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The alpha-fetoprotein enhancer region activates the albumin and alpha-fetoprotein promoters during liver development

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The four members of the albumin gene family encode the serum transport proteins albumin, α-fetoprotein, α-albumin, and vitamin D-binding protein. These genes are transcribed primarily in the liver with each having a different pattern of developmental expression. The tight linkage of these genes, particularly that of albumin, α-fetoprotein and α-albumin, and their liver-specific expression, has led to the suggestion that these genes share common regulatory elements. To directly examine whether the α-fetoprotein enhancer region could regulate the albumin gene family, expression of these genes was monitored in mice in which this region was deleted by homologous recombination. Our data indicate that this enhancer region is required for α-fetoprotein and albumin activation early in liver development and α-fetoprotein reactivation during liver regeneration, but that albumin, α-albumin, and vitamin D-binding protein expression later in hepatic development is not affected by the absence of these enhancers. We also demonstrate that RNA polymerase II loading on the α-fetoprotein and albumin promoters is reduced in the absence of this enhancer region, indicating a direct role for these enhancers in the assembly of the RNA Polymerase II complex during liver development.

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Introduction

The albumin, α-fetoprotein (AFP), α-albumin (afamin; AFM) and vitamin D-binding protein (DBP) genes comprise a small gene family (ALB gene family) with a highly conserved intron/exon organization that encodes proteins with similar structural and functional properties (Belanger et al., 1994; Cooke and David, 1985; Lichtenstein et al., 1994; Tilghman, 1985; Yang et al., 1985). Through evolution, a series of duplications formed the genes within the ALB family, with DBP being the most distantly related (Gibbs et al., 1998; Haefliger et al., 1989). All four genes are expressed primarily in the liver with different patterns of developmental regulation (Belanger et al., 1994). AFP expression is low in the foregut endoderm and dramatically increases in progenitor hepatoblasts that differentiate from this tissue; albumin is highly induced in parallel with AFP in these progenitor hepatoblasts that form the liver bud (Gauldi et al., 1996; Tilghman and Belayew, 1982). Both AFP and albumin continue to be expressed at high levels in the fetal liver. AFP is also abundantly expressed in the visceral endoderm of the yolk sac; albumin is expressed at low levels in this extraembryonic tissue (Tilghman, 1985). DBP expression initiates midgestation in the fetal liver (Song et al., 1998a). After birth, AFP transcription is rapidly repressed to barely detectable levels while albumin and DBP transcription increase slightly. Temporal activation of AFM parallels AFP silencing during the perinatal period. Albumin, AFM and DBP continue to be expressed in the adult liver. AFP is normally silent after birth, but can be reactivated during periods of liver regeneration and in hepatocellular carcinomas (Abelev, 1971; Belanger et al., 1994; Belayew and Tilghman, 1982; Tilghman and Belayew, 1982).

Albumin, AFP and AFM are tandemly arranged in the same transcriptional orientation in all mammals examined, including humans and rodents, with albumin being ~14 kilobases (kb) upstream of the 5′ end of AFP and AFM being ~25 kb downstream of the 3′ end of AFP (Belanger et al., 1994; Chevrete et al., 1987; Ingram et al., 1981; Nishio et al., 1996; Urano et al., 1984) (Fig. 1A). The more distantly related DBP is linked, albeit less tightly, to the other three genes (Buetow et al., 1991); in humans and mice, DBP is 1.5 and 1.0 Mb upstream of the 5′ end of albumin, respectively, and in the opposite transcriptional orientation (Song et al., 1999).

Several enhancers have been identified that are required for tissue-specific expression of genes within this locus in mice. These include the albumin enhancer, which lies ~10 kb upstream of the albumin gene and is able to direct high-level transgene expression in...
adult liver but has modest activity in fetal liver (Pinkert et al., 1987; Postic and Magnuson, 2000), and three AFP enhancers, E1, E2 and E3, which are located within the albumin-AFP intergenic region (Godbout et al., 1986, 1988) (Fig. 1A). In transgenic animals, each AFP enhancer is sufficient to activate transgenes in the yolk sac, fetal liver and gut and continues to be active in the adult liver and gut (Camper and Tilghman, 1989; Hammer et al., 1987; Ramesh et al., 1995). In contrast to rodents, the human genome contains E2 and E3 but does not have an E1 (Long et al., 2004). Enhancers for AFM have not yet been identified, whereas multiple enhancers that govern DBP expression have been well characterized (Hiroki et al., 2007, 2006; Song et al., 1998b).

