Postnatal repression of the α -fetoprotein gene is enhancer independent

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The mammalian liver undergoes a number of dramatic changes in gene expression during development. One of these is typified by the α -fetoprotein (AFP) gene, which is activated in the fetal liver but undergoes a transcriptional decline at birth. In contrast, although activated at the same time during fetal development, albumin gene transcription is maintained at high levels in adult animals. To determine whether the postnatal decline in AFP gene transcription is mediated through its distal enhancers or through more proximal elements surrounding the promoter or structural gene, chimeric genes bearing substitutions of albumin gene *cis*-acting elements for the equivalent AFP gene elements were introduced into the germ line of mice. The expression of the transgenes was then analyzed at various stages of development. Our results indicate that the AFP gene enhancers are not involved in the postnatal decline in AFP transcription. Rather, a region within the first kilobase of DNA upstream of the AFP gene, including its promoter, and/or portions of the structural gene is sufficient to direct postnatal repression of the gene.

[*Key Words*: Mammalian liver; α -fetoprotein gene; transcription]

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The mammalian liver serves as an excellent model to explore the process of regulated gene activity in development. It is discernible by day 10 of the 20-day gestation period in the mouse (Barth et al. 1982; Thielier 1972). However, not all liver-specific genes are activated simultaneously at this time. For example, at birth, the animal becomes responsible for generating its own supply of glucose, as well as eliminating its waste, and a dramatic change in liver gene expression is required to fulfill these new metabolic requirements. Some of the genes that are activated at this time [e.g., phosphoenolpyruvate carboxykinase, tyrosine aminotransferase, amylase and transferrin (Garcia-Ruiz et al. 1978; Panduro et al. 1987; Samuelson et al. 1988)] are influenced by hormonal changes that occur at birth, such as the increasing levels of glucocorticoids and the shift in the ratio of insulin to glucagon (Dalle et al. 1978; DiMarco et al. 1978). An equally critical change at birth is the concomitant repression of a collection of genes, which include α -fetoprotein (AFP), γ -glutamyl transpeptidase, and H19 (Jalanko 1979; Pachnis et al. 1984; Tilghman 1985). The decline in AFP gene transcription is dramatic: Its mRNA level is reduced 10,000-fold during the first 4 weeks of life to a low basal level that is maintained in adult animals (Tilghman and Belayew 1982).

A simple model to explain the loss of AFP gene activity is that one or more of the factors necessary for its transcription are fetal specific, i.e., their activity and/or

concentration declines at birth. Alternatively, the postnatal decline in AFP could result from the activation of a dominant repressor, which interferes with the action of the continued presence of positive transcription factors. The first step in distinguishing these models is to localize the region of the AFP gene that is required to direct its postnatal decline.

Transient expression assays in tissue culture cells have been used to identify *cis*-acting sequences necessary for murine AFP gene transcription (Godbout et al. 1986, 1988; Widen and Papacostantinou 1986). These studies identified a large distal enhancer domain between 2.5 and 6.5 kb of DNA upstream of the AFP gene, which contained three separate enhancers and a proximal promoter within the first 125 bp of the 5'-flanking region DNA (Godbout et al. 1986; M. Feuerman, R. Godbout, and S.M. Tilghman, in prep.). Equivalent regulatory regions have been identified in the rat (Muglia and Rothman-Denes 1986; Guertin et al. 1988) and human (Watanabe et al. 1987) AFP genes. Using germ line transformation, we showed that these domains—contained within the first 7.6 kb of DNA flanking the AFP gene—were both necessary and sufficient to activate and subsequently to repress transcription of the gene (Krumlauf et al. 1985; Hammer et al. 1987). To locate more precisely the sequences responsible for repression, we now show that the AFP enhancer domain cannot direct postnatal repression when placed on a gene that normally is expressed in adult liver. Rather one or more elements within 1 kb of the 5' end of the AFP gene or a limited portion of the structural gene are sufficient for repression. Thus, complete repression functions indepen-

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dently of the AFP enhancers and does not require cooperation or interaction of the AFP enhancers with its promoter.

