promoter and 5′ sequences extending to −1 kb did not promote expression in any tissue; however, when it was combined with any of three large regions that cover the region from −1 to −7 kb, there was substantial, but not equivalent, expression in all three target tissues. In addition to appropriate tissue-specific expression, the α-fetoprotein gene was repressed after birth and activated by liver damage in the adult, in concert with the endogenous genes (Hammer et al. 1987). In contrast to the results in transgenic mice, the α-fetoprotein promoter does stimulate expression in a cell-specific manner when transfected into cultured cells, and a region located between −52 and −85 appears to be essential for this property (Godbout et al. 1986; Muglia and Rothman-Denes 1986; Widen and Papaconstantinou 1986). Each of the upstream regions behaved like a typical enhancer (Serfling et al. 1985) in that its orientation and position relative to the promoter could be changed. Furthermore, they could activate a heterologous promoter, when tested by transfection into cultured liver cells (Godbout et al. 1986).

Previous studies, in which the rat albumin promoter

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and 5′-flanking region were tested by transfection or infection into hepatoma cells, revealed that there is an important, tissue-specific element located within 400 bp of the cap site (Ott et al. 1984; Friedman et al. 1986). Furthermore, the mouse albumin promoter is transcribed more efficiently in cell-free extracts prepared from liver than from other tissues (Gorski et al. 1986). Using this in vitro transcription assay, the DNA elements required for preferential expression in liver extracts were localized to a region between 55 and 170 bp upstream of the cap site (Gorski et al. 1986). However, previous studies have not addressed the question of whether there is an albumin enhancer as well.

Considering that the albumin and α-fetoprotein genes are related, linked, and regulated similarly, one might expect the organization of the regulatory domains to be conserved. Therefore, we set out to ascertain whether the albumin gene has enhancers and, if so, where they lie in relation to the structural gene. We show here that the albumin promoter is tissue-specific in transgenic mice and there appears to be only one region with enhancer-like activity that is located much further upstream than any of the α-fetoprotein gene elements.

Results

Albumin-growth hormone fusion genes are expressed in liver

Because we wished to examine the control of the mouse albumin gene in transgenic mice, we selected human growth hormone (hGH) as a heterologous reporter gene. This choice was based on the facts that the hHG gene has been successfully used in combination with a number of other regulatory regions, its mRNA appears to be relatively stable in a variety of cells, and it stimulates growth if sufficient amounts of GH are secreted into the bloodstream (Palmiter et al. 1983; for review, see Palmiter and Brinster 1986). To construct albumin–human growth hormone (alb–hGH) fusion genes, a synthetic *Bam*HI linker was inserted 22 bp downstream from the albumin cap site in an albumin subclone that spans the promoter region (see Materials and methods). This subclone was then fused to the hGH gene at its natural *Bam*HI site at +3. Thus, the albumin promoter and 5′-flanking region were joined to the hGH structural gene near the beginning of the first exon of each gene such that the transcription start site would be determined by albumin sequences and the resulting hGH mRNA would have 22 extra nucleotides contributed by albumin sequences. Additional albumin 5′-flanking region was inserted into this clone to create a fusion gene with about 12 kb of albumin 5′ sequence fused to the hGH gene (Fig. 1). The proper reconstruction of the albumin sequences was verified by Southern blot comparison of the plasmid clone and genomic DNA after digestion with several enzymes.

Restriction fragments containing about 0.3, 0.9, 3.8, 8.5, or 12 kb of albumin 5′-flanking region and the hGH structural gene (top line, Fig. 1) were separated from the remaining plasmid DNA by agarose gel electrophoresis. A few hundred copies of each of these DNA fragments were microinjected into pronuclei of fertilized eggs to produce transgenic mice (Brinster et al. 1985). Groups of 3–21 transgenic mice were produced with each of these constructs. With the exception of those mice containing the 0.3 alb–hGH construct, some mice grew significantly larger than normal, indicating that the alb–hGH genes were being expressed in some tissues of those mice.

To determine in which organs the genes were being expressed, the mice were sacrificed and hGH mRNA levels were measured in a variety of organs by a solution hybridization assay using an oligonucleotide complementary to a sequence in exon 4 of hGH mRNA. hGH mRNA was detected in livers of all mice that grew larger than normal as well as in a few other mice in each group; it was also detected in the kidneys of a few mice, but not in any other tissue that was assayed, including spleen, intestine, pancreas, brain, heart, muscle, lung, and testis. Figure 2 shows the levels of hGH mRNA detected in liver and kidney of each of the founder mice and indicates which mice grew larger than normal. The mean level of hGH mRNA in the liver of those mice that expressed the transgene is indicated by the histograms. The frequency of detecting expression was low (only 2 of 10 transgenic mice), with the construct containing only 0.3 kb of albumin sequence, but was high for the constructs with 0.9, 3.8, 8.5, and 12 kb of albumin sequence (Fig. 2). The extreme variability in the level of expression from one mouse to another is often observed in transgenic mouse experiments and probably reflects influences from the chromosomal site of integration, as well as variable transgene copy number and mosaicism of some of the founders (Palmiter and Brinster 1986).