The tight linkage of albumin and AFP has led to the hypothesis that both genes require the AFP enhancers for proper activation during hepatogenesis (Godbout et al., 1986, 1988; Spear, 1999). This possibility is supported by the fact that the albumin enhancer exhibits only weak activity in the yolk sac, fetal liver and gut and continues to be active in the adult liver and gut (Godbout and Tilghman, 1989; Hammer et al., 1987; Ramesh et al., 1995). In contrast to rodents, the human genome contains E2 and E3 but does not have an E1 (Long et al., 2004). Enhancers for AFM have not yet been identified, whereas multiple enhancers that govern DBP expression have been well characterized (Hiroki et al., 2007, 2006; Song et al., 1998b).

The targeting vector comprised of the 5.8 kb AFP 5′ homologous region (from BglII site at -12.6 to Apal site at -6.8), Neo-TK selection cassette flanked by loxP sites (gray arrows), with a HindIII site at the end of the 3′ loxP site), 4.5 kb AFP 3′ homologous region (from SphI site at -2.0 to EcoRI site at +2.5), and the DT-A gene. The next two structures represent the targeted locus before and after transient transfection with Cre recombinase, which removes the Neo-TK cassette and leaves behind a single loxP site; distances between diagnostic EcoRI and HindIII sites for the primary and secondary ES cell clones are shown.

Materials and methods

Targeting vector, ES cells, and production of chimeric mice

A BAC clone containing the AFP gene from a 129/Sv genomic BAC library (Research Genetics) was used to obtain AFP fragments for constructing the AFP enhancer targeting construct. A 4.8-kb region from an Apal site at -6,643 to a SphI site at -1,880 (relative to the AFP transcription start site) containing the three AFP enhancers was replaced with a floxed Neo/TK cassette (details regarding the targeting vector will be provided upon request). The targeting vector was electroporated into R1 ES cells and Southern analysis was used to screen for correctly targeted clones. The Neo/TK cassette was subsequently removed by transient transfection of Cre recombinase. Chimeric mice were generated by aggregation of targeted R1 cells with CD1 blastocysts, followed by mating to obtain germ-line transmission of the targeted allele. For mouse genotyping, the primer set for the wild type (enh<sup>+</sup>) allele is: 5′ GAATCTCAACGGTACAGCC and 5′ CCACATGACTTTGTGTGAGG, which will generate a 220 bp fragment; the primer set for the knock-out (enh<sup>−</sup>) allele is 5′ ...
GTGTCATTGTCCAGATCTGG and 5’-CAATCTGCAAGGCTAAGTGC-3’, which will generate a 450 bp fragment.

RNA isolation and analysis

Total RNA was isolated from embryonic day 14.5 (e14.5) livers or yolk sac using Trizol following manufacturer’s instruction (Invitrogen, Inc.). One μg of total RNA was used for reverse transcription to cDNA using the iScript cDNA synthesis kit (Invitrogen, Inc) in a 20 μl reaction volume. After RT, cDNA was dilute in sterile water to 100 μl.

Real time PCR was carried out using a MyQ real time PCR detection system (Bio-Rad Laboratories), using 1 μl of cDNA, 12.5 μl of Sybergreen Mix (Bio-Rad) and 5 μM of each primer in a 25 μl volume. Each sample was analyzed in triplicate. For each run, amplifications with water alone was a control to monitor for contamination. The following primers were used for real-time RT-PCR: AFP, 5’-AGCGAGG-GGA-3’ and 5’-AGCGAGTGTTCCGATACTC-3’; ALB, 5’-TCGTGCTGCTGCTGATCCAC-3’ and 5’-AGCGAGGAATTCCGACT GTA-3’; DBP, 5’-TCCTGCTGAAGTCCTTTTATATCC-3’ and 5’-TCCTGCTGAAGTCCTTTTATATCC-3’; AFM, 5’-TCCTGCTGAAGTCCTTTTATATCC-3’ and 5’-TCCTGCTGAAGTCCTTTTATATCC-3’; albumin promoter, 5’-CTGCTGCTGCTGCTGATCCAC-3’ and 5’-AGCGAGGAGTTCCGATACTC-3’; AFP promoter, 5’-TCCTGCTGCTGCTGATCCAC-3’ and 5’-TCCTGCTGCTGCTGATCCAC-3’; TTR, 5’-GTCGAGTTCCCGTCTGCGTCCGA-3’ and 5’-CTCATCTGTCCGCTGCGTCCGA-3’.