Results

Experimental strategy

The strategy adopted to map the elements required for postnatal repression of the AFP gene was to link AFP transcriptional control sequences to those of another liver-specific gene that does not exhibit postnatal repression. We selected the albumin gene for several reasons. First, like the AFP gene, two distinct regulatory domains comprising a promoter-proximal element from -150 bp to the cap site and a distal enhancer at approximately -10 kb (stippled box in Fig. 1) had been mapped (Gorski et al. 1986; Heard et al. 1987; Lichtsteiner et al. 1987; Pinkert et al. 1987). Second, the two genes are linked tightly in the mouse, as illustrated in Figure 1, and arose from a common ancestor 300–500 million years ago (Ingram et al. 1981; Young and Tilghman 1985). This, together with their temporal coactivation and tissue distribution of expression, suggested that the regulatory elements would likely be compatible. Both genes are activated simultaneously in the developing liver, but albumin expression is not silenced at birth; rather, its expression is maintained at high levels in adult liver (Tilghman and Belayew 1982).

Are the AFP enhancers fetal specific?

The first chimeric gene tested was AFP_EALB , (Fig. 1) in which the three AFP enhancers, spanning from -1 to -7.6 kb of the AFP gene, were placed upstream of the albumin gene and its promoter at a restriction site at -600 bp. The albumin gene had been deleted internally, generating a 3-exon minigene consisting of exons 1, 2, and 15 fused within introns 2 and 14. If the AFP en-

Figure 1. The structure of genes introduced into the mouse germ line. The first line depicts the organization of the mouse albumin (*alb*) and α -fetoprotein (*AFP*) genes in the mouse genome. The albumin gene enhancer, between -9 and -12 kb, is indicated by the stippled box, and the three AFP enhancers at -6.5 , -5 , and -2.5 kb by black boxes. The genes are indicated in the open boxes, with the positions of the exons drawn as lines. The ALB_EAFP construct contains the *alb* enhancer ligated to ZE.5, an internally deleted AFP minigene (Scott et al. 1984). ALB_EALB contains the entire 12 kb of DNA flanking the 5' end of the albumin gene linked to an albumin minigene, as described in Materials and Methods. ALB is the albumin minigene, along with 0.6 kb of 5'-flanking DNA. AFP_EALB is ALB, together with the three AFP enhancers between -1 and -7.6 kb.

hancers are involved in repression, then AFP_EALB would be expected to exhibit the developmental pattern of expression characteristic of AFP rather than albumin.

With this construct, four lines of transgenic mice were produced and bred, and progeny of each were analyzed at various times prior to or after birth. Poly(A)⁺ RNA from individual tissues of fetuses derived from founder lines was analyzed by Northern blotting to determine whether the albumin minigene was expressed prior to birth. As shown in Figure 2 for three such lines, endogenous albumin mRNA (*alb*) is present at high levels in the fetal liver and at lower levels in the visceral endoderm of the yolk sac and fetal gut. Albumin minigene mRNA levels in fetal liver ranged from 26% of the endogenous albumin mRNA in line E-645 to 410% in line N-331, correlating roughly with the copy number of the transgene (see Table 1). The concentration of the minigene transcript was also high in the yolk sac, where it ranged from 110 to 650% of endogenous levels. In the fetal gut, 50–100% of normal mRNA levels were attained. Interestingly, although two of the lines, N-331 and L-354, exhibited significantly higher levels of the transgene transcript in the fetal liver than the other two tissues, characteristic of the endogenous albumin gene, line E-645 expressed the transgene at the highest level in the yolk sac, which is more in keeping with the pattern for the endogenous AFP gene. In all instances, expression of AFP_EALB was restricted to the three appropriate tissues (Fig. 2).

Adult progeny of the three lines were then examined to determine whether the AFP enhancers contained information to direct postnatal repression. As shown in Figure 3, transcripts of the albumin minigene were at least as abundant in the livers of adults as in the livers of fetal mice in each of the three lines. Steady-state levels of the minigene mRNA remained constant throughout development in line N-331 and L-354, whereas an increase was noted in the E-645 line. In no instance was there evidence of a loss of transcriptional competence of the AFP_EALB with age.

