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# Individual Mouse α-Fetoprotein Enhancer Elements Exhibit Different Patterns of Tissue-Specific and Hepatic Position-Dependent Activities

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**Transcription of the mouse α-fetoprotein (AFP) gene, which is expressed at high levels in the visceral endoderm of the yolk sac and fetal liver and at low levels in the fetal gut, is regulated by three distinct upstream enhancer regions. To investigate the activities of these regions, each enhancer was individually linked to a heterologous human β-globin promoter fused to the mouse class I *H-2D$^d$* structural gene. When tested in transgenic mice, the β-globin promoter alone has minimal activity. We find that all three enhancers activate the β-globin promoter in an AFP-like pattern; i.e., activity is detected in the yolk sac, fetal liver, and fetal gut. The enhancers remain active in the livers and guts of adult mice, consistent with previous studies showing that postnatal AFP repression is due not to the loss of enhancer activity but to a dominant repressor region. Enhancer III also functions in the brain. In addition, these studies reveal that the three enhancers exhibit different position-dependent activities in the adult liver. Enhancers I and II are most active in hepatocytes surrounding the central vein, with a gradual decrease in activity along the hepatic plates toward the portal triad. Enhancer III is active exclusively in hepatocytes surrounding the central vein. These data represent the first examples of individual control elements exhibiting positionally regulated activity in adult liver.**

The mammalian liver is an ideal organ for studying tissue-specific transcriptional control. A number of genes are expressed solely in the liver, while others have a restricted but slightly broader range of expression. The products of these genes include many of the serum proteins, metabolic enzymes, acute-phase reactants, and detoxifying enzymes that serve to maintain homeostasis in an organism. The coordinate regulation of numerous genes is required within the liver, and many genes must be expressed in response to developmental, hormonal, and environmental signals. In addition to these modes of gene regulation, an interesting phenomenon observed in the adult liver is the zonal control of gene expression. Whereas some genes are expressed in all hepatocytes, others are restricted either to hepatocytes surrounding the portal triad or encircling the central vein (for a review, see reference 18). The molecular basis for positional regulation of hepatocyte gene expression is not known (16).

The *cis*-acting control regions of numerous genes expressed in the liver have been well characterized and factors interacting with these regions have been identified (8). These factors include hepatocyte nuclear factor 1α (HNF-1α) and HNF-1β, HNF-4, and members of the CCAAT/enhancer-binding protein (C/EBP) and HNF-3 families. Interestingly, the expression of these factors is not restricted to the liver. For example, HNF-1β protein is also found in the kidney, lung, and ovary (26), and C/EBPα is also present in lung, intestine, and adipose tissue (8). These results suggest that liver-specific transcription is not mediated solely by the activity of any one of these factors. Rather, it is likely that the interaction of multiple transcription factors with *cis*-acting sites will result in liver-restricted gene expression. This notion is supported by the finding that many *cis*-acting control regions contain multiple binding sites for different factors (9). One prediction of these results is that individual regulatory elements of liver-expressed genes, when tested separately, may be active in a broader range of tissues.

The mouse α-fetoprotein (AFP) gene is an excellent model for studying tissue-specific and developmentally regulated transcriptional control (34). AFP is transcribed at high levels in the visceral endoderm of the yolk sac and fetal liver and at low levels in the fetal gut. Expression is dramatically repressed at birth, primarily at the transcriptional level, leading to low but measurable levels of mRNA in the adult liver and gut (4, 32). This repression is reversible, however, because AFP can be reactivated in hepatocellular carcinomas and during liver regeneration after partial hepatectomy or treatment with acute hepatotoxins. Five distinct *cis*-acting control regions that govern AFP expression have been well characterized in cultured cells and transgenic mice (13, 14, 19, 32, 33, 38, 39). The AFP promoter, located within the first 250 bp upstream of the AFP cap site, is at least partially regulated by HNF-1 and C/EBP (5, 12, 41). Promoter activity is limited to the tissues where AFP is normally transcribed, suggesting that this region contributes to tissue-specific expression. Postnatal shutoff of AFP transcription is due to a repressor region located between the AFP promoter and the upstream enhancers; removal of this region leads to continued AFP expression in adult liver and gut (36). Three distinct upstream enhancer regions, each several hundred nucleotides long, have been defined (13, 14). Enhancer I (EI), located most proximal to the promoter, binds several nuclear proteins including C/EBP (42). Proteins that bind to EII and EIII have not been well characterized, although HNF-3 contributes to EIII activity (17, 25).

The three AFP enhancers have been analyzed in transgenic mice by linking each region individually to the mouse AFP promoter-repressor. These studies demonstrated that each enhancer could activate the AFP promoter, although at various

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levels, in the yolk sac, fetal liver, and fetal gut (19). Whether the activities of these enhancers were restricted to these three tissues could not be determined from these studies, since the AFP promoter itself is tissue specific (13, 33). To further characterize the AFP enhancer elements in transgenic mice, we have linked these elements to the human β-globin (β-gl) promoter. This promoter alone has very weak activity in cultured cells and transgenic mice but is highly responsive to linked enhancer elements. We find that all three enhancers activate the heterologous β-gl promoter in an AFP-like pattern, i.e., activity is detected in the yolk sac, liver, and gut. In addition, enhancer III activates the β-gl promoter in the brain. Consistent with previous studies suggesting postnatal AFP repression is not due to the loss of enhancer activity but rather due to a distinct repressor region located between EI and the AFP promoter, the enhancers are active in the livers and guts of both fetal and adult mice. Finally, these studies reveal that the three enhancers exhibit different position-dependent activities in the adult liver.

## MATERIALS AND METHODS

**Plasmids.** The mouse *H-2D*$^d$ (*D*$^d$) structural gene was fused to the human β-gl promoter to generate βgl-D$^d$ (Fig. 1) (31). To accomplish this, a 1.7-kb fragment from pSVαBGcat(X) (21) containing the β-gl promoter was inserted 5′ to the *H-2D*$^d$ structural gene. This hybrid gene contains roughly 55 bp of untranslated β-gl sequence downstream of the β-gl transcription start site and 16 bp of polylinker sequence upstream of the *H-2D*$^d$ 5′ *Bam*HI site. AFP EI and EII, contained within 2.8- and 1.5-kb *Bam*HI fragments (13), respectively, were inserted in their natural orientation into a *Bgl*II site directly 5′ of the β-gl promoter. A 2.3-kb *Eco*RI-*Bam*HI fragment containing AFP EIII (13) was inserted into *Bam*HI-*Eco*RI-linearized pSP72 (Promega Biotec), excised as a 2.3-kb *Bam*HI-*Bgl*II fragment, and inserted in its natural orientation into the *Bgl*II site directly 5′ of the β-gl promoter.