For liver regeneration, adult mice (4–6 weeks of age) were injected i.p. with 50 μl of a 10% (v:v) solution of carbon tetrachloride (CCl4) dissolved in mineral oil alone. After 3 days, livers were removed and RNA was prepared using the lithium chloride procedure (Spear, 1994). cDNA was prepared as described above, and standard PCR was performed; products were resolved on polyacrylamide gels and visualized by ethidium bromide staining. The following primer pairs were used for real-time RT-PCR: AFP, 5’-AGCGAGG-GGA-3’ and 5’-AGCGAGTGTTCCGATACTC-3’; ALB, 5’-TCGTGCTGCTGCTGATCCAC-3’ and 5’-AGCGAGGAATTCCGACT GTA-3’; DBP, 5’-TCCTGCTGAAGTCCTTTTATATCC-3’ and 5’-TCCTGCTGAAGTCCTTTTATATCC-3’; AFM, 5’-TCCTGCTGAAGTCCTTTTATATCC-3’ and 5’-TCCTGCTGAAGTCCTTTTATATCC-3’; albumin promoter, 5’-CTGCTGCTGCTGCTGATCCAC-3’ and 5’-AGCGAGGAGTTCCGATACTC-3’.

Chromatin immunoprecipitation (ChIP) assays

Livers were removed from e14.5 embryos and snap frozen in liquid nitrogen. After PCR genotyping, enh+/+ and enh−/− mice were used to establish a colony of mice in e18.5 livers, when both AFP and DBP levels in these embryos were significantly decreased (data not shown). The second major site of AFP synthesis is the yolk sac, and DBP levels in yolk sac were not signiﬁcantly different (data not shown). The second major site of AFP synthesis during development is the extraembryonic yolk sac. Here, we saw a significant, 4-fold decrease in AFP expression in the absence of the AFP enhancer-dependent mice.

Production of AFP enhancer-deﬁcient mice

The three AFP enhancers located within the albumin-AFP intergenic region have been identiﬁed and extensively characterized using in vitro cell transfections and transgenic mice. To determine the role of these enhancers during liver development within their native chromosomal context, the enhancer region was deleted in ES cells using homologous recombination (Fig. 1). A 4.8 kb region encompassing the three enhancers was replaced with a floxed Neo-TK cassette. Three of ninety-six clones that were screened by Southern blot using fragment “A” as a probe (Fig. 1) exhibited correct targeting based on the presence of a 13.2 kb band after digestion with EcoRI (data not shown). These primary clones were expanded and transiently transfected with a Cre recombinase expression vector, followed by gancyclovir selection; Southern analysis using fragment “B” as a probe (Fig. 1) indicated that all secondary resistant clones had deleted the Neo-TK cassette, leaving behind a single loxP site, based on the presence of a 1 kb band after digestion with HindIII (data not shown).

Targeted ES cells were aggregated with CD1 embryos, and the aggregates were transferred into pseudopregnant females. The most extensively chimeric pups (as judged by coat color and PCR/Southern analysis of tail biopsy DNA) were bred to wild-type C57BL/6 mice and germ-line transmission of the enhancer-deleted allele (enh−) was achieved. These offspring were used to establish a colony of these mice. The frequency of enh−/− offspring was 1:2:1, indicating that the absence of the AFP enhancer region did not result in embryonic lethality.

Expression of ALB family genes in e14.5 liver and yolk sac

We monitored expression of genes in the ALB family at embryonic day 14.5 (e14.5). At this mid-gestational time, the liver is still undergoing rapid proliferation and is a mixture of hepatic and hematopoietic cells. The albumin and AFP genes are actively transcribed at this time, whereas this is prior to maximal AFM and DBP expression. The livers and yolk sac were removed from embryos derived from the mating of enh+/− parents; genotyping was performed with tail DNA to identify enh+/+ and enh−/− embryos. RNA was prepared from liver, AFP, AFM, and DBP levels were analyzed by real-time RT-PCR. We chose to use transthyretin (TTR) to control for variations in RNA. TTR is highly expressed in fetal hepatocytes and yolk sac, but is unlinked to the ALB gene family and therefore would not be influenced by the presence or absence of the AFP enhancers. Since the e14.5 liver is comprised of a variety of hepatic and non-hepatic cell types, with a possible variability in the cellular composition between animals, a hepatocyte-specific control would be more appropriate than that which was expressed in all cell types. The absence of the AFP enhancers resulted in a dramatic 12-fold reduction of steady-state AFP mRNA levels in the fetal liver (Fig. 2A). We also saw a modest, ∼30% reduction in albumin expression, but this change was not statistically significant. We also analyzed AFM and DBP expression in e14.5 livers. As expected, both genes were expressed at lower levels than AFP and albumin at this timepoint although mRNA from both genes could be detected (Fig. 2B). Even though both showed a modest reduction in expression in the absence of the AFP enhancer region, these changes were not significant. AFM and DBP levels in enh+/− and enh−/− mice in e18.5 livers, when both genes are expressed at higher levels, also were not significantly different (data not shown).