Although there was a progressive increase in the average level of hGH mRNA detected in the liver as more albumin-flanking DNA was included in the constructs (Fig. 2), the level of hepatic expression was dramatically higher in most of the mice with 12 kb of albumin sequence. The average level of hGH mRNA in these mice was about 5000 molecules/cell and in some mice the level was comparable to that of endogenous albumin mRNA, estimated to be about 10,000 molecules/cell (Tilghman and Belayew 1982). These data suggest that there is an enhancer-like element located 8.5–12 kb upstream of the promoter. Moreover, the albumin promoter, which is contained within the 0.3-kb construct, is tissue-specific but it has a propensity not to be expressed unless additional 5′ sequence is present, as in the 0.9-, 3.8-, and 8.5-kb alb–hGH constructs. Curiously, the constructs with 0.3–8.5 kb of albumin sequence also promoted a low level of expression in the kidney of some mice. However, this activity appears to be extinguished when the enhancer-like element between −8.5 and −12 kb is present, since no kidney expression was detected in any of the mice with the 12-kb construct (Fig. 2).

Because many of the mice grew larger than normal, we knew that functional hGH mRNA was produced, indi-

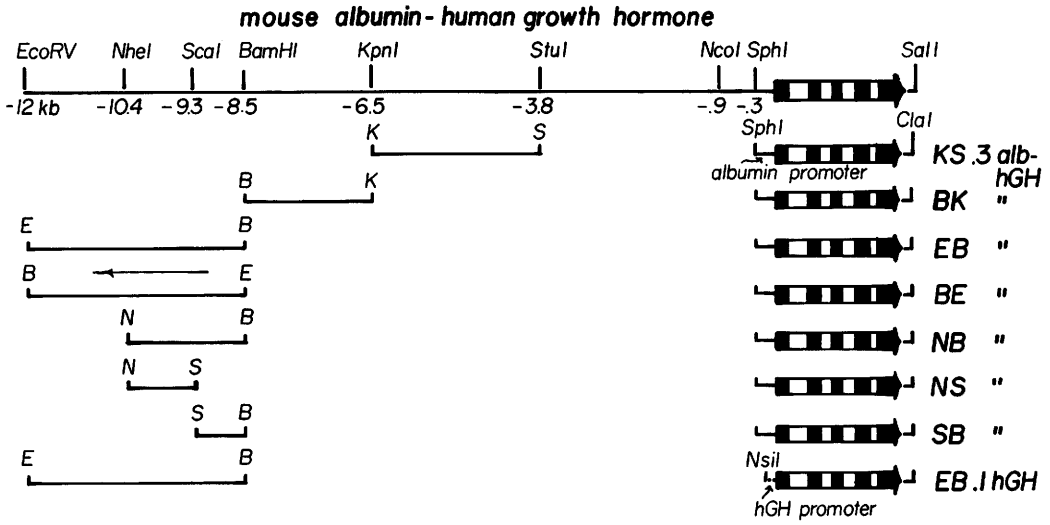


Figure 1. Maps of the alb–hGH gene constructs tested in transgenic mice. The 12-kb alb–hGH construct (*top line*) was prepared as described under Materials and methods. The 12-, 8.5-, 3.8-, 0.9-, and 0.3-kb alb–hGH DNA fragments were isolated using unique sites in the albumin sequence and the *Sal*I site at the 3′ side of the hGH gene. The internal deletions were created as described under Materials and methods; in each case the upstream albumin fragments were joined to the albumin promoter at the *Sph*I site that lies at −0.3 kb; the letter designations refer to the restriction enzymes used to isolate the albumin fragments. The EB 0.1–hGH construct has the albumin EB fragment joined to an *Nsi*I site located at −90 in the hGH gene. Each of these latter fragments was isolated with *Kpn*I (in the polylinker) and *Cla*I for microinjection.

cating that normal splicing occurred. To ascertain whether transcription was starting at the proper site and whether the same site was used for each of the constructs, we performed primer extension assays. Total RNA was hybridized with a ^{32}P-labeled oligonucleotide complementary to a sequence in the first exon of hGH mRNA and extended with reverse transcriptase. We expected an extension product of 72 nucleotides if the normal albumin cap site was being used. Indeed, as shown in Figure 3, a prominent extension product of that length was observed with all samples tested, including liver samples from transgenic mice with the 0.3-, 0.9-, 3.8-, and 12-kb constructs and a kidney sample from a mouse with the 0.9-kb construct.

The enhancer-like element between −8.5 and −12 kb is sufficient for high-level hepatic expression

To ascertain whether there might be multiple enhancer-like elements in the 12 kb of albumin 5′ flanking region,