The albumin promoter requires an enhancer for activity in animals

The high-level expression of pAFP_EALB in adult liver argues that the AFP enhancers are not fetal specific but capable of enhancing transcription throughout development. However, this conclusion requires that AFP_EALB expression was, in fact, dependent on the presence of the AFP enhancers. Transfection experiments with 400 bp of the 5'-flanking region of the rat albumin gene fused to the bacterial chloramphenicol acetyltransferase reporter gene in hepatoma cell lines indicated that the albumin promoter alone exhibits strong transcriptional activity relative to the SV40 and RSV promoters (Ott et al. 1984), suggesting the possibility that the gene could function well without additional enhancer sequences.

In contrast, little or no mRNA was detected in transgenic mice carrying the mouse albumin promoter (300 bp, 5' to the cap site) fused to a human growth hormone

Figure 2. Tissue specificity of expression of AFP_EALB in fetal mice. Transgenic male progeny of the founders N-331, E-645, and L-354 were mated to BALB/c females. Fetuses and yolk sacs were collected prior to birth. DNA from yolk sacs were subjected to Southern blotting to determine which fetuses were transgenic (Krumlauf et al. 1985). Subsequently, poly(A)⁺ RNA was prepared from the yolk sacs (Y), livers (L), guts (G), kidneys (K), hearts (H), and brains (B) and analyzed by agarose gel electrophoresis and blotting with an albumin gene-specific probe. The migration of authentic albumin mRNA (alb) and the minigene (MG) are indicated.

reporter gene (Pinkert et al. 1987). Reasonable expression of the reporter gene required the inclusion of an upstream enhancer-like element between -9 and -12 kb. A similar result was obtained recently by Lores et al. (1988), who tested an albumin minigene, along with 2.2 kb of 5'-flanking DNA, in transgenic mice and found no expression.

Our experiments also suggest that the albumin gene requires an enhancer element for expression in transgenic mice. Two lines of mice (M-802 and M-805 in Figs. 4 and 5) were generated using the albumin minigene and 600 bp of its 5'-flanking domain alone. Neither line expressed the albumin minigene significantly in either the fetal or adult liver. Fetuses from lines M-802 and M-805 had no detectable levels of minigene transcripts in yolk sac, brain, heart, liver, or gut, but transcripts were detected in the fetal kidney of M-802 progeny. Overexpression in the kidney was observed in albumin-growth hormone transgenic mice lacking the albumin enhancer element as well (Pinkert et al. 1987) and also has been noted occasionally with AFP minigenes (Krumlauf et al. 1985; Hammer et al. 1987). A trace of albumin minigene mRNA was detected in the livers of adult animals of the M-805 line on very long exposures, but the levels were too low for accurate quantitation. These results support the contention that the high level of minigene expression in 4 of 4 of the AFP_EALB mice was dependent on the presence of the AFP enhancers.

Four transgenic lines were generated also with ALB_EALB, which contains 12 kb of 5'-flanking DNA of the albumin gene, including an enhancer at -10 kb, to compare the efficacy of the enhancer domains of the two genes. The efficiency of producing transgenic mice with ALB_EALB was less than that observed with other DNAs (9% versus 18%), and the copy number was also low.

This could be due to technical reasons; the gene was contained on a large 15-kb restriction fragment, larger than any of the other constructs. A similarly sized AFP fragment (YZE, 17 kb) also proved to be a low-frequency transformant (10%) (Hammer et al. 1987). Two of the four lines, J-635 and J-632, exhibited evidence of a loss of a restriction site just 3' to the end of the minigene, but the constructs apparently were intact otherwise. Only one of the lines, J-634, expressed a significant amount of albumin transgene transcript at any stage in development. The sporadic expression achieved with ALB_EALB is in contrast to the high-level expression of AFP_EALB and underscores the assertion that transcription of the albumin minigene in AFP_EALB mice is dependent on the AFP enhancers.

