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Genetics

α-Fetoprotein gene sequences mediating *Afr2* regulation during liver regeneration

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ABSTRACT **α-fetoprotein (AFP) gene expression occurs in the yolk sac, in the fetal liver and gut, and in the adult liver during regeneration and tumorigenesis. Two unlinked genes determine the level of AFP gene expression in adult mice: *Afr1* regulates the basal level of expression in the normal adult liver, and *Afr2* regulates the increase in expression during liver regeneration. It has been shown that AFP-derived transgenes, including the sequences between −1,010 and −838 bp and between −118 bp and the transcriptional start site were induced appropriately during liver regeneration and were *Afr2*-regulated. To assess the role of the distal sequence in gene expression during liver regeneration, a new transgene with 7.6 kilobases of 5′-flanking sequence deleted between −1,010 and −838 bp was designed. We show that this transgene was subject to characteristic AFP tissue-specific and developmental regulation, in that it was highly expressed in the yolk sac and the fetal liver and gut but not in normal adult tissues. Expression was induced in response to liver regeneration as observed for the endogenous gene. The genetic regulation of the basal level of AFP gene expression in adult liver by the *Afr1* gene was undisturbed. However, transgene expression was not regulated by *Afr2* during liver regeneration. Our data suggest that *Afr2* regulation of AFP gene expression during liver regeneration requires the sequence between −1,010 and −838 bp and is independent of other regulatory mechanisms.**

Gene expression can be regulated on the basis of tissue, developmental stage, pathological condition, and genetic background. Expression of the α-fetoprotein (AFP) gene is subject to regulation from each of these sources. AFP is a classic oncofetal antigen expressed at high levels in the yolk sac and the liver and gut of the fetus but not in normal adult tissues (1, 2). However, AFP gene expression can be induced in the adult liver in response to liver regeneration or tumorigenesis (1, 3).

The level of AFP gene expression in the normal adult mouse liver and in regenerating liver has been shown to be regulated by two unlinked genetic loci, *Afr1* and *Afr2* (formerly known as *raf* and *Rif*, respectively) (4–6). BALB/cJ mice, homozygous for the recessive *Afr1*b allele, have higher levels of AFP mRNA in the normal adult liver as compared with C3H/HeJ and C57BL/6J mice, which are homozygous for the dominant *Afr1*A allele (5, 6). The increase in AFP gene expression during liver regeneration is regulated by *Afr2*. Two codominant alleles have been identified at the *Afr2* locus. The rare *Afr2*B allele found in C57BL/6J mice leads to 8- to 10-fold lower levels of AFP protein and mRNA when liver regeneration is induced as compared with C3H/HeJ mice carrying the more prevalent *Afr2*A allele (4, 6). B6C3F1 mice, the F1 progeny of C57BL/6J and C3H/HeJ, express an intermediate level of AFP. It has been shown that the level of AFP gene expression during liver regeneration reflects the genotype of both genes, with *Afr1* specifying the level of expression in the normal liver and *Afr2* specifying the increase in expression (4).

The molecular mechanisms regulating AFP gene expression have been studied *in vitro* and *in vivo*. The AFP gene is located on mouse Chromosome 5 as part of an evolutionarily related gene family including the albumin gene, an albumin-like gene, and the vitamin D-binding protein gene (7–9). Tissue-specific regulation of AFP gene expression is mediated by three distal enhancers (10–14) and a complex proximal promoter element (15–20) 5′ of the AFP transcriptional start site as shown in experiments with transgenic mice and tissue culture cells. *In vitro* studies have suggested that the activities of the enhancers and the promoter are coupled by the activity of a factor recognizing a sequence associated with the promoter (21, 22).

The complex developmental, pathological, and genetic regulation of the AFP gene has been studied in transgenic mice. It has been shown that 3.8 kilobases (kb) of AFP 5′-flanking sequence was sufficient to confer AFP gene regulation on two unrelated reporter genes (23, 24). Further studies using transgenic mice showed that deletion of sequences between −838 and −250 bp led to persistent AFP gene expression in adult mice, suggesting that these sequences acted as an adult-specific repressor (25–27). In addition, using the same lines of transgenic mice, we have shown that, even in the absence of complete repression in the adult, expression from these transgenes was induced in response to liver regeneration. This suggests that induction of AFP gene expression during liver regeneration is not simply a reversal of postnatal repression and that sequences outside of the repressor region respond to this stimulus. Increased gene expression was detected in all transgenes where there were at least two tracts of proximal 5′-flanking sequence, a distal sequence of 172 bp between 1,010 and 838 bp upstream of the AFP structural gene, and a proximal sequence within 118 bp of the transcriptional start site (28).