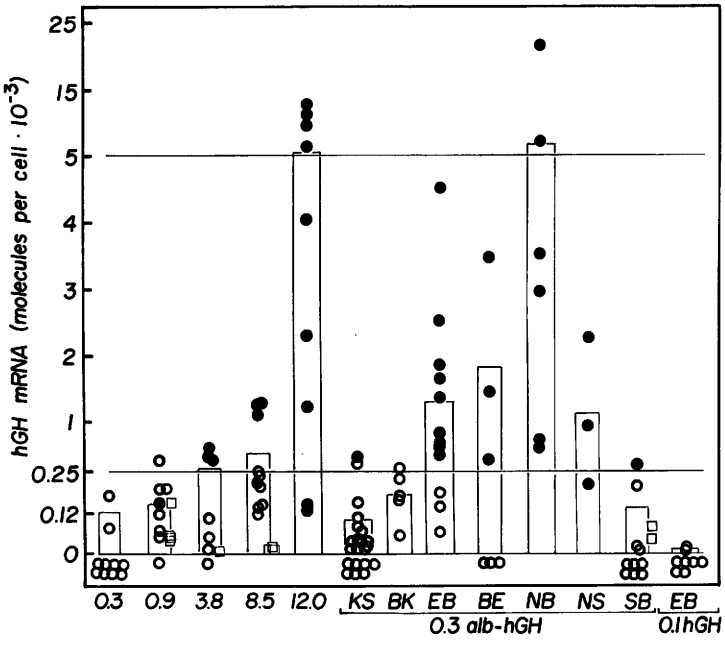


Figure 2. hGH mRNA levels in liver and kidney of transgenic mice bearing the alb–hGH constructs shown in Fig. 1. hGH mRNA was measured by solution hybridization as described under Materials and methods. Each value corresponds to an individual founder transgenic mouse; values below the origin represent mice in which no hGH mRNA could be detected (less than 10 molecules/cell); solid symbols correspond to liver values of mice that grew more than 1.3-fold larger than control littermates; open symbols are used for those mice that did not grow larger than normal. Liver values are presented as circles, and kidney values as squares. Histograms represent the mean levels of liver hGH mRNA, based on only those mice that expressed the gene. Note the change in scale at 250 and 5000 molecules/cell.

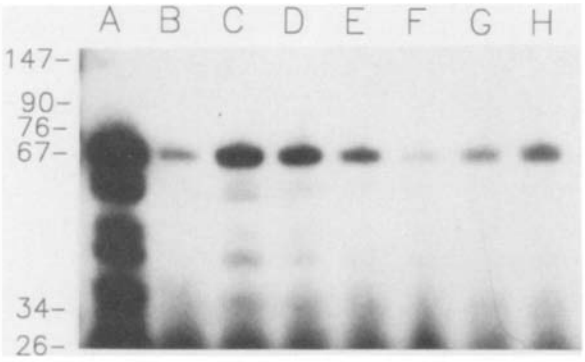


Figure 3. Primer extension analysis of alb–hGH transcripts. Total RNA was prepared, 50 μg was hybridized with an end-labeled oligonucleotide and extended with reverse transcriptase, and the extension products were displayed on an acrylamide gel as described under Materials and methods. Markers are end-labeled *Hpa*II fragments from pBR322. (Lane *A*) Liver RNA from a mouse bearing a 12-kb alb–hGH construct (this RNA sample was from a mouse with 12,000 hGH mRNA molecules/cell; consequently, the lane is overexposed and reveals prominent bands of smaller size which probably represent premature termination products due to excess template); (lanes *B* and *C*) liver RNA from mice bearing a 3.8-kb alb–hGH construct; (lanes *D* and *E*) liver RNA from mice bearing the 0.9-kb alb–hGH construct; (lane *F*) kidney RNA from a mouse with 0.9-kb alb–hGH construct; (lanes *G* and *H*) liver RNA from mice with 0.3-kb alb–hGH construct. The EB 0.3 alb–hGH construct also gives a prominent band similar to that shown in lanes *B–H* (data not shown).

as is the case for the closely related and closely linked α-fetoprotein gene (Hammer et al. 1987), we tested three large regions for activity. We chose convenient restriction sites to generate fragments of about 3 kb each and positioned each fragment, in its normal orientation, directly upstream of the 0.3 alb–hGH construct. We tested these upstream fragments in the context of the albumin promoter region because previous data suggested that the albumin promoter has tissue-specific properties (see Introduction).

Only one of the three upstream fragments (the *Eco*RV–*Bam*HI fragment; EB-0.3 alb–hGH) stimulated hepatic expression to levels higher than that observed with the 0.3 alb–hGH construct alone. Whereas only one of the 26 mice with either of the two proximal fragments grew larger than normal, 9 of 12 mice with the far-upstream EB fragment grew large and they had hepatic hGH mRNA levels that averaged about 1200 molecules per cell (Fig. 2). Moreover, no expression in kidney, or in any other tissue, was observed with this construct. Thus, the albumin sequence between −0.3 and −8.5 kb is unnecessary for enhanced levels of hepatic-specific expression in the adult.

The far-upstream albumin element functions in the reverse orientation but does not activate a heterologous promoter

One property of enhancers is that their orientation relative to a promoter can be reversed without affecting their function (Serfling et al. 1985). To test this aspect of enhancer function, we reversed the orientation of the *Eco*RV–*Bam*HI fragment upstream of the 0.3 alb–hGH construct. This reverse orientation (BE 0.3 alb–hGH) worked just as well as the normal orientation (Fig. 2).

Another property of many enhancers is that they will activate a heterologous promoter. We have shown that the immunoglobulin, elastase, or metallothionein enhancers, when placed 90 bp upstream of the hGH gene with its own promoter, can direct expression to appropriate tissues, whereas the enhancerless hGH gene is not expressed anywhere in transgenic mice (Hammer et al. and Ornitz et al., in prep.). Hence, we tested the EB fragment upstream of the enhancerless 0.1 hGH vector. Eight transgenic mice were produced with this construct, but none of them grew larger than normal and only two of them had a trace of hGH mRNA in the liver. Thus, it appears that the far-upstream albumin element does not function well with the hGH promoter and may require its own promoter.