The second major site of AFP synthesis during development is the extraembryonic yolk sac. Here, we saw a significant, 4-fold decrease in AFP expression in the absence of the AFP enhancer-dependent mice.
enhancers at e14.5. We also observed a ∼3-fold increase in albumin expression but this increase was not statistically significant.

The mechanism by which enhancers influence the developmental control of promoter activation is not fully understood. Enhancer-bound factors can directly or indirectly influence the binding and assembly of factors on promoters and influence chromatin structure. Evidence for direct interactions between enhancers and promoters has come from chromosome conformation capture (3C) assays, which demonstrate physical association of these elements (Carter et al., 2002; Dekker, 2003; Tolhuis et al., 2002). We investigated whether the AFP enhancer region could affect RNA pol II binding to the AFP and albumin promoters. Chromatin immunoprecipitation (ChIP) analysis was performed using antibodies against the RNA Polymerase II large subunit with formaldehyde-fixed nuclei from e14.5 enh+/+ and enh−/− livers; as controls, ChIP was carried out with non-specific Ig. The precipitated DNA was amplified with primers for the promoters of the AFP, albumin (ALB) and transthyretin (TTR) genes. For each of the six anti-RNA Pol II precipitations, the amount of DNA precipitated shown is relative to the control Ig, which was arbitrarily set at 1. These data are from pooled embryos in a single experiment and is representative of three independent experiments; each real-time PCR reaction performed in triplicate. (B) Quantitation of the fold difference in RNA Pol II loading on the AFP and albumin promoters in enh+/+ and enh−/− mice. This represents data compiled from three independent experiments with different litters, and indicates that there is a roughly 4-fold and 2.5-fold decrease in RNA Pol II loading on the AFP and albumin promoters, respectively.

AFP and albumin expression in e10.5 livers

Although albumin mRNA levels were not significantly different between enh+/+ and enh−/− livers at e14.5, ChIP analysis suggested that the AFP enhancer region could influence RNA Pol II binding at the albumin promoter. This led us to consider whether a more dramatic affect on albumin expression would be observed at an earlier developmental timepoint. To test this possibility, we analyzed...
AFP expression in adult liver regeneration

The normally silent AFP gene in the adult liver is reactivated during liver regeneration in response to liver damage and partial hepatectomy. To test whether the AFP enhancer region is required for AFP re-expression in the adult liver, enh+/+ and enh−/− adult mice were treated with carbon tetrachloride (CCl4) dissolved in mineral oil; control mice were injected with mineral oil alone. CCl4 is an acute hepatotoxin that specifically kills hepatocytes in pericentral regions of the liver lobule. AFP expression increases transiently during the period of regeneration subsequent to liver damage, with peak expression seen 3 days after treatment. Low but detectable levels of AFP were present in adult livers of wild-type mice (Fig. 5A). As expected, AFP expression was increased in wild-type livers roughly 6-fold after CCl4 treatment (Fig. 5B). In contrast, AFP levels in control enh−/− adult livers were lower than in enh+/+ livers, demonstrating that these enhancers continue to function in the adult liver for basal AFP expression; these data are consistent with previous studies indicating that the AFP enhancers continue to be active in the adult liver (Ramesh et al., 1995). However, AFP levels did not increase in the livers of adult enh−/− mice, indicating that these enhancers are involved in AFP reactivation during regeneration. Expression of H19, a gene unlinked to AFP that is also reactivated during liver regeneration, increased in both enh+/+ and enh−/− mice; demonstrating that transcriptional changes associated with regeneration were occurring in both groups of mice (Fig. 5). These data indicate that the AFP enhancer region is also required for reactivation of the AFP gene in regenerating adult liver.