The genetic regulation of AFP gene expression in adult mice also was studied by using these same lines of transgenic mice. Vacher *et al.* (29) studied transgene expression and performed nuclear run-on assays, which suggested that post-transcriptional mechanisms contributed to the genetically determined high basal level of AFP gene expression in BALB/cJ mice. Because Spear (24) subsequently showed that AFP 5′-flanking sequences were sufficient for *Afr1* regulation of AFP gene expression, he suggested that the coupling of transcriptional and post-transcriptional regulatory mechanisms may be involved in *Afr1* regulation of AFP gene expression (24). Finally, using the same strategy employed above, we have shown that

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Abbreviations: AFP, α-fetoprotein; kb, kilobase; MG, minigene; HNF-3, hepatocyte nuclear factor-3.

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the same sequences required for induction of AFP gene expression during liver regeneration also led to *Afr2* regulation of the level of AFP gene expression (30).

To assess the contribution of the distal 172 bp of AFP 5′-flanking sequence between −1,010 and −838 bp to the regulation of AFP gene expression, we generated a new transgene deleted for this sequence. This transgene, Δ10, includes the three AFP enhancers as well as the AFP promoter driving the expression of an AFP minigene (AFP MG) and is directly comparable to transgenes studied (12, 13, 25, 26, 29, 31, 32). We found that the Δ10 transgene was expressed in the appropriate tissues during development and was not expressed in the normal adult but was induced in response to liver regeneration. We also found that transgene expression in the normal liver was higher when the transgene was introduced into the BALB/cJ genetic background, consistent with *Afr1* regulation of expression. However, the level of transgene expression during liver regeneration did not reflect the *Afr2* phenotype, as determined by the level of endogenous AFP gene expression. Therefore, *Afr2* regulation of AFP gene expression requires the sequence between −1,010 and −838 bp and is independent of the other sources of regulation observed for the AFP gene.

MATERIALS AND METHODS

Construction of the Transgene. pAFP 5′ with 7.6 kb of AFP 5′-flanking sequence was digested with *Bam*HI (New England Biolabs), and the 5′-overhangs were filled in by the Klenow fragment of DNA polymerase I (Boehringer Mannheim). *Xba*I linkers (New England Biolabs) were ligated, and the DNA was digested with both *Xba*I and BsteII (New England Biolabs). The BsteII to *Xba*I (formerly the *Bam*HI site at the 1.0 kb upstream of the transcriptional start site, which is at the 3′-end of AFP enhancer element I) was isolated from a gel and was ligated into the original plasmid digested with BsteII and *Xba*I. The result is a deletion of the sequence lying between the *Bam*HI site at −1.0 kb and the *Xba*I site at −838 bp, as shown in Fig. 1. This fragment was inserted into a plasmid to drive expression of the AFP MG, which encodes a 500-nt transcript that is easily distinguished from the endogenous 2.2-kb mRNA by RNase protection assay (27, 28).

Animals. C3H/HeJ, BALB/cJ, and B6C3F1 mice were obtained from The Jackson Laboratory. Transgenic founders were derived from C3H/HeJ × C57BL/6J F1 embryos that were mated to C3H/HeJ mice for propagation of the line. Transgenic progeny were identified by Southern blot analysis of DNA obtained from tail biopsies as described (23, 24, 28). Liver regeneration was induced in 6- to 8-week-old mice by either CCl_4 administration or 70% partial hepatectomy. A single necrotizing dose of CCl_4 was administered as an i.p. injection (10 μl/gm weight) of a 10% solution in mineral oil. Control mice were injected with mineral oil alone (28). Partial hepatectomies were performed as described by Higgins and Anderson (33). Under methoxyflurane anesthesia, the medial and left lateral lobes of the liver were ligated at the vascular stalk and removed. Livers were manipulated, but no tissue was removed in sham operations.