**Production and identification of transgenic animals.** To prepare DNA for microinjection, hybrid genes were released from vector sequences by digestion with *Eco*RI. The resulting fragments were purified by agarose gel electrophoresis and CsCl ultracentrifugation (20). The quality and quantity of DNA were monitored by agarose gel electrophoresis, and the purified fragments were diluted to a final concentration of 5 ng/μl in phosphate-buffered saline (PBS). $F_2$ hybrid embryos from C57BL/6 × C3H parents were microinjected and transferred to pseudopregnant ICR/HSD females (20). All mice were generated at the University of Kentucky Transgenic Mouse Facility except lines A.1 and A.2, which were produced by Jean Vacher at Princeton University. Two weeks after birth, the progeny were screened for the presence of the transgene in tail DNA by Southern blot analysis (31). The DNA was digested with *Hin*dIII and probed with the 1.7-kb human β-gl promoter fragment. Three to four founder mice were generated with each transgene.

**Analysis of mRNA.** Total RNA was prepared from mouse tissues by the lithium chloride procedure as described previously (2) or using RNazol B (Tel-Test, Inc., Friendswood, Tex.) following the manufacturer's instructions. The quality of the RNA samples was monitored by formaldehyde gel electrophoresis and ethidium bromide staining; these procedures also confirmed the RNA concentration.

The RNAs were analyzed by RNase protection assays essentially as described previously (32). Total RNA (50 to 100 μg) and radiolabelled RNA probe ($10^5$ cpm) were ethanol precipitated in the presence of 1.5 M ammonium acetate, pelleted by centrifugation, and dried. When samples contained less than 50 μg of cellular RNA, tRNA was added to bring the total RNA to 50 μg. Samples were resuspended in 30 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% formamide], heated to 90°C for 6 to 10 min, and incubated overnight at 42°C (AFP probe) or 55°C (βgl-D$^d$ probe). Digestions with RNase A (18 μg/sample; Sigma Biochemicals) and RNase $T_1$ (270 U/sample; GIBCO/BRL) were performed in 330 μl for 1 h at 30°C in the presence of 0.3 M NaCl, 10 mM Tris (pH 7.5), and 5 mM EDTA. Proteinase K (50 μg/sample; GIBCO/BRL) and sodium dodecyl sulfate (20 μl of a 10% solution) were added to each sample, and then the samples were incubated for 30 min at 37°C. Samples were extracted with phenol-chloroform-isoamyl alcohol (50:48:2) and ethanol precipitated. Dried pellets were suspended in 80% formamide–TBE (Tris-borate-EDTA) buffer–0.1% bromophenol blue–0.1% xylene cyanol, denatured for 2 min at 90°C, and electrophoresed on a 7 M urea–7.5% acrylamide gel in TBE buffer. Radioactive bands were visualized by autoradiography with Kodak X-Omat AR film. All autoradiographic exposures were for 14 to 18 h.

The AFP RNase probe was generated by subcloning a 665-bp fragment spanning the AFP transcription start site (from a *Pvu*II site at −610 to a *Sau*3AI site at +55) into *Eco*RV-*Bam*HI-digested Bluescript II KS− (Stratagene). The βgl-D$^d$ RNase probe was prepared by inserting a 250-bp fragment of the βgl-D$^d$ hybrid gene (from a *Rsa*I site at −130 of the β-gl promoter to a *Not*I site at +49 of *D*$^d$) into *Eco*RV-*Not*I-digested Bluescript II KS−. Both vectors were linearized with *Xho*I, and riboprobes were generated with T7 polymerase.

**Immunohistochemistry.** Liver sections were mounted in Tissue-Tek and snap frozen in liquid nitrogen. Serial 8-μm-thick sections were prepared, collected on Superfrost Plus slides (Curtin Matheson Scientific, Inc.), and air dried for several hours. For antibody staining, sections were fixed in 100% ethanol for 20 min, washed with PBS for 10 min, and incubated overnight with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-D$^d$ monoclonal antibody (catalog no. 06134D; Pharmingen, San Diego, Calif.) that had been diluted 1/100 in PBS. Sections were washed three times for 3 min each time in PBS, mounted with Vectashield (Vector Laboratories, Inc., Burlingame, Calif.), and photographed by fluorescence microscopy. Alternative serial sections were stained with hematoxylin and eosin, dried, mounted, and photographed.

## RESULTS

Transcriptional control of the AFP gene during mouse development is mediated by multiple *cis*-acting regulatory domains, including three distinct enhancer elements (Fig. 1A). To determine whether any of these three enhancer regions has unique or novel patterns of activity in transgenic mice when tested independently of the other AFP regulatory elements, each AFP enhancer was individually inserted upstream of a heterologous human β-gl promoter (Fig. 1B). Previous transgenic studies showed that the human β-gl promoter exhibited low levels of transcriptional activity when assayed in the absence of linked enhancers (3, 7). In addition, this promoter had little activity when tested by transient transfections in human hepatoma HepG2 cells (33). However, we demonstrated that this promoter could be activated by AFP enhancers in HepG2 cells (33), indicating that the β-gl promoter was responsive to linked AFP regulatory elements in cells other than those of the erythroid lineage, in which β-gl is normally synthesized. We therefore reasoned that this promoter would enable us to determine AFP enhancer activity in numerous tissues and that any transcription would likely be due to the action of the linked enhancer elements. To monitor the activities of these control regions, the β-gl promoter was fused to the mouse major histocompatibility complex-encoded class I *H-2D*$^d$ structural gene. We previously used *D*$^d$ as a reporter gene in cultured cell lines and transgenic mice, enabling us to monitor transgene expression at the RNA level, and in intact cells with D$^d$-specific monoclonal antibodies (32, 33).

To ensure that the 1.5-kb human β-gl promoter linked to the *H-2D*$^d$ structural gene in transgenic mice has minimal activity in the absence of linked enhancer elements, three founder lines with βgl-D$^d$, designated C.1, C.2, and C.3, were generated. Embryonic day 18 offspring from each founder were sacrificed to obtain yolk sac, fetal liver, and fetal gut RNAs. Two- to four-month old animals were sacrificed for adult tissue RNA samples. An RNase protection assay was employed to measure steady-state transgene mRNA levels. In lines C.1 and C.2, βgl-D$^d$ transcripts were not detected in any of the fetal or adult tissues (Fig. 2). In the third line, C.3, moderate levels of transgene mRNA were observed in the fetal and adult gut. This was not expected, since the β-gl gene is normally inactive in the gut. Because activity was observed in only one of the three βgl-D$^d$ lines, it is likely that this aberrant expression is due to the site of transgene integration; the β-gl promoter is responding to a nearby enhancer that is active in the gut (1). In all three lines, transgene mRNA was not detected in the fetal liver and adult spleen, the two tissues where the β-gl promoter would most likely be active. Overall, these results showed that the human β-gl promoter has little, if any, transcriptional activity in transgenic mice and validates the further use of this promoter to test the activity of linked enhancer regions.