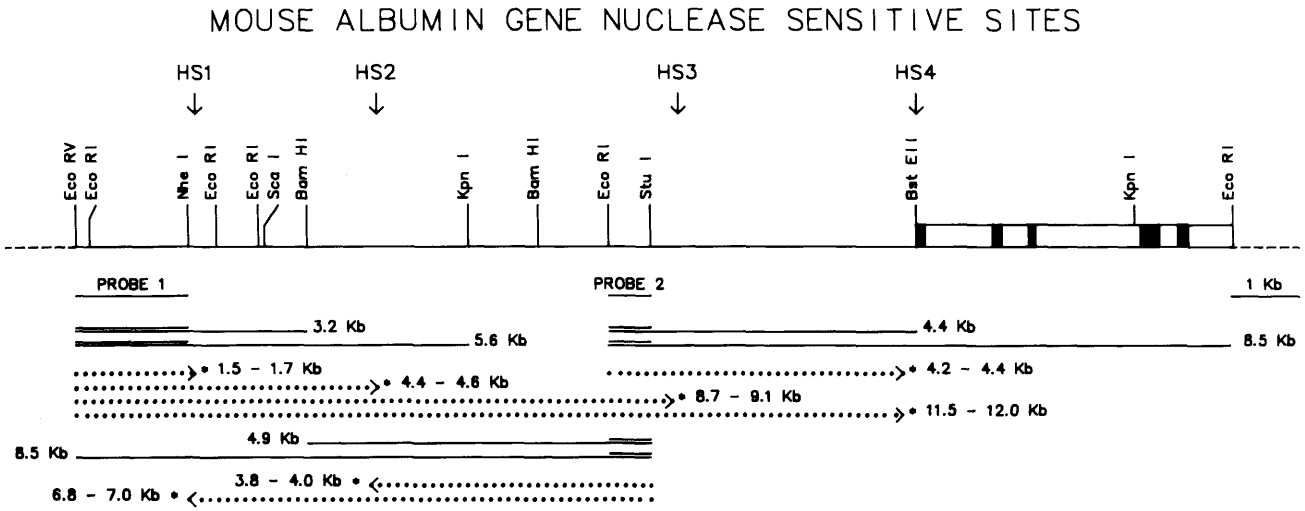
More precise localization of the albumin enhancer-like elements

To localize the far-upstream element further, we tested various smaller fragments derived from the EB region, each upstream of the 0.3 alb–hGH construct. A *Nhe*I–*Bam*HI fragment that encompassed the proximal half of the region (see Fig. 1) gave the highest average level of expression of all the constructs tested and all of the mice with this construct grew large (Fig. 2). This deletion narrowed the enhancer region down to about 1.9 kb. We then cut this region in half with *Sca*I and tested the distal NS fragment and the proximal SB fragment in the same manner. Four of 10 mice with the proximal SB region gave a low level of expression, comparable to that observed with 0.3 alb–hGH alone, and two of them expressed a low level of hGH mRNA in kidney as well. All three of the mice with the distal NS region had hepatic hGH mRNA levels averaging 10-fold higher than that observed with the promoter alone (Fig. 2). However, low expression (less than 20 molecules/cell) was also observed in tissues such as pancreas, intestine, and lung that had never been positive with other constructs. Thus, it appears that the *Nhe*I–*Sca*I region still has enhancer-like activity, but that elements within the adjacent region also play a role in determining hepatic-specific expression.

One of four DNase hypersensitive sites is located within the upstream enhancer region

The choice of restriction sites for the previous dissection of the upstream region was guided in part by the results of a search for DNase-sensitive sites in the albumin 5′-flanking region. Such hypersensitive sites may reflect the binding of regulatory proteins to specific DNA sequences (Elgin 1981). Two unique probes isolated from the albumin-flanking region, one centered at about −4 kb (probe 2) and the other centered at about −11 kb (probe 1), were used to map DNase hypersensitive re-

gions of the endogenous albumin gene. Liver nuclei were isolated and incubated at 25°C for various times to allow an endogenous DNase to make preferential cuts. Then the DNA was isolated, restricted with *Stu*I or *Eco*RI to map 5′ and 3′ of probe 2, respectively, or with *Eco*RV to map 3′ of probe 1 (see Fig. 4). Four DNase hypersensitive sites were observed: a prominent one (labeled HS1) is located at about −10.5 kb within the *Nhe*I–*Sca*I region