J-634 fetal mice did not express minigene transcripts at detectable levels in the yolk sac (data not shown), but traces of expression were noted in brain, heart, gut, and kidney, indicating that the control restricting expression to the appropriate tissues is not completely functional in this line (Fig. 4). Expression in the fetal liver was detected easily, but levels were low compared to those observed in adult liver (Fig. 5). In day-1 neonates, the minigene was expressed at only 14% of the endogenous albumin gene, whereas in adult liver, the minigene mRNA levels had risen to 424% of the endogenous gene transcripts. Although this is a result from only one transgenic line, it opens the possibility that full expression of the albumin gene in utero is not achieved with the albumin control regions alone.

Postnatal repression in ALB_EAFP transgenic mice

The AFP minigene with 1 kb of 5'-flanking DNA, in the absence of an enhancer, had been shown previously to be

Table 1. Quantitation of transgene expression

Construct	Founder mouse	Transgene copy number	Yolk sac	Liver	Gut
albumin RNA (%)					
AFP _E ALB	N-331	22	220	410	100
	E-645	22	650	26	48
	L-354	~50	110	57	53
AFP mRNA (%)					
ALB _E AFP	951H	25	7.2	26	trace
	951L	1	4.2	30	trace
	962H	10	6.5	30	0
	962L	4	<0.5	3	0

Transgene copy number was determined by averaging the densitometric scanning values obtained from Southern blots of tail DNA from several progeny of each founder mouse on the assumption that the endogenous gene signal represents two copies per cell. Estimates of transgene mRNA levels relative to endogenous albumin (AFP_EALB) or AFP (ALB_EAFP) mRNA levels were based on averaging values obtained from several individuals by densitometric scanning of autoradiograms or direct counting of filters from Northern blots of poly(A)⁺ RNA.

silent in transgenic mice (Hammer et al. 1987). By combining this minigene with the albumin gene enhancer between -9 and -12 kb, (ALB_EAFP, Fig. 1), we could test whether the sequences within this segment were sufficient to direct postnatal repression. Fourteen lines of transgenic mice carrying intact copies of ALB_EAFP were produced. One was sterile, but 13 were bred, and liver RNA from day-1 neonates was analyzed by Northern blotting. Only 4 of 13 lines of mice expressed ALB_EAFP at detectable levels in the neonate, 3 of which are shown in Figure 6. The lack of expression in the other nine lines (e.g., see 964A in Fig. 6) could not be attributed to either obvious mutations in the transgenes or low copy numbers because a range of copy numbers were represented in both the expressing and nonexpressing transgenic lines.

The lines that expressed the chimeric transgene ALB_EAFP were analyzed late in gestation for expression. Representative Northern blots from individual fetuses of three lines are shown in Figure 6. The transgene was activated very weakly in the visceral endoderm of the yolk sac, representing <18% of the endogenous AFP mRNA levels (see Table 1). This low level of expression approximates the level of expression expected for the endogenous albumin gene. In the fetal liver, the level of transgene expression was, at most, 30% of the endogenous AFP levels. In the fetal gut, no expression was detected for the 962H or 962L lines, whereas traces of transgene expression were detected in guts of the 951L and 951H lines but were too low for accurate quantitation. In contrast, transgenic mice bearing the AFP enhancers coupled to the AFP minigene consistently expressed the minigene at very high levels in all three tissues (Hammer et al. 1987).

When adult mice carrying ALB_EAFP were examined,

all had extinguished expression of the AFP minigene (Fig. 7, lanes A). Thus, the signals to direct postnatal repression of the AFP gene are encoded in 1 kb of 5'-flanking DNA or in the 18% of the structural gene that is retained in the minigene.