Analysis of RNA. RNA was prepared by lithium chloride-urea extraction and was analyzed for specific species by RNase protection assay by using RNase One (Promega) as described (28, 30, 34). Fragments protected from digestion were subjected to denaturing gel electrophoresis, and the results were analyzed by using a PhosphorImager (Molecular Dynamics). The following probes were used: (*i*) AFP probe spanning exons 3 and 4 such that the endogenous AFP mRNA protected a fragment 191 nt long whereas AFP MG mRNA protected a fragment 120 nt long (27, 28); (*ii*) albumin probe containing exons 1–4 protecting a fragment 275 nt long (28, 30); and (*iii*) glyceraldehyde-3-phosphate dehydrogenase protecting a fragment 170 nt long. Unless otherwise noted, 20 μg of total RNA was used in assays with the AFP probe, 1 μg was used with the albumin probe, and 10 μg was used with the glyceraldehyde-3-phosphate dehydrogenase probe.

RESULTS

Derivation of Transgenic Mice. Previous work performed by us (28) and others (23, 24) suggested that the sequences required for induction of AFP gene expression during liver regeneration and *Afr2* regulation resided within two regions of AFP 5′-flanking sequence: a distal 172 bp between 1,010 and 838 bp upstream of the transcriptional start site or a proximal 118 bp immediately adjacent to the gene. To assess the importance of the distal sequence, a new transgene designated Δ10 was constructed by using 7.6 kb of AFP 5′-flanking sequence deleted between the *Xba*I site at −838 bp and the *Bam*HI site at −1,010 bp to drive expression of the AFP MG. The AFP MG consists of exons 1, 2, 3, 14, and 15 and encoded a 500-bp mRNA that can be differentiated readily from the endogenous gene in RNase protection assays (27, 28, 30). The AFP 5′-flanking sequence includes the three enhancer elements, the site required for repression of AFP gene expression in adult mice, and the proximal promoter (12, 25–27, 32). Two independently derived founders, Δ10A and Δ10B, were generated.

Tissue-Specific and Developmental Regulation of the Δ10 Transgene. High level AFP gene expression normally is confined to the yolk sac, the liver, and the gut of the developing mouse, decreasing to below detectable levels in the adult (1, 2). To determine whether the Δ10 transgene is subject to this aspect of AFP gene regulation, various tissues were assayed for the presence of AFP MG mRNA by RNase protection during development. As shown in Fig. 2, both AFP and AFP MG mRNA were detected in RNA isolated from the yolk sac and fetal liver and gut tissues but not from other tissues assayed from litters sired by transgenic males on approximately the 16th day of gestation. Under standard assay conditions (20 μg of RNA), endogenous AFP mRNA was detected in liver samples from mice up to 2 weeks of age whereas transgene mRNA was detected in mice up to 4 weeks of age. These results were observed for multiple samples in both lines of transgenic mice (data not shown). The persistence of AFP MG expression is most likely caused by the high transgene copy number (between 10 and 20) in both founder lines. Therefore, the Δ10 transgene is subject to the same type of developmental and tissue-specific regulation as the endogenous AFP gene.

Δ10 Transgene Expression During Liver Regeneration. To determine whether the Δ10 transgene includes sequences

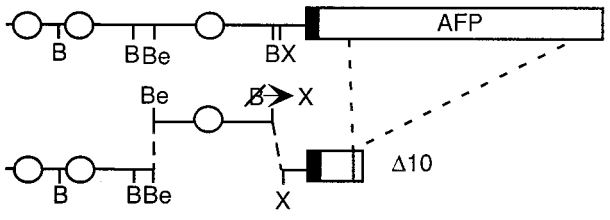

FIG. 1. Structure of the Δ10 transgene. A cartoon of the endogenous AFP gene locus illustrating the positions of the enhancer elements and the promoter along with selected restriction enzyme recognition sites is shown in the upper portion of the figure. Insertion of the 2.5 kb BsteII (Be) to *Bam*HI (B) (at −1.0 kb) via *Xba*I (X) linkers into a plasmid containing 7.6 kb of AFP 5′-flanking sequence digested with BsteII and *Xba*I led to the deletion of the 172-bp sequence between the *Bam*HI and *Xba*I sites. The three AFP enhancers (ovals), the proximal promoter (filled box), and the postnatal repressor sequence downstream of the *Xba*I site were included in the construct. These transcriptional control elements drove the expression of the internally deleted 5-exon AFP MG.