Discussion

The tight linkage throughout mammalian evolution, liver-enriched expression and overlapping developmental expression profiles of the ALB gene family has led to the idea that this locus may contain one or
more shared regulatory elements. Here, we have used a targeted deletion approach to directly test whether the absence of the AFP enhancers affected the expression of ALB family genes. Our data indicate that the AFP enhancer region is required for normal AFP expression in the yolk sac, fetal liver, and regenerating adult liver. Our data also demonstrate that the AFP enhancer region is required for albumin activation early during liver development. However, while delayed, albumin expression does eventually reach normal levels. Action of the albumin enhancer presumably is responsible for this activation later in gestation. AFM expression was normal in enh−/− mice, demonstrating that activation of this gene occurred independently of the AFP enhancers. While these data do not rule out the possibility of coordinated AFP repression and AFM activation during the perinatal period, we can conclude that the AFP enhancers are not involved in such control if it does exist.

Previous studies have identified each of the three AFP enhancers as distinct ∼300 bp elements in the 4.8 kb region that was deleted in the enh−/− mice. It is possible that regulatory elements other than the three known enhancers are present in the deleted region, and that the loss of these elements contribute to the changes in AFP and albumin expression. However, we believe this is unlikely. Extensive deletion analysis of this region from the mouse and rat AFP gene in both transgenic mice and tissue culture cells failed to identify regulatory elements other than the known enhancers (Godbout et al., 1986; Godbout et al., 1988; Hammer et al., 1987; Millonig et al., 1995; Wen et al., 1991). Thus, we are confident that the changes in AFP and ALB expression are due to the specific loss of the three AFP enhancers.

Eukaryotic genes with common evolutional origins and related functions are often organized into multigene loci in which genes can be coordinately regulated during development by virtue of shared cis-regulatory elements such as locus control regions (LCR). Multigene loci exhibiting this type of control include those for the β-globin, growth hormone, MHC class II and TNFα cytokines (Greaves et al., 1989; Grosveld et al., 1987; Ho et al., 2002; Masternak et al., 1998). A hallmark of LCRs is their hypersensitivity to cleavage with DNase I. Previous studies by Godbout and Tilghman (1988) demonstrated that the AFP enhancers, particularly enhancer E2, were highly sensitive to DNase I digestion in the fetal and adult liver. These data are consistent with the AFP enhancers acting as an LCR for genes in this region. However, while AFP expression is dramatically reduced in the fetal and regenerating adult liver when the AFP enhancer region is deleted, albumin expression in the liver does reach normal levels after a delay in activation. Furthermore, AFM mRNA levels are not affected by the absence of the AFP enhancers. Taken together, these data indicate that the AFP enhancer region does not function as an LCR for this locus, although we cannot rule out the possibility of an LCR elsewhere in this gene cluster.

Based on our data, we propose a model in which the AFP enhancer region is active early in hepatogenesis and required for AFP and albumin activation during this time. The albumin enhancer can activate the albumin promoter later during liver development, but does not influence AFP promoter activity. The possibility that the albumin enhancer becomes active after the albumin gene is turned on is supported by transgenic studies in our lab (T. Ramesh and B.T.S., unpublished data) and albumin-Cre recombinase transgenic mice in which Cre expression is first detected later in fetal development (Postic and Magnuson, 2000).

Elegant studies by Zaret and colleagues have shown that Foxa and GATA factors bind the albumin enhancer in gut endoderm tissue prior to albumin activation during hepatogenesis (Bassord and Zaret, 1998; Gauldi et al., 1996). Binding of these pioneer factors is required for the subsequent binding of other factors and albumin gene activation. Our data showing a dramatic reduction in albumin expression in en−/− mice in the absence of the AFP enhancer region indicate that the albumin enhancer by itself is not sufficient for albumin activation during early stages of hepatogenesis. Whether the AFP enhancers directly influence the albumin promoter or influence the assembly of factors, including Foxa and Gata 4, on the albumin enhancer will require further study.

Our ChIP data provide direct evidence that enhancers are required for the proper assembly of RNA polymerase on to a promoter during mammalian development. Factors that bind the AFP and albumin promoters have been identified and include HNF1, C/EBP and NFI. Further studies can evaluate the binding of these factors in the presence or absence of the AFP enhancers. Changes in chromatin can also be analyzed. These studies will help elucidate the events involved in promoter activation during development and in liver regeneration, and the role enhancers play in these events.

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