Discussion

AFP enhancers are not required for postnatal repression

The fact that there is a basal level of AFP expression in adult mice raised the possibility that its postnatal repression proceeds via a loss of activity of the AFP enhancers such that the low adult level of AFP mRNA is due to unenhanced transcription from the AFP promoter. We have shown that this is not the case. A chimeric transgene consisting of the three AFP enhancers, albumin promoter, and albumin minigene (AFP_EALB) is expressed at high levels in both fetal and adult mice. Thus, if enhancers are required continuously for high-level transcription and do not function only to activate the gene during differentiation (Wang and Calame 1986; Vogt et al. 1988; Grosschedl and Marx 1988), the AFP enhancers must be active throughout adult development and cannot play a central role in repression.

This argument was reinforced when transgenic mice carrying the AFP minigene and 1 kb of its 5'-flanking domain, activated by the albumin enhancer, were shown to undergo postnatal repression, indistinguishable from that of the endogenous gene. This result eliminated another plausible model, whereby repression required the combination of both the AFP enhancers and promoter. Rather, the results argue that the information specifying postnatal repression is contained in the immediate 5'-flanking region or within the elements of the AFP structural gene that are contained in the minigene.

An alternative means for studying regulatory regions is to probe their structure in the chromatin of intact cells. DNase I-hypersensitive sites have been found associated with the regulatory regions of transcribed genes and are thought to occur as the consequence of specific protein-DNA interactions (Wu 1980; Eissenberg et al. 1985). The three AFP enhancers have been shown recently to exhibit DNase I hypersensitivity in fetal liver, and this structure is maintained in adult liver (Godbout and Tilghman 1988), consistent with our finding that the enhancers remain active in adult mice. In contrast, the pattern of DNase I-hypersensitive sites around the AFP promoter and structural gene changes in development (Godbout and Tilghman 1988). In the fetus, a DNase I-hypersensitive site exists at -120 bp, a site that has been shown to bind a liver-specific protein *in vitro* (M. Feuerman, R. Godbout, and S.M. Tilghman, *in prep.*). This DNase I-hypersensitive site is no longer evident in adult liver; instead, new DNase I-hypersensitive sites are detected at approximately -300 bp, at the transcriptional start site itself and at +1.5 kb within the structural gene. These three regions are contained within the chimeric transgene ALB_EAFP and will be the focus of future studies to localize the DNA sequences required for postnatal repression.

Figure 3. Developmental regulation of AFP_EALB in transgenic mice. Three transgenic founder mice, N-331, E-645, and L-354, each carrying varying numbers of copies of AFP_EALB, were established as lines through matings to BALB/cJ mice. Progeny were sacrificed at the indicated days, before (indicated by a negative number) or after birth. Poly(A)⁺ RNA was prepared from individual livers, analyzed by formaldehyde agarose gel electrophoresis, and hybridized after blotting to an albumin gene-specific probe. The migration of authentic albumin mRNA (alb) and the albumin minigene (MG) are noted. Founder E-645 contained two independently segregating integrations with identical characteristics of expression. (Lane -4*) A 2-week exposure of lane -4, to emphasize the presence of the minigene mRNA. The other exposures are 12–18 hr. (Bottom) The structure of AFP_EALB contained in these mice.

The developmental regulation of the albumin–AFP locus proceeds via the simultaneous activation of both genes, followed by their independent regulation after birth (Belayew and Tilghman 1982), and does not involve a reciprocal switch. This program of gene expression is different from the β -globin gene cluster where the fetal gene is extinguished at the same time that the adult gene is activated. The chicken β -globin genes are organized in tandem, similarly to the albumin and AFP genes, with the adult gene upstream of the fetal gene. An enhancer has been mapped to the intergenic region, and recent evidence suggests that the promoters of the two genes compete for this intergenic enhancer (Choi and Engel 1988). In addition, it also has been suggested that in adults the *trans*-acting factors that recognize the fetal promoter may be reduced relative to those that the adult promoter requires, and this may facilitate the competition (Nickol and Felsenfeld 1988). Our studies on the AFP and albumin genes suggest a very different type of

control, wherein specific elements that map close to or within the AFP gene are sufficient to accomplish postnatal regulation, in the absence of the albumin gene altogether.