Previous transgenic studies indicated that AFP EI, when

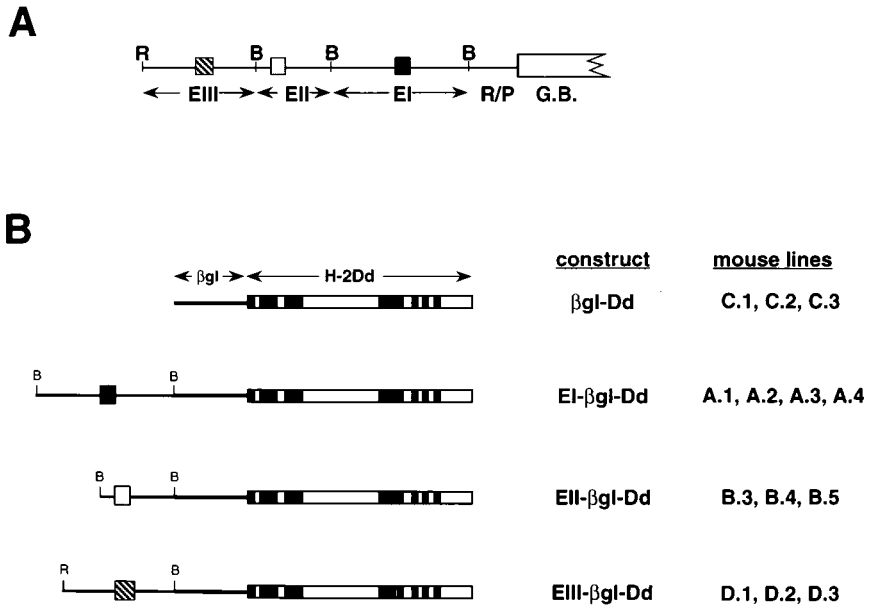


FIG. 1. Structures of AFP gene and constructs used for generating transgenic mice. (A) The entire AFP transcriptional control region flanks the 5′ end of the AFP structural gene body (G.B.) and contains several distinct control sites. The promoter-repressor region (R/P) is localized between the transcription start site and the *Bam*HI site (B) at −1 kb (transcription start site is defined as +1). AFP EI, adjacent to the R/P region, is within a 2.8-kb *Bam*HI fragment extending from −1.0 to −3.8. EII is within a 1.5-kb *Bam*HI fragment from −3.8 to −5.3; EIII is within a 2.3-kb *Bam*HI-*Eco*RI (E) fragment from −5.3 to −7.6. *Bal* 31 deletional analysis localized the activity of each enhancer fragment to a 300- to 400-bp MER (13). (B) The transgenes used in this study. All constructs contained the human β-gl promoter linked to the mouse *H-2D*$^d$ structural gene. Each of the three AFP enhancers was fused 5′ of the β-gl promoter in its natural orientation. The construct names and lines generated from transgenic founder mice are shown. Symbols: ■, MERI; ▨, MERII; ▧, MERIII.

linked to the AFP promoter, was active in all three AFP-expressing fetal tissues (19). To characterize EI activity in the absence of the tissue-specific AFP promoter, four founder lines containing EI-βgl-D$^d$, A.1 to A.4, were generated. RNA was prepared from fetal and adult tissues as described above and analyzed by RNase protection. In fetal tissue from all four lines, the pattern of transgene expression is similar to that of the endogenous AFP gene in that βgl-D$^d$ mRNA levels were high in the yolk sac, moderate in the fetal liver, and low (A.1, A.3, and A.4) or undetectable (A.2) in the fetal gut (Fig. 3). These results confirm that EI is active in all three fetal tissues where AFP is expressed.

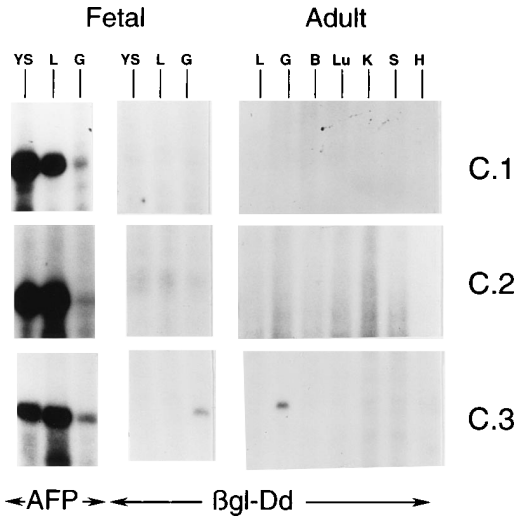


FIG. 2. Expression of βgl-D$^d$ mRNA in the three C mouse lines which contain the βgl-D$^d$ transgene. RNA was prepared from the three AFP-permissive fetal tissues, the yolk sac (YS), liver (L), and gut (G), at embryonic day 18. RNA was also prepared from adult liver (L), gut (G), brain (B), lung (Lu), kidney (K), spleen (S), and heart (H). Transgenic fetuses and adult mice were identified by Southern blot analysis of tail DNA. The quality and quantity of RNA in each sample were monitored by formaldehyde gel analysis. RNase protection analysis was performed with either 5 μg of fetal RNA or 50 μg of adult RNA. The three leftmost lanes were analyzed with the mouse AFP riboprobe; correctly initiated AFP transcripts protect a 55-nucleotide fragment. All other lanes were analyzed with the βgl-D$^d$ probe; correctly initiated hybrid gene transcripts protect a 115-nucleotide fragment.