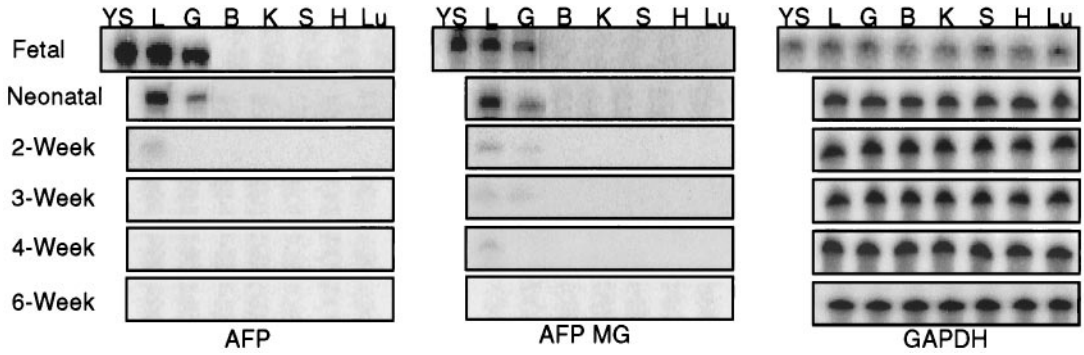

FIG. 2. Tissue-specific and developmental regulation of Δ10 transgene expression. RNase protection assays of RNA isolated from organs of fetal, neonatal, 2-, 3-, 4-, and 6-week-old mice by using probes for AFP or glyceraldehyde-3-phosphate dehydrogenase. Fetal and neonatal samples were pooled from single litters. (AFP, endogenous AFP mRNA; AFP MG, transgene mRNA; Alb, albumin mRNA; YS, yolk sac; L, liver; G, gut; B, brain; K, kidney; S, spleen; H, heart; Lu, lung.)

required for induction of gene expression during liver regeneration, transgenic mice were injected with CCl_4 or were subjected to partial hepatectomy and transgene expression assessed by RNase protection assay. Adult transgenic mice (6 weeks old) were injected with a necrotizing dose of CCl_4 in mineral oil or with mineral oil alone as control. As shown in Fig. 3, mineral oil-injected mice expressed neither the endogenous AFP gene as expected nor the transgene. However, 1 day after liver regeneration was induced, both transgene and endogenous AFP mRNAs were detected. Expression of both reached peak levels 2 days after injection. Transgene expression returned to undetectable levels within 5 days. Partial hepatectomy also was tested as a stimulus for liver regeneration with similar results (data not shown).

Genetic Regulation of Δ10 Transgene Expression in Adult Mice. The level of AFP gene expression in normal adult liver is regulated by the *Afr1* gene. To determine whether the Δ10 transgene was subject to *Afr1* regulation, transgenic mice were mated to BALB/cJ mice for two consecutive generations. The progeny of second generation should be divided equally between *Afr1*Ab and *Afr1*bb expressing low and high levels of AFP, respectively. Transgenic mice (4 weeks old) were killed, and 100 μg of liver RNA was analyzed for the presence of both AFP and AFP MG mRNA. As shown in Fig. 4, mice expressing high and low levels of endogenous AFP mRNA were identified. As shown in Fig. 4*B*, in the 67 mice analyzed from both transgenic lines, mice expressing high levels of endogenous AFP mRNA also expressed high levels of transgene mRNA. Thus, the transgene is *Afr1*-regulated in the same fashion as the endogenous gene.