Tissue specificity of albumin and AFP gene expression

The mechanism underlying restriction of a gene's expression to one or a few tissues must account for the absence of transcription in most cell types, as well as the means by which each expressing tissue establishes its characteristic rate of transcription. Insight into how restriction can occur has been provided by the demonstration that *trans*-acting factors such as the B-cell-specific octamer binding factor (Staudt et al. 1986), the pituitary-specific binding proteins pit-1 (Ingraham et al. 1988) and GHF-1 (Bodner et al. 1988), and a variety of liver-specific *trans*-acting factors (Courtois et al. 1987; Grayson et al.

Figure 4. Tissue specificity of expression of ALB_EALB and ALB in fetal mice. Transgenic progeny of founder mice carrying either ALB_EALB (J-634) or ALB (M-802 and M-805) were sacrificed before birth, and poly(A)⁺ RNA was prepared from the tissues identified as in the legend to Fig. 2. These were analyzed by agarose gel electrophoresis and blotting using an albumin gene-specific probe. The migration of authentic albumin mRNA (alb) and minigene mRNA (MG) are indicated. The unusual hybridization noted in this illustration of M-802 brain was not seen routinely. This is a shorter exposure than the one in Fig. 2; consequently, the endogenous albumin mRNA in the gut is not detected.

Figure 5. Expression of ALB_EALB and ALB in adult mice. Transgenic founder mice carrying either ALB_EALB (J-634, J-635, J-632, and J-211) or ALB (M-802 and M-805), as indicated, were mated to BALB/cJ or ICR:Ha (icr) mice, and F_1 progeny were sacrificed at 18 days of gestation (-2), at 1, 3, 5, 7, and 15 days after birth or at 1–2 months of age (A). Poly(A)⁺ RNA was prepared from their livers and analyzed as described in the legend to Fig. 2 for authentic albumin (alb) and minigene (MG) RNA. The copy number of the transgene for each line was as follows: J-634, 2 copies; J-635, J-632, and J-211, 1 copy; M-802, ~100 copies; and M-805, 2 copies.

1988; Lichtsteiner et al. 1987; Schaul and Ben-Levy 1987) are distributed tissue specifically. Less is known about how individual tissues establish specific rates of transcription for a gene, which is a particular problem for the AFP gene whose transcripts accumulate to very different levels in the yolk sac, liver, and gut of the developing fetus. Although it was tempting to attribute such differences to different concentrations of *trans*-acting factors, our studies using transgenic mice argued that this could not be the case for the AFP gene (Hammer et al. 1987). Rather, these experiments supported a model for generation of differential transcrip-

tion rates through combinatorial action of multiple enhancers.

The chimeric genes that were analyzed in this paper provide new insight into the means by which levels of transcription are achieved for the AFP gene. The fact that the levels of expression of both the ALB_EAFP and AFP_EALB chimeric transgenes in the yolk sac generally are low, characteristic of the endogenous albumin gene, suggests that the high level of AFP gene transcription in that tissue requires *both* the AFP enhancers and promoter. Likewise, the inappropriately high level of AFP transgene expression in the fetal gut observed with the

Figure 6. Tissue specificity of expression of ALB_EAFP in fetal mice. Lines were established from transgenic founders carrying the ALB_EAFP construct. Founder 951 had two independently segregating integrations designated as 951L and 951H. Transgenic males were mated to BALB/cJ or CD1 females, and fetuses were collected from the pregnant females prior to birth. Poly(A)⁺ RNA was prepared from the tissues indicated in the legend to Fig. 3. The RNAs were analyzed by agarose gel electrophoresis and blotting and hybridized to an AFP-specific probe. The migration of authentic AFP mRNA and minigene mRNA (MG) is indicated.