Although AFP transcription declines dramatically at birth, several lines of evidence suggest that the postnatal decline in AFP levels is not caused by a decrease in enhancer activity (6, 15) but to a dominant repressor region located between the AFP promoter and EI (36). Therefore, we would predict that EI would continue to stimulate transcription of the linked β-gl promoter after birth. Analysis of adult tissues in all four A lines revealed that the transgene was still expressed in the liver and gut, suggesting that EI remains active in these adult tissues (Fig. 3). However, transgene levels in both tissues varied dramatically between lines. For example, transgene levels ranged from being high in the livers of A.1 mice to barely detectable in the livers of A.2 mice. This is in contrast to fetal levels of expression, where transgene levels in the liver and gut were relatively uniform when the four lines were compared. In general, βgl-D$^d$ mRNA levels decreased in the adult liver in all four lines (note that 5 μg of RNA was used for fetal samples, whereas 50 μg of RNA was used for adult samples). In the gut, transgene levels actually increased in lines A.2 and A.3. This variability was not observed within a particular line; different animals from the same founder had the same transgene levels in the liver and gut.

Several adult tissues where the AFP gene is not normally transcribed were also tested for transgene expression. These data revealed that low levels of βgl-D$^d$ transcripts were detected in the hearts of A.1 mice and in the brains of A.3 mice. Expression in these two organs was limited to a single line and

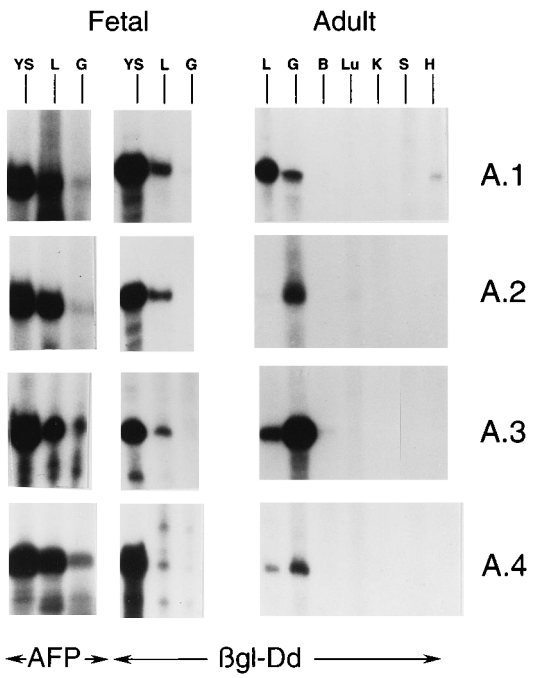


FIG. 3. Expression of βgl-D$^d$ mRNA in the four A mouse lines which contain the EI-βgl-D$^d$ transgene. RNA was prepared from embryonic day 18 and adult tissues as described in the legend to Fig. 2. Transgenic fetuses and adult mice were identified by Southern blot analysis of tail DNA. The quality and quantity of RNA in each sample were monitored by formaldehyde gel analysis. RNase protection analysis was performed with either 5 μg of fetal RNA or 50 μg of adult RNA. The three leftmost lanes were analyzed with the mouse AFP riboprobe; all other lanes were analyzed with the βgl-D$^d$ probe.

thus not likely due to enhancer activity in these tissues. This sporadic expression was similar to what was observed previously in C.3 mice and we again favor the notion that the aberrant expression in A.1 and A.3 mice is likely due to the site of transgene integration (1). Overall, these results suggest that AFP EI remains active in the adult but that this activity is restricted to tissues where AFP is normally synthesized.

To monitor the activity of EII, three transgenic lines, B.3, B.4, and B.5, were generated with EII-βgl-D$^d$. The transgene was expressed at high levels in the yolk sac and fetal liver in all three lines (Fig. 4). Transgene levels were higher in the liver than the yolk sac in lines B.3 and B.5, whereas levels were roughly equivalent in B.4 mice. This is in contrast to the four A lines, which showed higher transgene levels in the yolk sac. Transgene mRNA was found in the fetal gut at very low levels in B.3 and B.5 mice and was undetectable in B.4 mice. Analysis of adult tissues revealed that mRNA derived from EII-βgl-D$^d$ was still present at high levels in B.5 liver and moderate levels in B.3 and B.4 livers (Fig. 4). Transgene mRNA was also detected in the guts of B.3 and B.5 mice with a clear increase over fetal gut levels in B.5; no mRNA was seen in B.4 adult gut samples. Transgene expression was seen in B.3 lung.

Previous studies indicated that EIII was active in the yolk sac but had very low activity in the fetal liver and gut (19). However, EIII had high levels of activity in hepatoma HepG2 and gut Caco-2 cultured human cell lines (35). To further investigate EIII activity, three transgenic lines, D.1, D.2, and D.3, were generated with EIII-βgl-D$^d$. A variety of expression patterns were seen in fetal samples from these three lines (Fig. 5). The βgl-D$^d$ mRNA levels ranged in the yolk sac from very high in D.2 to low in D.3, in fetal liver from very high in D.1 to low

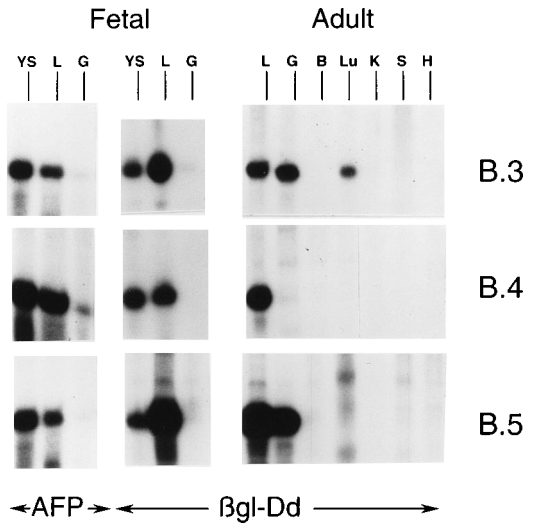


FIG. 4. Expression of βgl-D$^d$ mRNA in the three B mouse lines which contain the EII-βgl-D$^d$ transgene. RNA was prepared from embryonic day 18 and adult tissues as described in the legend to Fig. 2. The quality and quantity of RNA in each sample were monitored by formaldehyde gel analysis. Transgenic fetuses and adult mice were identified by Southern blot analysis of tail DNA. RNase protection analysis was performed with either 5 μg of fetal RNA or 50 μg of adult RNA. The three leftmost lanes were analyzed with the mouse AFP riboprobe; all other lanes were analyzed with the βgl-D$^d$ probe.

in D.3, and in fetal gut from high in D.1 to very low in D.2 and D.3. This broad range in patterns of transgene expression between the different D lines is in contrast to the results with A and B mouse lines, where transgene levels were quite similar in fetal tissues from different lines. These results suggest that EIII activity is more sensitive to the site of chromosomal integration than is either EI or EII.