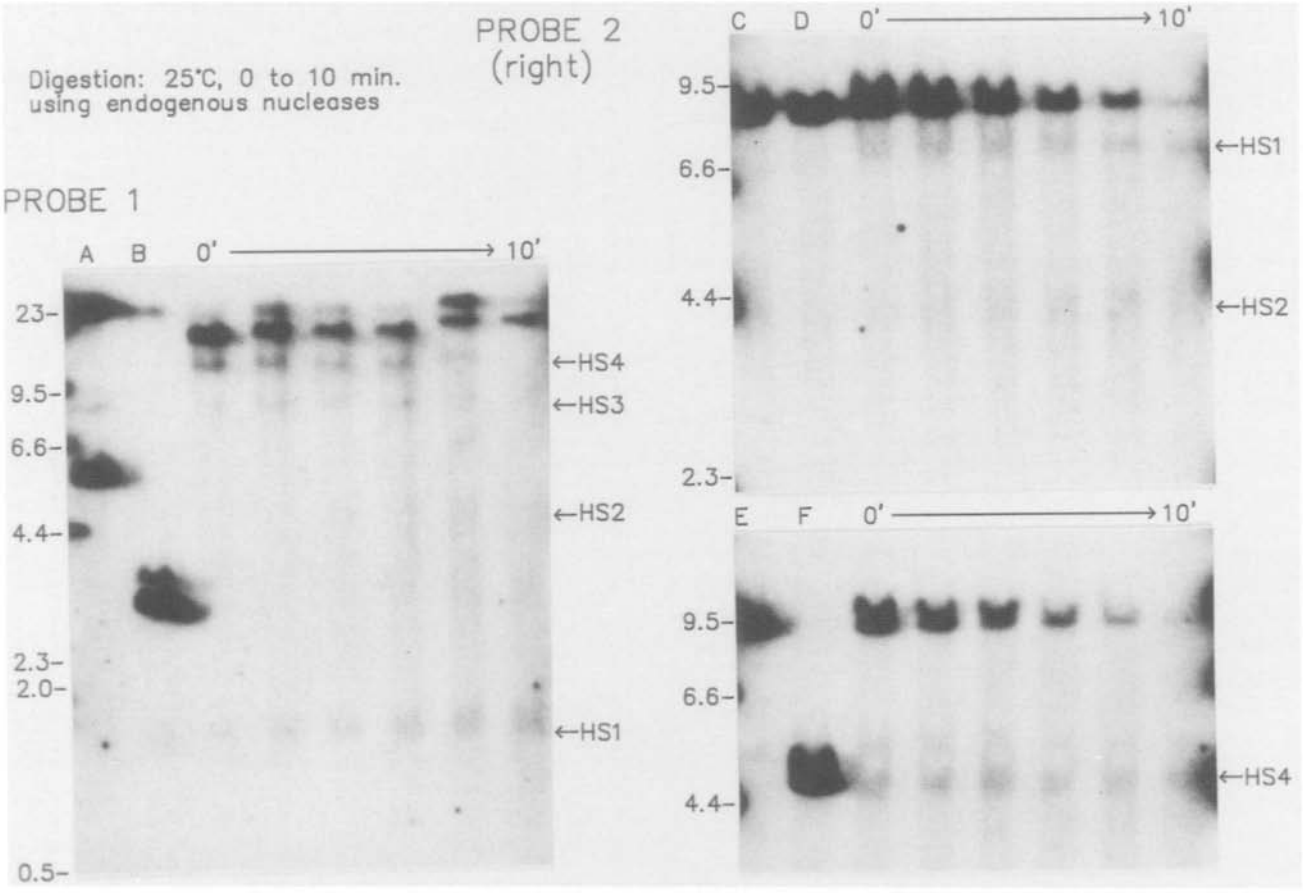


Figure 4. *(See facing page for legend.)*

that has enhancer-like activity, two less intense sites (HS2 and HS3) are located at about −7.5 and −3.1 kb, and a fourth intense site (HS4) is located in the vicinity of the promoter. Partial digestion by the endogenous nucleases during nuclei isolation probably accounts for the presence of most of these hypersensitive sites in the zero-time controls. Note, however, that HS1 and HS4 are absent in kidney nuclei (lanes C, D, and E).

Discussion

The 12 kb of mouse albumin 5′-flanking region appears to contain all the *cis*-acting elements necessary for appropriate expression of the albumin gene in the adult. Based on the analysis presented here, two regions are critical for liver-specific expression in the adult. One is the 300-bp promoter region, which had been shown previously to be a tissue-specific promoter (see Introduction), and the other is an enhancer-like element, or elements, that lie within the *Nhe*I–*Bam*HI region, 8.5–10.4 kb upstream of the promoter. When these two regions are juxtaposed, as in the NB 0.3 alb–hGH construct, they stimulate the production of hGH mRNA levels comparable to endogenous albumin mRNA (see Fig. 2). Although the high level of expression achieved with this construct suggests that all the essential *cis*-acting elements are present within the 12 kb of albumin 5′-flanking region, we cannot rule out the possibility that there may be other elements either further upstream, within, or downstream of the albumin gene that also contribute to appropriate expression. This uncertainty stems from the comparison of hGH and albumin mRNA levels, which may have different half-lives, and the uncertainty of knowing how many transgenes contribute to hGH mRNA accumulation. All of the transgenic mice studied in this report were founder animals, hence some of them were surely mosaics with the transgene in only a fraction of their cells (Wilkie et al. 1986). Moreover, most of the mice have multiple copies of the transgene and, like most other constructs tested in mice, there is little relationship between gene copy number and the level of expression; thus, we cannot know how many of the transgene copies are actually contributing to expression. Nevertheless, these results indicate that important control elements can lie great distances away from the stuctural genes that they regulate. Because the distance is about 10 kb, we favor the idea that this region is brought into proximity to the promoter by DNA looping rather than by some propagation of information along the DNA from the upstream element to the promoter (Serfling et al. 1985; Ptashne 1986).

The far-upstream albumin region has some of the properties of an enhancer, in that its position and orientation relative to the promoter do not seem to be critical. However, it failed to promote significant levels of expression when combined with the heterologous hGH promoter, which has responded to several other enhancers. Although it is possible that the albumin enhancer might function in conjunction with other promoters, it is also possible that it can only function in conjunction with its own or other liver-specific promoters. Thus, at present, we can only say that it enhances the function of the albumin promoter about 50-fold. It is curious that the albumin sequences between −8.5 and −12 kb also appear to suppress the kidney expression observed in some transgenic mice bearing constructs that contain the region between −0.3 and −0.9 kb.

The smallest, most effective albumin region tested thus far is about 1.9 kb. Although other truncated versions remain to be tested, it seems likely that the albumin element may not be as compact as other enhancer elements. This conclusion is based on the observation that the 1.9-kb *Nhe*I–*Bam*HI region is very effective whereas the smaller *Nhe*I–*Sca*I region is less effective quantitatively and allows expression in other tissues. Furthermore, the neighboring *Sca*I–*Bam*HI fragment does not stimulate hepatic expression but it does promote nonhepatic expression.