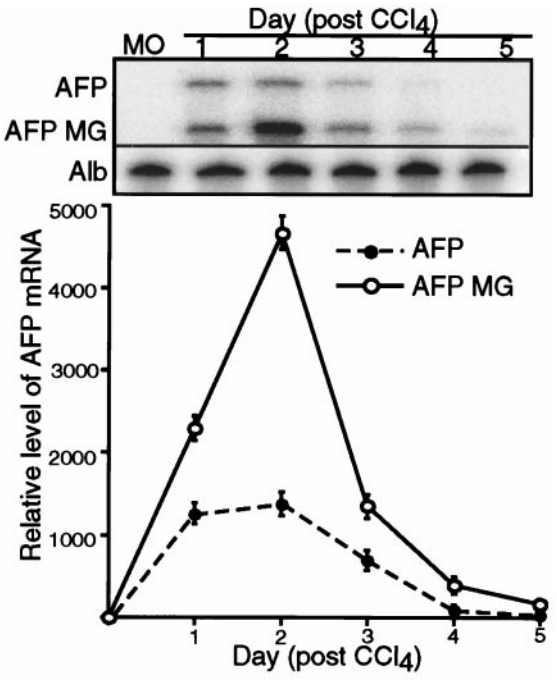

FIG. 3. Expression of the Δ10 transgene during liver regeneration. Liver regeneration was induced in transgenic mice by CCl_4 injection, and the levels of AFP MG and endogenous AFP mRNA were determined by RNase protection assay. The levels of the AFP-related mRNAs were normalized against the level of albumin mRNA and were plotted. (MO, mineral oil; $n = 4$ for each graph point.)

The level of AFP gene expression during liver regeneration is regulated by a second genetic locus, *Afr2*. The *Afr2* gene originally was recognized by the different levels of AFP gene expression during liver regeneration observed between C3H/HeJ and C57BL/6J mice. To determine whether the Δ10 transgene was subject to *Afr2* regulation, transgenic mice from C3H/HeJ matings were mated to B6C3F1 mice. Half of the transgenic progeny should have the *Afr2*AA genotype expressing higher levels of AFP during liver regeneration than the other half, with the *Afr2*AB genotype. Transgenic mice (6 weeks old) were injected with CCl_4 to induce liver regeneration, and the levels of both endogenous AFP and AFP MG mRNA were determined by RNase protection assay. As shown in Fig. 5*A*, mice expressing high and low levels of the endogenous AFP gene were identified readily. However, transgene mRNA was present at similar levels in all treated transgenic mice. Quantitation of the levels of expression for both mRNAs in Fig. 5*B* showed that, although mice 3 through 11 clearly expressed lower levels of endogenous AFP mRNA than mice 12 through 21, no statistically significant differences were observed in transgene expression. All mice tested expressed similar levels of transgene mRNA regardless of the level of endogenous AFP expression, which shows that the Δ10 transgene expression during liver regeneration is not *Afr2* regulated.

DISCUSSION

Using newly derived transgenic mice, we have identified the region of the AFP gene locus, between 1,010 and 838 bp upstream of the transcriptional start site, that is required for *Afr2* regulation of gene expression during liver regeneration. The loss of *Afr2* regulation did not lead to changes in other aspects of gene expression. The transgene was expressed in the appropriate tissues—the yolk sac and the fetal liver and gut—and was repressed properly in all adult tissues. Transgene expression in response to liver regeneration was induced by either CCl_4 or partial hepatectomy. Finally, the genetic regulation of the basal level of AFP gene expression in the normal adult liver of the transgene by the *Afr1* gene also was unaffected by deletion of this sequence.