Figure 7. Developmental regulation of ALB_EAFP in transgenic mice. Lines were established from three founder transgenic mice carrying ALB_EAFP by mating to BALB/cJ or CD1 mice. Progeny from subsequent matings were sacrificed 1–3 days (N) or 1–2 months (A) after birth. Founder 962 carried two integrations that segregated independently in the F₁ progeny, which are designated 962L and 962H. Poly(A)⁺ RNA was prepared from individual transgenic livers and was analyzed by agarose gel electrophoresis and blotting using an AFP gene-specific probe. The migration of authentic AFP and AFP minigene (MG) mRNA are indicated. The same RNA samples also were hybridized to an albumin gene-specific probe, as shown at bottom.

intact AFP gene (Hammer et al. 1987) was not achieved with either AFP_EALB or ALB_EAFP. Previously, we had attributed this overexpression to the presence of the proximal enhancer at -2.5 kb but now can conclude that one or more elements in the vicinity of the AFP promoter and/or minigene contribute as well.

The role of the intergenic AFP enhancers in the activity of the albumin gene

The albumin and AFP genes are estimated to have been generated from a gene duplication between 300 and 500 million years ago. Nevertheless, they have remained tightly linked in both rodents and humans (Ingram et al. 1981; Urano et al. 1984; Chevrette et al. 1987). The conservation of the 15-kb intergenic distance led us to suggest that the two genes share regulatory elements and that this has provided the evolutionary pressure to retain linkage (Ingram et al. 1981). If this is so, evidence presented here suggests that the intergenic AFP enhancers are the best candidates for such common elements.

Virtually every transgenic mouse that carries the intergenic AFP enhancers free of prokaryotic vector DNA expresses the reporter gene at high levels in the fetal liver [14 of 15 lines in Hammer et al. (1987) and 4 of 4 in this paper]. The albumin enhancer, on the other hand, is a much weaker element, both qualitatively and quantitatively, in that transgenes dependent on this enhancer for expression often are silent or are expressed at low,

variable levels (Figs. 5 and 6; Chisari et al. 1987; Pinkert et al. 1987). If this is a true reflection of the activity of the enhancer in vivo, then the AFP enhancers must be the dominant regulatory domain in the locus. Alternatively, it is possible that there are additional elements in the locus that have not been identified as yet, which are critical for albumin gene transcription.

The relative strengths of the albumin and AFP enhancers are reminiscent of two enhancers identified in the human β -globin gene cluster. Several laboratories had identified an enhancer 3' to the adult β -globin gene, which conferred upon the β -globin gene low level and sporadic expression in transgenic mice (Townes et al. 1985; Trudel and Costantini 1987). More recently, Grosfeld et al. (1987) have shown that a segment of DNA, 5' to the ϵ -globin gene, which is >50 kb upstream of the β -globin gene, generated position-independent and copy-number-dependent expression on the β -globin gene. We propose that the AFP enhancers are analogous to the 5'-distal enhancer of the β -globin cluster, acting bidirectionally to activate the albumin as well as AFP genes during fetal life.

Several additional pieces of evidence support this view. First, in the single ALB_EALB line that expressed the transgene, its activity in fetal liver was low and only increased dramatically in the postnatal period. This is in contrast to the majority of the AFP_EALB mice, where expression in utero was as high as the endogenous albumin gene. In addition, all lines of albumin-HBV_sAg transgenic mice exhibit a steady increase in HBV_sAg mRNA over the first 4 months of life (Chisari et al. 1987). Because of the propensity of the position of integration of a transgene to influence its timing of activation (Adams et al. 1987), more albumin transgenic mice will have to be analyzed to confirm that the albumin enhancer is not sufficient for high-level transcription of albumin in the fetal liver. Second, the albumin enhancer does not exhibit hypersensitivity to DNase I in the fetal liver (S. Tilghman, unpubl.), but it clearly is hypersensitive in adult liver (Pinkert et al. 1987; Liu et al. 1988). Third, the albumin enhancer has no activity in a variety of hepatoma lines that express fetal markers such as AFP, but it is active in AFP-negative immortalized adult hepatocytes (S. Camper, T. Miller, and H. Isom, unpubl.).

Considering all of these lines of evidence, we suggest a model whereby the AFP enhancers activate both the albumin and AFP genes during fetal liver development. Subsequently, the AFP promoter is blocked specifically in its ability to use these enhancers through *cis*-acting sequences in the 1 kb of DNA immediately 5' to the gene, including the promoter and/or gene body. The albumin gene enhancer becomes fully activated at birth and acts in concert with the continued activity of the AFP enhancers to maintain high-level transcription of the albumin gene.