In the process of dissecting three different enhancers, we have noted that tissue specificity is lost before enhancing ability is lost. In addition to the example above, when the elastase element was trimmed from 134 bp to 80 bp it allowed promiscuous expression (Hammer et al., in prep.) and when a *Dra*I–*Pst*I fragment that includes the core of the immunoglobulin heavy-chain enhancer was tested, it allowed expression in several tissues in addition to B cells (Ornitz et al., in prep.). These observations suggest that each of these enhancers may contain core elements that bind general enhancing factors and that enhancing activity is restricted to specific cell

Figure 4. DNase hypersensitive sites in albumin 5′-flanking region. (*Top*) Map of the albumin gene extending from −12 kb to +4 kb (in the 5th intron). Albumin exons are solid boxes, introns are open boxes. Arrows labeled HS1 to HS4 indicate the positions of the hypersensitive sites mapped as described below. Probe 1 is a unique *Eco*RV–*Nhe*I fragment; probe 2 is a unique *Eco*RI–*Stu*I fragment. Genomic restriction fragments used as internal size markers are shown as solid lines paired with the appropriate probes. Dotted lines represent the size of the bands mapped with probes 1 and 2 and visualized on the autoradiographs shown below. (*Left*) Probe 1 was used to map nuclease-sensitive sites downstream of *Eco*RV. (Lane *A*) DNA from liver nuclei restricted with *Eco*RV and *Kpn*I; (lane *B*) DNA from liver nuclei restricted with *Eco*RV and *Bam*HI; (lanes *0′–10′*) digestion products from liver nuclei treated for 0–10 min at 25°C and then restricted with *Eco*RV. (*Center right*) Probe 2 was used to map nuclease-sensitive sites upstream of the *Stu*I site. (Lane *C*) DNA from kidney nuclei restricted with *Stu*I; (lane *D*) DNA from kidney nuclei restricted with *Stu*I and *Eco*RV; (lanes *0′–10′*) liver nuclei treated for 0–10 min at 25°C and then restricted with *Stu*I. (*Bottom right*) Probe 2 was used to map hypersensitive sites downstream of *Eco*RI. (Lane *E*) DNA from kidney nuclei restricted with *Eco*RI; (lane *F*) DNA from kidney nuclei restricted with *Eco*RI and *Bst*EII; (lanes *0′–10′*) liver nuclei treated at 25°C for 0–10 min and then digested with *Eco*RI. Markers are λ DNA restricted with *Hin*dIII.

types by factors that bind to adjacent regions. In some cases, all of the binding sites may be arranged in a very compact region, as in the case of the elastase enhancer, whereas in other cases the binding sites may be dispersed over several hundred base pairs as in albumin and immunoglobulin genes.

The binding of proteins to the albumin promoter and the far-upstream albumin region is consistent with the presence of DNase hypersensitive sites in these regions (Fig. 4). We noted two other hypersensitive regions between the promoter and the far-upstream regions, although we cannot ascribe any function to them. They might be involved in developmental or environmental control of albumin gene expression. Babiss et al. (1986) also noted DNase hypersensitive sites in the rat albumin gene located near the promoter and about 2.8 kb upstream; they did not look more then 7 kb upstream.

At this level of analysis, it appears that the organization of the regulatory elements for albumin and α-fetoprotein genes is dissimilar. The only similarity is that both promoters appear to be tissue specific, although neither functions very well in transgenic mice in the absence of upstream enhancer elements (Hammer et al. 1987). Whereas the enhancer elements for the α-fetoprotein gene are spread out in at least three regions spanning about 6 kb (Hammer et al. 1987), the albumin enhancer lies in a 1.9-kb region positioned nearly 10 kb upstream of the gene. However, it is important to recall that we are comparing the sequences that are important for expression of the albumin gene in the adult and expression of the α-fetoprotein gene in the developing fetus. Perhaps the sequences responsible for fetal expression of the albumin gene are the same ones that are used for α-fetoprotein gene expression; alternatively, they might be duplicated in the region between −0.3 and −8.5 kb. Thus, it is conceivable that the elements involved in the fetal expression of the two genes are similar and the far-upstream albumin elements are critical for maintenance of expression in adult liver. Ultimately, it will be informative to compare the DNA sequences of the enhancers for these two genes and the proteins that interact with them.

We originally planned to generate lines of transgenic mice that express alb-hGH fusion genes so that offspring could be used to study developmental and environmental control of transgene expression. However, the use of hGH as the reporter gene has precluded the generation of any lines that express significant amounts of hGH because the reproductive performance of both transgenic males and females was impaired. We anticipated some problem with females, since transgenic females that express metallothionein–hGH fusion genes are relatively infertile (Hammer et al. 1985), but the problem seems to be more severe with the alb-hGH constructs and both sexes are affected. The liver is also a prime site of synthesis of hGH when the metallothionein promoter is used; nevertheless, MT–hGH lines have been established and maintained for many generations (R. Hammer and R. Brinster, unpubl.). Perhaps some critical differences in the expression of these two transgenes during fetal development affects reproductive function.