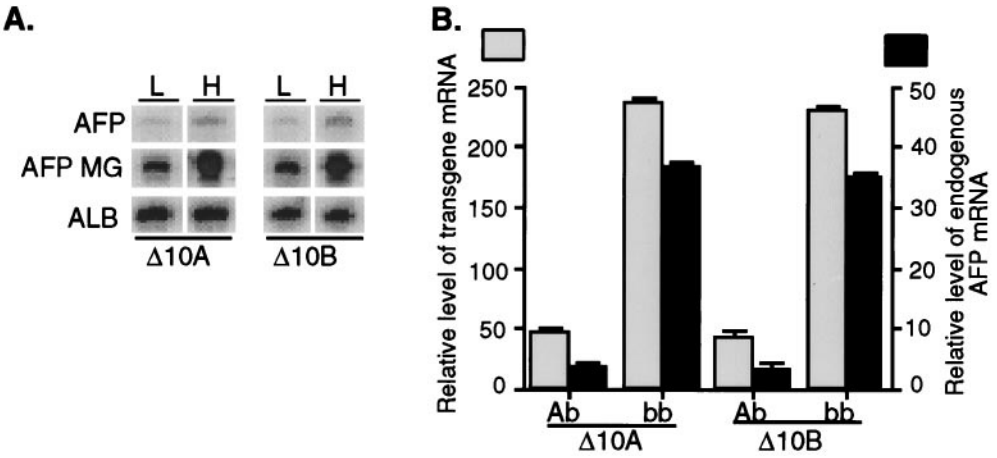

FIG. 4. *Afr1* regulation of Δ10 transgene expression. RNA was isolated from livers of 4-week-old transgenic mice from BALB/cJ matings. (*A*) Typical results of RNase protection assays using 100 μg of total RNA. (*B*) Transgenic mice were grouped by *Afr1* phenotype as determined by the level of endogenous AFP gene expression and the level of AFP MG mRNA plotted. (Δ10A: Ab, $n = 19$; bb $n = 10$; Δ10B: Ab, $n = 21$; bb $n = 17$.)

Polymorphism at the *Afr2* locus correlates with the differential susceptibility to liver tumorigenesis of C3H/HeJ and C57BL/6J mice. C3H/HeJ mice express higher levels of AFP during liver regeneration and are more susceptible to liver tumors than C57BL/6J (35). It has been suggested (36) that hepatocytes in C3H/HeJ mice have a greater proliferative potential than in C57BL/6J because preneoplastic foci in C3H/HeJ grow faster than in C57BL/6J. Another indication of this difference is that partial hepatectomy is an effective tumor promoter in C57BL/6J mice but not in C3H/HeJ, suggesting that the growth stimulus provided by the surgery is present endogenously in C3H/HeJ mice (37). At least six independent chromosomal locations have been associated with the increased genetic susceptibility to liver tumorigenesis (38, 39). *Afr2* may be a gene associated with the strain-specific differences in tumor susceptibility. We have mapped genetically the *Afr2* gene to ≈60 centimorgans distal to the centromere of mouse Chromosome 2 (40). One tumor susceptibility