Materials and methods

Construction of albumin and AFP minigenes

The albumin minigene was constructed from palbE, a 7-kb

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EcoRI fragment containing the first five exons of the albumin gene and 3.2 kb of 5'-flanking DNA, and *palbB*, a 1.1-kb *EcoRI* fragment containing its 3' end (Kioussis et al. 1981). The *EcoRI* fragment from *palbE* was cleaved partially with *XbaI* in the second intron and ligated to an *XbaI-EcoRI* fragment derived from *palbB*, which contains the last exon and 0.3 kb of 3'-flanking DNA. This generated an albumin minigene *pEB.1* composed of the first two and the last exons of the natural 15 exon gene plus 3.2 kb of 5'-flanking DNA. *pALB_EALB* was generated by ligating a partial *EcoRI* fragment consisting of 5'-flanking DNA between -12 and -3.2 kb to the 5' end of *pEB.1*. *pALB* was derived from *pEB.1* by cleavage with *AvaI* at -600 bp and attachment of a *BamHI* linker. *pAFP_EALB* consists of *pALB* ligated to the AFP enhancer domain between -1 and -7.6 kb contained on an *EcoRI/partial BamHI* fragment.

pALB_EAFP was generated by combining the AFP minigene *pAFP ZE.5* (Scott et al. 1984), along with 1 kb of AFP 5'-flanking DNA, with a *BamHI-EcoRV* fragment that contains the albumin gene enhancer domain between -9 and -12 kb (Pinkert et al. 1987).

Microinjection of mouse zygotes

The DNA fragments were prepared for microinjection by cleavage with a restriction enzyme, which liberated the eukaryotic DNA from the plasmid vector, and separation by agarose gel electrophoresis. Following electroelution, the fragment was purified by either binding to glass beads or by CsCl centrifugation (Hogan et al. 1986). Gel-eluted DNA fragments not subjected to such purification steps yielded variable results. For example, in one case, 0 of 24 mice analyzed were transgenic.

F₂ hybrid embryos from C57BL/6J × SJL/J parents were microinjected as described by Hogan et al. (1986) and transferred to pseudopregnant ICR:Ha (icr) or CD1 females. The efficiency of successful implantation was somewhat better for those done on the same day (60%) versus those done on the day following microinjection (40%). Approximately 70% of the zygotes survived injection, 15% of the implanted embryos resulted in live mice, and 18% of the mice were transgenic.

Detection of transgenic animals

Progeny were tested at 2 weeks after birth for the presence of the transgene by Southern blot analysis (Southern 1975) of tail DNA, using restriction enzyme cleavages, which distinguished the endogenous albumin and AFP genes from the transgenes. In *HindIII* digests of genomic DNA the *AFP_EALB* transgenes were detected using a 1.2-kb *HindIII-BamHI* fragment of the AFP enhancer at -2.5 kb as a probe, *ALB* and *ALB_EALB* transgenes were detected using an albumin exon 15-probe (*palbB*), and *ALB_EAFP* transgenes using a 2.4-kb *HhaI-EcoRI* genomic segment encompassing the second and third exons of the AFP gene.

Analysis of tissue RNA

Poly(A)⁺ was prepared from a variety of fetal and neonatal tissues of transgenic animals by phenol extraction (Krumlauf et al. 1985) or by guanidinium isothiocyanate extraction and oligo(dT)-cellulose chromatography (Aviv and Leder 1972). The RNAs were analyzed by electrophoresis in 1.5% agarose-6%

formaldehyde gels (Thomas 1980), blotted and UV cross-linked onto Hybond (Amersham) filters, and hybridized to Sp6 probes containing either exon 1 of the albumin gene (*pGEMalB*) on an *AvaI-HindIII* fragment or exon 1 of the AFP gene (*pGEMAFP*) on a 0.44-kb *HincII* fragment.

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