Analysis of adult tissues showed that EIII remained active in the adult liver and gut. In addition, low levels of βgl-D$^d$ mRNA

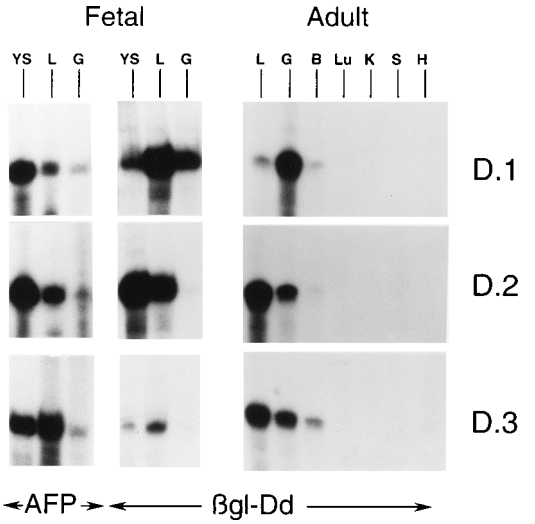


FIG. 5. Expression of βgl-D$^d$ mRNA in the three D mouse lines which contain the EIII-βgl-D$^d$ transgene. RNA was prepared from embryonic day 18 and adult tissues as described in the legend to Fig. 2. Transgenic fetuses and adult mice were identified by Southern blot analysis of tail DNA. RNase protection analysis was performed with either 5 μg of fetal RNA or 50 μg of adult RNA. The three leftmost lanes were analyzed with the mouse AFP riboprobe; all other lanes were analyzed with the βgl-D$^d$ probe.

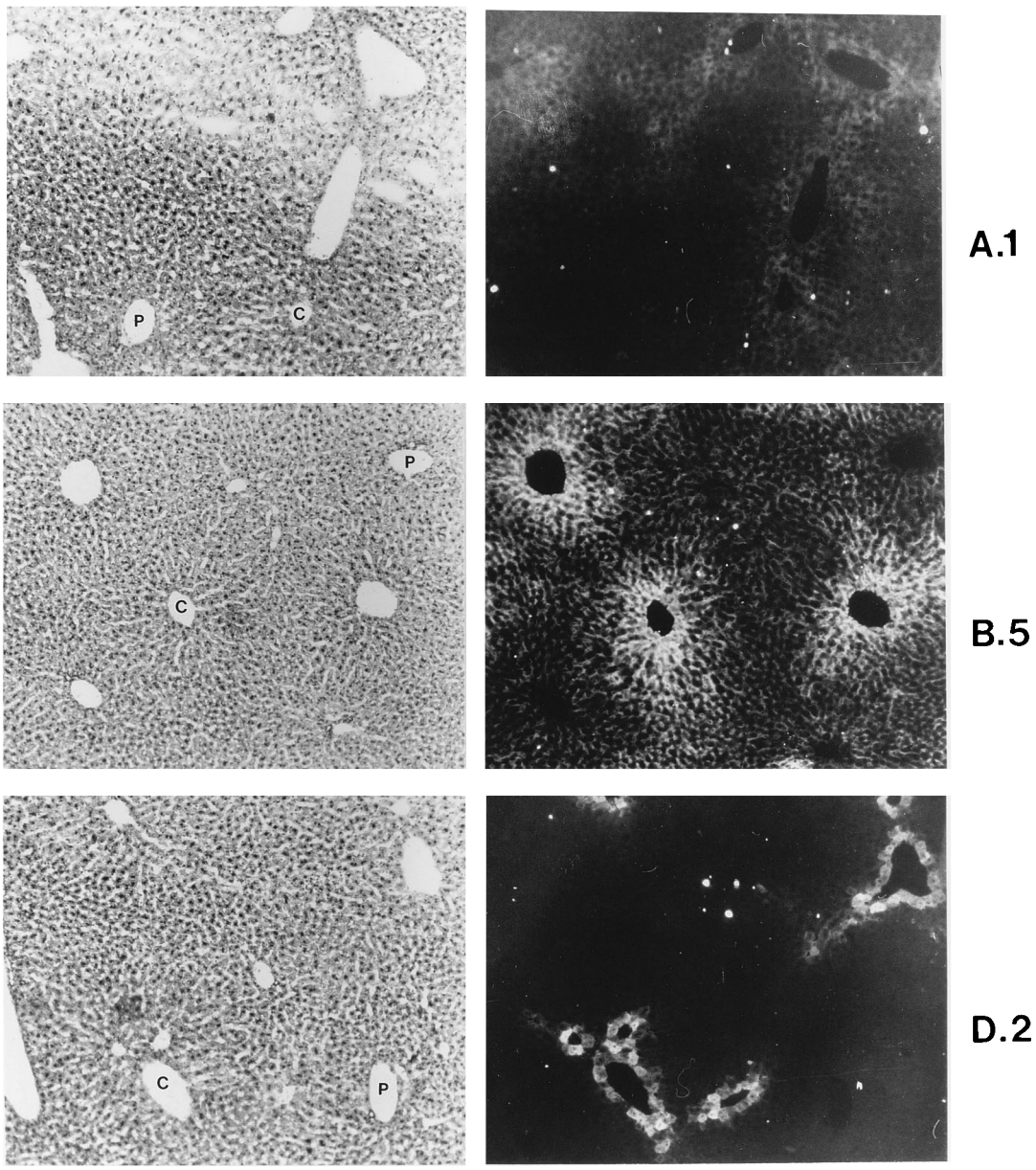


FIG. 6. Immunohistochemical localization of $D^d$ proteins in the livers of adult transgenic A.1, B.5, and D.2 mice. Sections were stained with hematoxylin and eosin (left panels); adjacent sections were stained in situ with an FITC-conjugated anti-$D^d$ monoclonal antibody (right panels). An example of a central vein (c) and portal triad (p) is denoted in each left panel. Identical exposure times were taken for each immunofluorescence photomicrograph; thus, $D^d$ protein levels can be compared between the three transgenic livers shown. In all three lines, staining is most intense in hepatocytes surrounding the central vein; in D.2 liver, the staining is largely restricted to hepatocytes surrounding the central vein. Magnification, ×75.

were observed in brain samples from all three D lines (Fig. 5). Although transgene mRNA levels were low, since expression was observed in all three lines, EIII must activate the β-gl promoter in the brain and this is not an artifact due to site of transgene integration. Of the three AFP enhancers, this represents the only example of enhancer activity in a tissue where AFP is not normally synthesized.