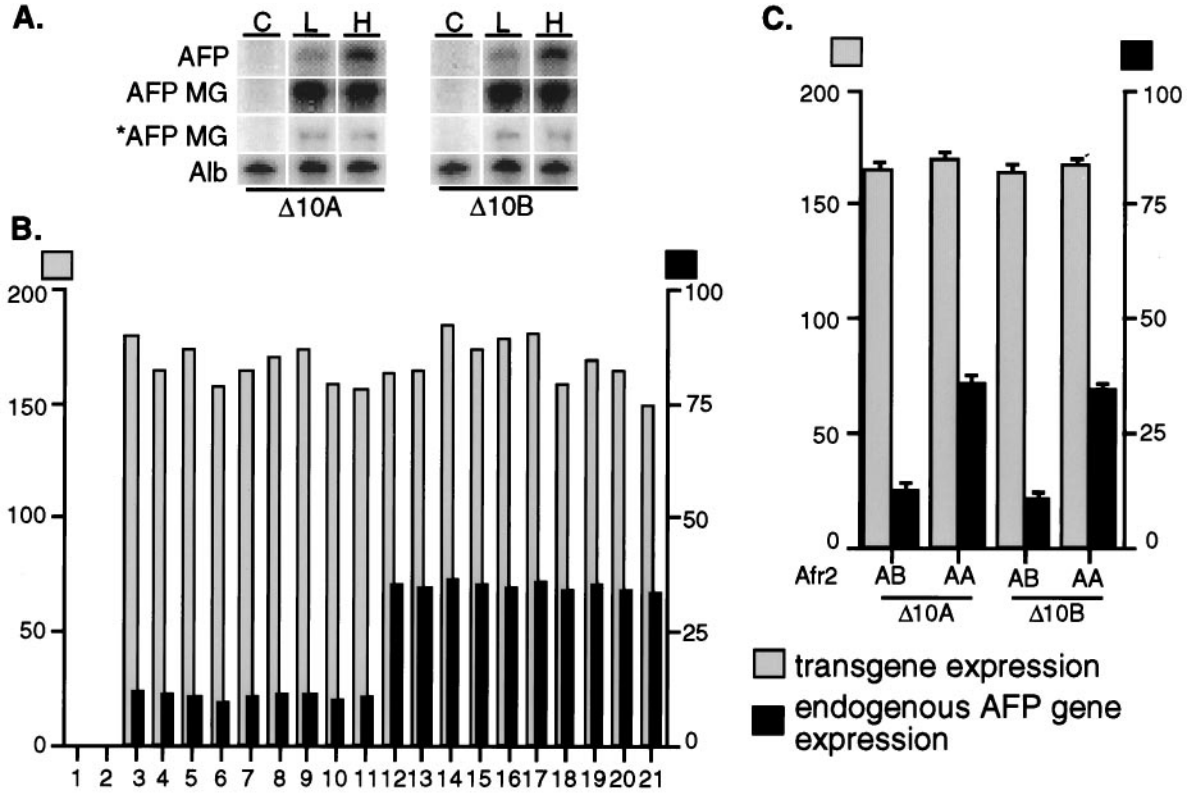

FIG. 5. *Afr2* regulation of Δ10 transgene expression during liver regeneration. Progeny of transgenic mice were mated with B6C3F1 mice and were assayed for the level of AFP and transgene expression after CCl_4 injection. (*A*) Typical RNase protection assays for the two lines of transgenic mice tested. (H and L, high and low levels of endogenous AFP mRNA, respectively; *AFP MG, RNase protection assays carried out with 1 μg of input RNA.) (*B*) Relative levels of endogenous and transgene mRNA in individual Δ10A mice. Mice 1 and 2 were injected with mineral oil, and mice 3 through 21 were injected with CCl_4. (*C*) Summary of the levels of AFP and transgene expression of all of the mice tested in both lines. Δ10A: AA, $n = 10$; AB, $n = 9$; Δ10B: AA, $n = 11$; AB $n = 9$. There were no statistically significant differences in the levels of transgene expression for any of the groups represented ($P < 0.05$).

locus has been mapped to Chromosome 2, but its reported location is 30 centimorgans distal to the *Afr2* gene, making it unlikely to be the *Afr2* gene (39).

One other *Afr2*-regulated gene identified thus far is *H19* (41). The *H19* gene is located on mouse Chromosome 7 in a region of imprinted genes including *Igf2* (42). The *H19* gene encodes an untranslated RNA, which, when the expressed maternal allele is deleted, leads to a loss of the imprinted regulation of *Igf2* gene expression. The increase in *Igf2* expression leads to an increase in cellular proliferation (43, 44). It has been suggested that *H19* may function either directly or indirectly as an antioncogene because over-expression *in vitro* causes growth arrest in cells (45), and the expressed allele has been deleted selectively in liver tumors (46–48). It is unclear, however, what role *Afr2* regulation of *H19* gene expression plays in the regulation of growth.

Afr2 regulation is highly specific for liver regeneration in adult mice. Strain-dependent differences in AFP and *H19* gene expression are not observed in fetal liver; therefore, *Afr2* regulation is not simply a phenomenon associated with hepatocyte growth (4). In addition, *Afr2* regulation is liver-specific because *H19* gene expression in muscle is not subject to *Afr2* regulation (40).

Although nothing is known about the function of the *Afr2* gene product, its activity must be transmitted through the AFP sequence deleted from the Δ10 transgene. The *Afr2* gene product could either enhance or repress AFP gene expression. The dependence on and the ability of the AFP 5′-flanking sequence to confer *Afr2* regulation on an unrelated reporter gene (23, 24) suggest that *Afr2* regulation occurs at the level of transcription. It is unlikely that the *Afr2* phenotype results from a difference in the level of a DNA-binding protein (transcription factor) between C3H/HeJ and C57BL/6J mice as transgenes present in high copy number are still subject to *Afr2* regulation (30). The codominance of the *Afr2* alleles also makes it unlikely that the allelic difference in the *Afr2* gene product leads to a difference in the DNA-binding affinity. However, the *Afr2* gene product could encode a transcription factor in which the allelic difference leads to a difference in transcriptional activation or association with other proteins involved in transcription. It is just as likely that *Afr2* could encode an activity that acts indirectly in a cascade of events modifying transcriptional activity.