These results reveal how the interpretation of results from enhancer studies depends on the reporter gene and the frame of reference. For example, albumin–CAT constructs have been instructive in terms of demonstrating that the albumin promoter functions better when transfected into hepatocytes than when tested in a few other cell types (Ott et al. 1984). However, because CAT enzyme activity was measured, it was not possible to know whether the level of albumin–CAT expression was quantitatively appropriate. Moreover, most hepatocyte cell lines do not make normal amounts of albumin mRNA, perhaps due to depletion of active enhancer-binding factors; thus, these cell lines may be unsuitable for detecting enhancer function. In conclusion, one cannot feel confident that all of the *cis*-acting elements have been included in a construct unless expression is developmentally appropriate, cell-specific, and quantitatively normal. The last parameter is difficult to measure and can only be satisfactorily answered by using a reporter gene that produces a mRNA with the same half-life as the endogenous gene or by measuring transcription rates. In both cases, one would ideally choose animals or cells in which only one gene was integrated so there would be no ambiguity about how many transgenes were functional.

The 12-kb albumin 5′-flanking region has been used effectively to direct the expression of other structural genes to the liver. For example, it will direct hepatitis B surface antigen (HBsAg) to hepatic cells, as visualized by immunofluorescence (Chisari et al. 1986). It has also been used to direct the expression of SV40 T-antigen and human c-H-*ras* genes to liver (E. Sandgren et al., unpubl.). Expression of each of these gene products leads to characteristic liver pathology. Expression of the hepatitis gene leads to accumulation of hepatitis large-envelope polypeptide in the endoplasmic reticulum and this is associated with progressive liver cell injury (Chisari et al. 1986 and unpubl.), whereas expression of either T antigen or an activated form of c-H-*ras* leads to hepatocellular tumors (E. Sandgren et al., unpubl.). Even the expression of hGH in the liver appears to be deleterious in that characteristic dysplasia is evident by 3 months, and by 7 months there is an obvious disorganization of normal lobular architecture (C. Pinkert et al., unpubl.). These examples serve to illustrate that the albumin promoter/enhancer can be used to generate animal models of human diseases in which the effects of various gene products on hepatic function can be studied.

Materials and methods

Construction of alb–hGH fusion genes and production of transgenic mice

A mouse albumin subclone spanning the promoter region was digested with *Bst*EII, which cut at a unique site about 50 bp downstream of the cap site. The DNA was digested with *Exo*III for varying times, treated with S1 nuclease and Klenow fragment of DNA polymerase to produce blunt ends, and then li-

gated with phosphorylated *Bam*HI linkers. A clone with a *Bam*HI linker positioned between the cap site and initiation codon was identified by acrylamide gel electrophoresis and the exact position was determined by DNA sequencing. The albumin promoter and about 4 kb of 5′-flanking region was fused to the hGH structural gene at its unique *Bam*HI site located at +3. A unique *Sal*I linker was introduced beyond the polyadenylation site of hGH, then the albumin 5′ region was extended to 12 kb by inserting a *Stu*I fragment isolated from a λ clone carrying albumin 5′-flanking sequences. Unique *Eco*RV and *Bss*HII sites lie at about −12 kb so that the entire alb–hGH region can be isolated with either of these enzymes and *Sal*I. To construct internal deletions, the alb–hGH gene was digested with *Sph*I, which cuts at −0.3 kb in the albumin gene and beyond the polyadenylation site of hGH, and this piece was cloned into the *Sph*I site of pUC18 to create a vector with a polylinker upstream of the albumin promoter. The 3′ *Sph*I site was converted to a unique *Cla*I site and then various 5′ albumin fragments were cloned into the polylinker (Fig. 1). An *Nsi*I site located at −90 in the hGH gene was used for the construction of the EB-O.1–hGH construct.

DNA fragments for microinjection were separated from remaining plasmid DNA by agarose gel electrophoresis, isolated by dissolving the gel in $NaClO_4$ and binding the DNA to glass (Whatman GF/C filters), and eluting with 1 mM Tris-HCl. The DNA concentration was measured and diluted to 2 ng/μl for microinjection. About 2 pl of the DNA solution was microinjected into pronuclei of fertilized C57/SJL F_2 hybrid mouse eggs. Eggs that survived injection were transferred to pseudopregnant recipients for continued development. Transgenic pups were identified by dot analysis of tail nucleic acids using a nick-translated hGH probe (Brinster et al. 1985).

Analysis of hGH mRNA levels

Total nucleic acids were isolated by homogenizing 50–100 mg of tissue in 4 ml of SET buffer with 100 μ/ml of proteinase K, incubating the extract at room temperature for 24 hr, and then isolating total nucleic acids by phenol/chloroform extraction and ethanol precipitation (Durnam and Palmiter 1983). hGH mRNA levels were determined by solution hybridization using a 21-base oligonucleotide complementary to a sequence in the fourth exon of hGH and M13 single-stranded DNA as a standard (Ornitz et al. 1985a). This assay is sensitive enough to measure 10 molecules of mRNA/cell.

For primer extension, an oligonucleotide, GTGGACAGCTCACCTAGCTAGCTGCTGCAAT, complementary to a sequence in the first exon of hGH mRNA was end-labeled with ^{32}P using T4 kinase, hybridized to 50 μg RNA prepared from total nucleic acids by treatment of DNase I, and then extended with AMV reverse transcriptase. The extension products were analyzed on a denaturing acrylamide gel (Townes et al. 1985).

DNase hypersensitivity assays

Liver nuclei were isolated as described by Ornitz et al. (1985b) and incubated at 25°C for up to 10 min to allow endogenous DNases to nick the DNA; then DNA was isolated by treatment with SDS and proteinase K followed by phenol/chloroform extraction and ethanol precipitation. DNA was digested with an appropriate restriction enzyme, electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated probe (Ornitz et al. 1985).