The phenomenon of zonally restricted transcription in the adult liver has been well documented for a number of genes (18), although the molecular basis for this regulation remains poorly understood (16). Whereas some genes are expressed uniformly across the liver acinus, other genes are restricted to cells surrounding either the central vein or portal triad. Since the results from RNase protection studies indicated that the three AFP enhancers were active in the adult liver, we wished to determine whether the AFP enhancer elements showed unique patterns of zonal activity. To accomplish this, frozen liver sections were incubated with a fluorescein-conjugated $D^d$-specific monoclonal antibody. This antibody reacts with H-$2D^d$ class I molecules on the cell surface and, therefore, would bind only to cells expressing the βgl-$D^d$ hybrid gene. We previously used antibody staining to monitor AFP-$D^d$ hybrid gene expression in cultured cell lines (10, 33).

When liver sections from C.2 mice were incubated with the anti-$D^d$ antibody, no $D^d$-positive cells were observed. The level of fluorescence intensity was identical to liver sections from

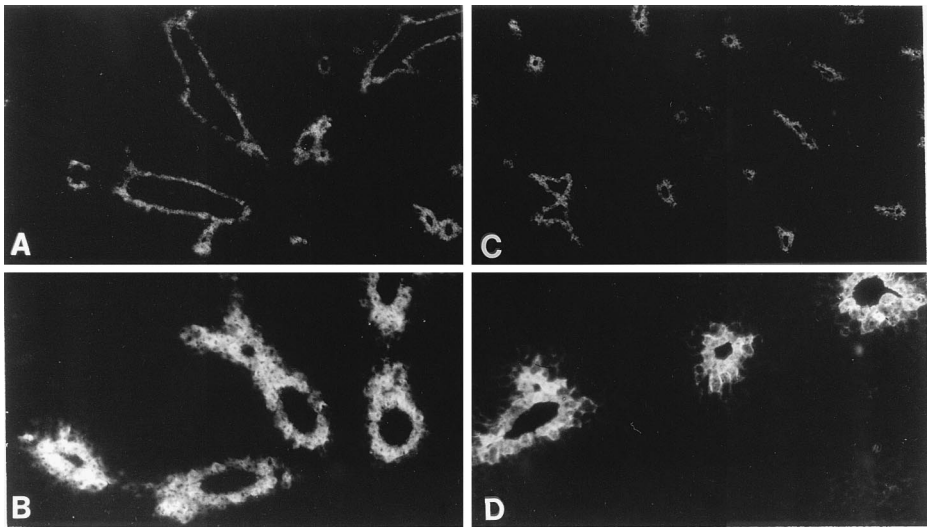


FIG. 7. Localization of $D^d$ proteins in the livers of adult transgenic D.1 (A and B) and D.2 (C and D) mice. In all panels, only cells surrounding the central veins and larger collecting hepatic veins are stained by the anti-$D^d$ antibody. Identical exposure times were used for each immunofluorescence photomicrograph. Magnifications, ×30 (A and C); ×75 (B and D).

nontransgenic mice that were incubated with antibody (data not shown), demonstrating that the degree of nonspecific antibody staining was very low. The absence of cell surface $D^d$ expression is consistent with the RNase protection data showing a lack of βgl-$D^d$ mRNA in C.2 liver. Analysis of adult liver sections from A, B, and D mice revealed significant antibody staining (Fig. 6). The level of staining was low but detectable in A.1 mice; cells surrounding the central vein showed the highest level of antibody binding. The level of staining decreased gradually across the liver acinus, and no staining was detected in hepatocytes surrounding the portal triad. The same zonal pattern of $D^d$ expression was observed in A.3 mice; no staining was seen in liver sections from A.4 mice, presumably because the transgene expression was so low as to be below the level of detection (data not shown).

$D^d$ protein expression in liver sections from adult B.5 mice (Fig. 6) revealed that all hepatocytes were stained by the $D^d$-specific antibody. However, cells surrounding the central vein expressed the highest amount of $D^d$ protein, with a gradual decrease in staining in cells more distant from the pericentral region. The same zonal pattern of $D^d$ staining was observed in B.3 mice (data not shown). A comparison of the results with the different A and B lines shows that the intensity of anti-$D^d$ antibody staining was in accordance with the amount of transgene mRNA, indicating that $D^d$ protein levels generally reflect the mRNA levels. Despite these quantitative differences, the general staining patterns in livers from A and B mouse lines appeared qualitatively to be very similar. This result suggests that EI and EII are similarly regulated in adult liver.

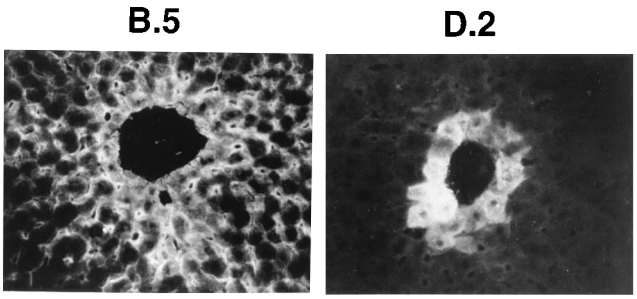


FIG. 8. Localization of $D^d$ proteins in the region surrounding a central vein in the livers of adult B.5 and D.2 mice, showing the highly restricted zonal activity of EIII. Identical exposure times were used for each immunofluorescence photomicrograph. Magnification, ×104.

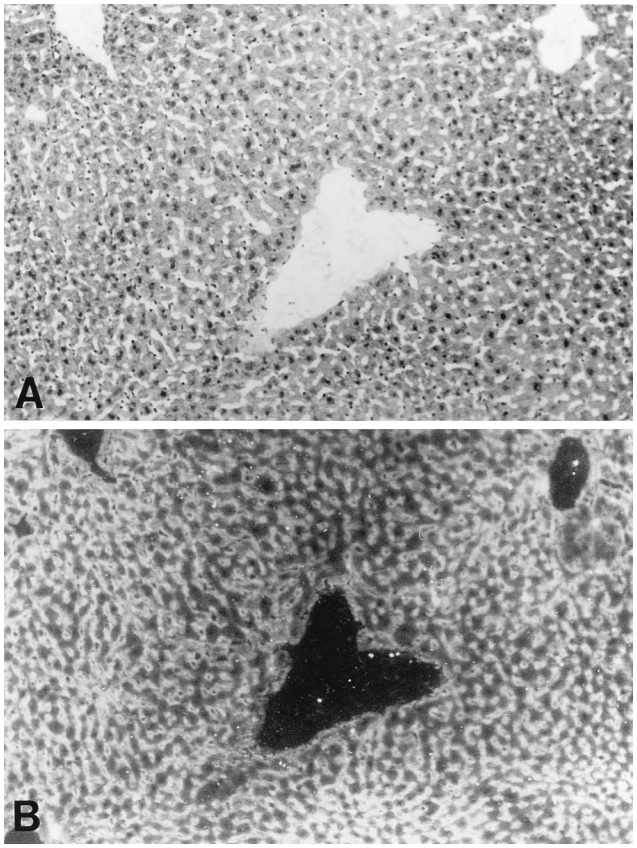


FIG. 9. Distribution of $D^d$ proteins in the livers of adult BALB/c mice ($H\text{-}2^d$). Adjacent sections were stained with hematoxylin and eosin (A) or in situ with the anti-$D^d$ monoclonal antibody (B). All hepatocytes across the liver acinus are uniformly stained. Magnification, ×75.