The sequence between 1,010 and 838 bp upstream of the AFP transcriptional start site was analyzed for known transcription factor binding sites and for homologies with the *H19* sequence. Located between 860 and 840 bp upstream of the AFP transcriptional start site are sequences that have been shown to be recognized by p53 (49) and hepatocyte nuclear factor 3 (50). There are also consensus binding sites for two transcription factors involved in the cell cycle-specific regulation of the histone genes *HiNF-A* and *HiNF-D* (51). Neither p53 nor the hepatocyte nuclear factor 3 isoforms could be the *Afr2* gene product because they have been mapped genetically to regions of the genome other than the *Afr2* locus (40, 52). However, the *Afr2* gene product could participate in a pathway regulating the activity of p53 or hepatocyte nuclear factor 3. Because, to our knowledge, the genetic map positions of *HiNF-A* and *-D* have yet to be reported, it is possible that *Afr2* regulation may stem from an allelic difference in activity of either of these factors.

Comparison of the available *H19* sequence, including 5.6 kb of 5′-flanking sequence (53), the entire coding region of the gene (54) and the 3′ enhancer elements (55) with the *Afr2* responsive sequence identified regions of homology, as shown in Fig. 6. Two of the putative transcription factors binding sites in the AFP locus, *HiNF-D* and p53, show homology with *H19* sequences. Both the AFP sequence and *H19* enhancer 1, located 3′ of the structural gene, contain p53 recognition sites. There are several *H19* sequences that show homology to the

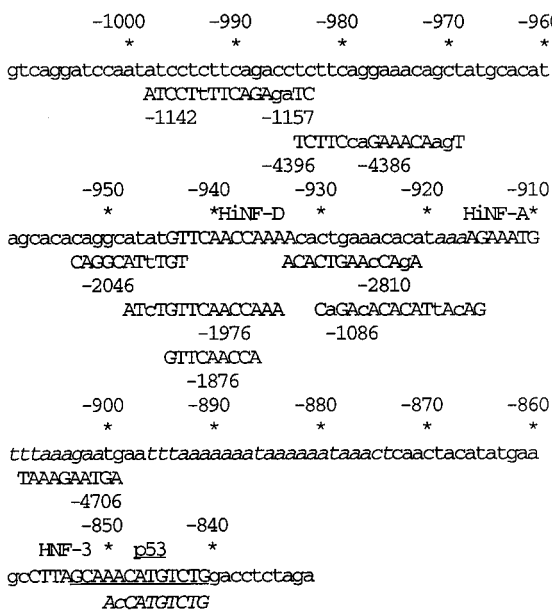

FIG. 6. Analysis of sequence required for *Afr2* regulation of transgene expression. AFP 5′-flanking sequence is listed in lowercase letters, with the position relative to the start of transcription indicated above. The repetitive DNA element is indicated by italics. Sequences homologous to known transcription factor binding sites are shown in capital letters, with the factor identified above. The p53 binding site that overlaps with the hepatocyte nuclear factor 3 binding site is underlined. H19 homologous sequences are shown below their AFP counterparts, with their position relative to the H19 transcriptional start site indicated. Homology between H19 enhancer 1 and AFP is indicated in upper case italics.

AFP sequence lying between the *HiNF-D* and *HiNF-A* sites. Computer analysis also identified a region located between −981 and −992 bp, where 11 of 12 bases are identical to *H19* sequence located on the opposite strand between −1,142 and −1,153 bp. The only difference is a pyrimidine transition, C in AFP and T in *H19*.

We have shown that *Afr2* regulation of AFP gene expression during liver regeneration depends on the AFP 5′-flanking sequence between 1,010 and 838 bp upstream of the transcriptional start site. The loss of *Afr2* regulation did not change other aspects of AFP tissue-specific, developmental, or pathological regulation. Of interest, transgene expression during liver regeneration was retained, suggesting that another mechanism, most likely requiring the sequence within 118 bp of the AFP mRNA cap site, mediated induction. These two mechanisms may work in concert to determine the final level of AFP gene expression.

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