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References

Babiss, L.E., A. Bennett, J.M. Friedman, and J.E. Darnell, Jr. 1986. DNase I hypersensitive sites in the 5′-flanking region of the rat serum albumin gene: Correlation between chromatin structure and transcriptional activity. *Proc. Natl. Acad. Sci.* **83:** 6504–6508.

Brinster, R.L., H.Y. Chen, M.E. Trumbauer, M.K. Yagle, and R.D. Palmiter. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc. Natl. Acad. Sci.* **82:** 4438–4442.

Chisari, F.V., P. Filippi, A. McLachlan, D.R. Milich, M. Riggs, S. Lee, R.D. Palmiter, C.A. Pinkert, and R.L. Brinster. 1986. Expression of hepatitis B virus large envelope polypeptide inhibits hepatitis B surface antigen secretion in transgenic mice. *J. Virol.* **60:** 880–887.

Durnam, D.M. and R.D. Palmiter. 1983. A practical approach for quantitating specific mRNAs by solution hybridization. *Anal. Biochem.* **131:** 385–393.

Elgin, S.C.R. 1981. DNAase-hypersensitive sites of chromatin. *Cell* **27:** 413–415.

Friedman, J.M., L.E. Babiss, D.F. Clayton, and J.E. Darnell, Jr. 1986. Cellular promoter incorporated into the adenovirus genome: Cell specificity of albumin and immunoglobulin expression. *Mol. Cell. Biol.* **6:** 3791–3797.

Godbout, R., R. Ingram, and S.M. Tilghman. 1986. Multiple regulatory elements in the intergenic region between the α-fetoprotein and albumin genes. *Mol. Cell. Biol.* **6:** 477–487.

Gorski, K., M. Carneiro, and U. Schibler. 1986. Tissue-specific *in vitro* transcription from the mouse albumin promoter. *Cell* **47:** 767–776.

Hammer, R.E., R.L. Brinster, and R.D. Palmiter. 1985. Use of gene transfer to increase animal growth. *Cold Spring Harbor Symp. Quant. Biol.* **50:** 379–387.

Hammer, R.E., R. Krumlauf, S.A. Camper, R.L. Brinster, and S.M. Tilghman. 1987. Diversity of α-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. *Science* **235:** 53–58.

Ingram, R., R.W. Scott, and S.M. Tilghman. 1981. α-fetoprotein and albumin genes are in tandem in the mouse genome. *Proc. Natl. Acad. Sci.* **78:** 4694–4698.

Krumlauf, R., R.E. Hammer, R.L. Brinster, V.M. Chapman, and S.M. Tilghman. 1985a. Regulated expression of α-fetoprotein genes in transgenic mice. *Cold Spring Harbor Symp. Quant. Biol.* **50:** 371–378.

Krumlauf, R., R.E. Hammer, S.M. Tilghman, and R.L. Brinster. 1985b. Developmental regulation of α-fetoprotein genes in transgenic mice. *Mol. Cell. Biol.* **5:** 1639–1648.

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.* **83:** 7653–7657.

Ornitz, D.M., R.D. Palmiter, R.E. Hammer, R.L. Brinster, G.H. Swift, and R.J. MacDonald. 1985a. Specific expression of an

elastase-human growth hormone fusion gene in pancreatic acinar cells of transgenic mice. *Nature* **313:** 600–602.

Ornitz, D.M., R.D. Palmiter, A. Messing, R.E. Hammer, C.A. Pinkert, and R.L. Brinster. 1985b. Elastase I promoter directs expression of human growth hormone and SV40 T-antigen genes to pancreatic acinar cells in transgenic mice. *Cold Spring Harbor Symp. Quant* **50:** 399–409.

Ott, M.-O., L. Sperling, P. Herbomel, M. Yaniv, and M.C. Weiss. 1984. Tissue specific expression is conferred by a sequence from the 5′ end of the rat albumin gene. *EMBO J.* **3:** 2505–2510.

Palmiter, R.D. and R.L. Brinster. 1986. Germ-line transformation of mice. *Annu. Rev. Genet.* **20:** 465–499.

Palmiter, R.D., G. Norstedt, R.E. Gelinas, R.E. Hammer, and R.L. Brinster. 1983. Metallothionein-human GH fusion genes stimulate growth of mice. *Science* **222:** 809–814.

Ptashne, M. 1986. Gene regulation by proteins acting nearby and at a distance. *Nature* **322:** 697–701.

Serfling, E., M. Jasin, and W. Schaffner. 1985. Enhancers and eukaryotic gene transcription. *Trends Genet.* **1:** 224–230.

Tilghman, S.M. and A. Belayew. 1982. Transcriptional control of the murine albumin/α-fetoprotein locus during development. *Proc. Natl. Acad. Sci.* **79:** 5254–5257.

Townes, T.M., J.B. Lingrel, H.Y. Chen, R.L. Brinster, and R.D. Palmiter. 1985. Erythroid-specific expression of human β-globin genes in transgenic mice. *EMBO J.* **4:** 1715–1723.

Widen, S.G. and J. Papaconstantinou. 1986. Liver-specific expression of the mouse α-fetoprotein gene is mediated by *cis*-acting DNA elements. *Proc. Natl. Acad. Sci.* **83:** 8196–8200.

Wilkie, T.M., R.L. Brinster, and R.D. Palmiter. 1986. Germline and somatic mosaicism in transgenic mice. *Dev. Biol.* **118:** 9–18.

An albumin enhancer located 10 kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice.

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