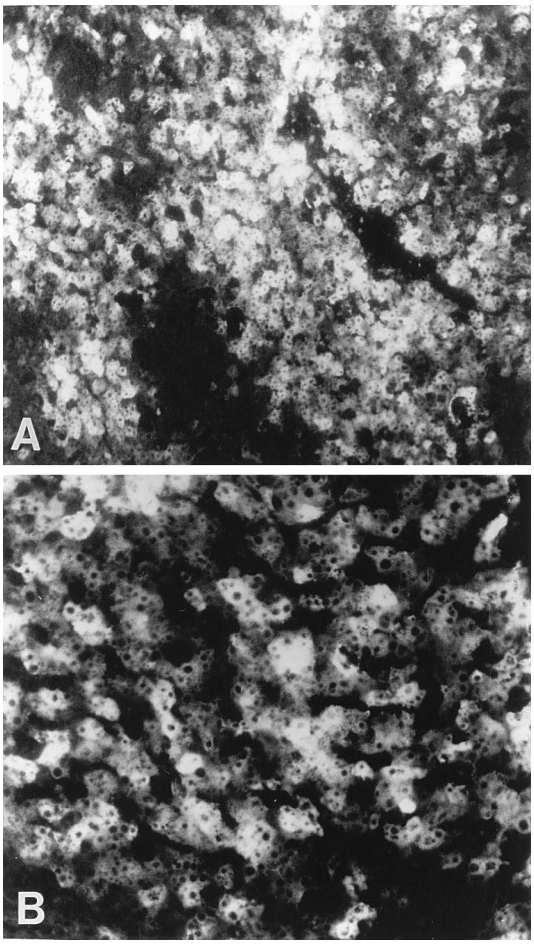


FIG. 10. Immunohistochemical analysis of $D^d$ expression in fetal (e18) D.2 liver sections. All hepatocytes were stained with the anti-$D^d$ antibody; regions that were not stained represent clusters of hematopoietic cells. Magnifications, ×31 (A) and ×78 (B).

A much more restricted pattern of transgene expression was observed in adult liver from D.2 mice, where cell surface $D^d$ proteins were found almost exclusively on hepatocytes surrounding the central vein (Fig. 6). In some cases, hepatocytes that are one cell removed from the central vein express low levels of $D^d$ protein. Additional analysis of D.1, D.2 (Fig. 7), and D.3 mice (data not shown) further illustrates the highly restricted pattern of EIII activity in the adult liver. These data reveal that all pericentral hepatocytes in D.1 and D.2 livers are stained by the anti-$D^d$ antibodies. Cells surrounding the larger hepatic veins also express high levels of $D^d$ proteins (Fig. 7A). The difference between the staining patterns in B.5 and D.2 mice is more striking at a higher magnification (Fig. 8). The gradual decrease in $D^d$ levels in cells more distant from the central veins in B.5 mice contrasts with the intense staining restricted to a thickness of 1 or 2 cells in D.2 mouse liver. This result indicates that EIII is active in a highly restricted zone around the central vein and argues that EIII regulation is distinct from EI and EII regulation.

Mouse histocompatibility class I molecules, such as H-$2D^d$, must be associated with $\beta_2$-microglobulin to be expressed on the cell surface (28). Thus, it was possible that the zonal pattern of $D^d$ expression in transgenic mice was not solely due to restricted transgene expression but resulted from limited $\beta_2$-microglobulin synthesis. To test this, liver sections from BALB/c mice were incubated with the anti-$D^d$ antibody. The BALB/c mouse strain expresses genes of the *H-2*$^d$ major histocompatibility complex, including *H-2D*$^d$. All hepatocytes in the BALB/c liver sections were uniformly stained by the anti-$D^d$ antibody (Fig. 9). This demonstrates that all hepatocytes have the capacity to express class I molecules and indicates that the zonal $D^d$ protein expression observed in transgenic mice is due to restricted transgene expression.

The structure of the adult liver parenchyma, along with the zonal pattern of hepatocyte gene expression, is organized in the first several weeks after birth (23). The architecture of the fetal liver is quite different, and the embryonic day 18 liver is composed of a mixture of hepatocytes and hematopoietic cells (27). Analysis of fetal liver sections in D.2 mice revealed that essentially all hepatocytes expressed substantial levels of $D^d$ proteins (Fig. 10). Hematoxylin and eosin staining of adjacent sections indicated that clusters of $D^d$-negative cells represent hematopoietic cells (data not shown). Thus, the zonal expression pattern of AFP EIII activity is established postnatally.

## DISCUSSION

The mouse AFP gene is an ideal system for studying tissue-specific and developmental transcriptional regulation. Of particular interest are the roles of the three distinct AFP enhancer elements. When tested by transient transfections in cultured hepatoma cells, these regions functioned in an equivalent and nonadditive fashion (13, 14). However, when monitored in transgenic mice, each enhancer exhibited different activities in the three tissues where AFP is normally expressed (19). In these previous studies, enhancer activity was measured when these regions were linked to the tissue- and fetal-specific AFP repressor-promoter region. In this study, each AFP enhancer element was linked individually to the heterologous human β-gl promoter, enabling us to monitor enhancer activity in a wide variety of tissues and at pre- and postnatal time points. By analyzing these hybrid gene constructs in transgenic mice, we have identified novel aspects of enhancer activity which further supports the notion that the three AFP enhancers have unique but overlapping patterns of activity.

In fetal mice, each AFP enhancer could activate the β-gl promoter in the yolk sac, liver, and gut. In general, these results are in agreement with a previous study (19). However, our results demonstrated that EIII has significant activity in the fetal liver and gut, in contrast to a previous study showing little EIII activity in these tissues (19). This discrepancy could be due to differences in the EIII fragment used in the two studies. Here, the 5′ end of the EIII fragment of the transgene was an *Eco*RI site located 1 kb from the 340-bp minimal enhancer region (MER) of enhancer III (MERIII). In the previous study, the 5′ end of the EIII fragment of the transgene was a *Cla*I site that is 14 bp away from a strong HNF-3 binding site at the 5′ end of MERIII (17); the proximity of the end of the transgene fragment could influence the activity of this HNF-3 site, and thus overall MERIII activity, in the liver and gut.

The pattern of EI-βgl-$D^d$ transgene expression (high levels in the yolk sac, intermediate levels in the fetal liver, and low levels in the fetal gut) closely paralleled that of the endogenous AFP gene. Whether these results indicate that EI is the dominant AFP enhancer in fetal tissues cannot be determined by this study. Of some interest was the finding of low transgene levels in the fetal gut. This is in contrast to what we (32) and Hammer and coworkers (19) previously observed; in both studies, EI activated the AFP promoter in the gut to levels that were dramatically higher than those of the endogenous AFP

gene. Taken together, these data indicate that the superactivation of fetal gut transcription depends upon specific interactions between enhancer I and the AFP promoter. Interestingly, a promoter coupling element has been identified in the rat AFP promoter and is required for strong stimulation by linked enhancers (37). This promoter coupling element could account for the different levels of AFP and β-globin promoter activation by EI in the fetal gut.

The continued presence of transgene mRNA in adult livers and guts from A, B, and D lines indicates that the three AFP enhancers remained active postnatally, consistent with previous studies showing that perinatal decline in AFP transcription requires a dominant repressor region upstream of the AFP promoter. Modified AFP transgenes containing all three enhancers and AFP promoter but lacking the repressor region and hybrid transgenes containing the AFP enhancer region linked to the albumin promoter continue to be expressed at high levels in the adult liver and gut (6, 36). In addition, studies with isolated adult liver nuclei indicated that all three enhancers were hypersensitive to cleavage with DNase, which is considered to be a measure of regulatory protein binding to specific DNA sequences (15). Interestingly, EII exhibited much greater DNase I sensitivity than EI and EIII did. These data are consistent with our results showing EII being the most active AFP enhancer in the adult liver. EIII is also highly active in this tissue, but activity is restricted to a very low percentage of adult hepatocytes. The apparent low DNase hypersensitivity of EIII in adult liver may reflect the fact that EIII is active in such a small number of cells.

The purification and characterization of transcriptional regulators have revealed that many factors involved in liver-specific gene control are in fact found at substantial levels in other tissues. For example, high levels of HNF-3 and C/EBP activities are found in the lung; HNF-1 and HNF-4 are present in the kidney (40). Therefore, we anticipated that the individual AFP enhancers, when tested in the absence of other AFP control regions, might be active in tissues where AFP is not synthesized. However, EI and EII activity remained limited to the tissues where AFP is normally expressed; EIII was additionally active in the brain. Since many transgenes are ectopically expressed in the brain, the significance of EIII activity in this tissue is not clear.

Sequence comparisons have revealed that the 200- to 300-bp MERs of EI and EII are more closely related to each other than either is to the MER of EIII (14). These findings might suggest that the activities of EI and EII would be similar to each other and distinct from EIII activity. Our results support this notion. First, EI and EII have similar patterns of zonal activity in the adult liver, while EIII-regulated transgenes have distinct, more highly restricted zonal activities. In addition, the activities of EI and EII are restricted to the yolk sac, liver and gut, whereas EIII also functions in the nervous system. The activities of EI and EII, however, can be distinguished in several ways. EI appears to have greater activity than EII in the yolk sac, whereas EII-regulated transgenes are expressed at higher levels in fetal and adult liver. Thus, subtle differences in these control regions may modulate their activity in different tissues. Additional expression studies, such as transgene induction during liver regeneration, may reveal additional differences or similarities between the AFP enhancers. In fact, preliminary studies indicate that the three enhancers have different activities during liver regeneration. When assayed 3 days after treatment with the hepatotoxin carbon tetrachloride, at the peak of AFP reexpression (4), EI appears to have little or no change in activity. At this same time, EII becomes active in all hepatocytes, whereas EIII activity is mainly restricted to cells adjacent to the necrotic regions (29). A more complete analysis of enhancer activity during liver regeneration is in progress. These studies, along with further characterization of the factors which regulate these enhancers, should help elucidate the basis of transcriptional changes during liver regeneration.

The most intriguing aspect of the current study is the zonally restricted activity of each enhancer. Previous studies showed that AFP transgenes containing all three enhancers and promoter but lacking the repressor region were expressed only in pericentral hepatocytes (11), indicating that the AFP repressor is required for postnatal AFP inactivation in pericentral hepatocytes. However, other mechanisms must account for the absence of AFP expression in intermediate and periportal regions; the data presented here suggest that restricted AFP enhancer activity may contribute to the postnatal decline of AFP mRNA levels in these areas.

Our results provide the first example of discrete regulatory domains having zonal regions of activity in the adult liver. That these enhancers can confer zonal expression upon a heterologous promoter-reporter gene indicates that the restricted expression is occurring at the transcriptional level. Our results cannot determine if the restricted pattern of enhancer activity is due to the action of zonally expressed positive or negative regulators. Additional studies, particularly with EIII, should help clarify the role of transcription factors in mediating zonal control. EIII is regulated by HNF-3 and C/EBP family members (17, 25). C/EBPα is uniformly distributed across the adult liver acinus (24); the zonal expression patterns of other C/EBP proteins and HNF-3 family members are not known. Whether these factors contribute to the zonal activity of EIII is under investigation by altering the binding sites for these factors by site-directed mutagenesis, followed by analysis in cultured cells and transgenic mice.

The phenomenon of positional control of liver gene expression has been well documented, but the basis for such control is poorly understood (18). One view is that hepatocyte stem cells differentiate as they slowly migrate across the hepatic plates from periportal to pericentral regions; this differentiation is accompanied by position-dependent changes in gene expression (30). A second model is that positional variation in gene expression is due to changes in the level of different metabolites in sinusoidal blood flowing from the portal triad toward the central vein and/or differences in cell matrix proteins across the liver acinus (22). Our results do not necessarily support one model over the other. However, the mice described here will be useful for further examining the basis of zonally restricted expression. By using $D^d$-specific monoclonal antibodies, flow cytometry could be used to enrich for viable populations of pericentral hepatocytes. This is particularly true with EIII-βgl-$D^d$ mice, where the distinction between $D^{d+}$ and $D^{d-}$ cells is so dramatic. Such enriched cells could be used to further characterize the unique properties of this subpopulation of hepatocytes.

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