MOLECULAR AND CELLULAR BIOLOGY, Oct. 1994, p. 6497–6505
0270-7306/94/$04.00+0

Vol. 14, No. 10

Mouse α-Fetoprotein Gene 5′ Regulatory Elements Are Required for Postnatal Regulation by *raf* and *Rif*

BRETT T. SPEAR*

Departments of Microbiology & Immunology and Pathology, University of Kentucky College of Medicine, Lexington, Kentucky 40536-0084

Received 14 April 1994/Returned for modification 25 May 1994/Accepted 29 June 1994

The mouse α-fetoprotein (AFP) gene is expressed at high levels in the yolk sac and fetal liver and at low levels in the fetal gut. AFP synthesis decreases dramatically shortly after birth to low levels that are maintained in the adult liver and gut. AFP expression can be reactivated in the adult liver upon renewed cell proliferation such as during liver regeneration or in hepatocellular carcinomas. Previously, two unlinked genetic loci that modulate postnatal AFP levels were identified. The *raf* locus controls, at least in part, basal steady-state AFP mRNA levels in adult liver. *Rif* influences the extent of AFP mRNA induction during liver regeneration. Transgenic mice were used to examine the role of 5′ AFP regulatory regions in *raf*- and *Rif*-mediated control. A fragment of the AFP 5′ region containing enhancer element I, the repressor, and the promoter was linked to the mouse class I *H-2D*^{d} structural gene. We demonstrate that this hybrid AFP-D^d transgene is expressed in the appropriate tissues. In addition, it is postnatally repressed and reactivated during liver regeneration in parallel with the endogenous AFP gene. Therefore, proper transcriptional control does not require the AFP structural gene. Furthermore, the AFP 5′ control region is sufficient to confer *raf* and *Rif* responsiveness to the linked *H-2D*^{d} structural gene, suggesting that *raf* and *Rif* act at the level of transcriptional initiation.

The proper development and function of mammalian organs require that specific genes be expressed in a temporally regulated and tissue-specific manner. Such control is often at the level of transcriptional initiation; the interaction of *trans*-acting transcription factors with *cis*-acting DNA elements serves to modulate the transcription rate of a gene. Our understanding of these factors and the control regions to which they bind has come primarily from DNA transfections into cultured cell lines and in vitro DNA-protein binding studies (23). These techniques, while powerful, are limited to studying events that can be monitored in vitro. Developmental changes in gene regulation requiring complex signaling pathways and/or cell-cell interactions that can be achieved only in an intact organism may not be amenable to in vitro analysis. Examples of such developmental regulation are seen in the postnatal control of the mouse α-fetoprotein (AFP) gene. AFP is expressed in a tissue-specific manner, being found at high levels in the visceral endoderm of the yolk sac and fetal liver and at low levels in the fetal gut (34). A dramatic postnatal decline in AFP levels in the liver and gut is seen; this decrease in the liver is controlled, at least in part, at the transcriptional level (4). However, repression is reversible, because AFP can be reactivated in the adult liver during periods of renewed cell proliferation such as during liver regeneration, induced experimentally with the hepatotoxin carbon tetrachloride, or in hepatocellular carcinomas (1).

Two unlinked genetic loci that modulate postnatal AFP levels have been identified. These loci probably encode *trans*-acting factors required for stage-specific gene regulation. The *raf* locus controls, at least in part, basal steady-state AFP mRNA levels in adult liver (4). The *raf* trait was initially observed in BALB/cJ mice (22, 28), which have 15- to 20-fold-higher AFP mRNA levels in adult liver than other mouse strains do. Two *raf* alleles exist; *raf*^{b} is found in the BALB/cJ strain and is recessive to the *raf*^{a} allele, which is found in all other mouse strains. The *raf* gene has been mapped to chromosome 15, 2 to 3 centimorgans proximal to the c-*myc* gene (7). A second locus, *Rif*, was originally defined by the presence of strain-specific differences in the level of steady-state AFP mRNA levels that are induced during liver regeneration (4). Mice of the C57BL/6 strain produce roughly 10-fold less AFP mRNA during liver regeneration than C3H mice do (1). The *Rif*^{b} allele found in C57BL/6 mice is dominant over the "wild-type" *Rif*^{a} allele present in C3H and other strains of mice (4). The activity of *raf* and *Rif* is not limited to AFP regulation; mRNA levels of a second gene, H19, in the adult liver are also controlled by *raf* and *Rif* (29). Understanding the basis of *raf* and *Rif* regulation should help elucidate regulatory mechanisms that act during mammalian development.

Experiments with transgenic mice, in addition to confirming the activity of *cis*-acting control elements that had been defined in transient-transfections assays (15, 16, 40), have allowed us to begin to characterize the basis for developmental changes in AFP expression. Transgenes containing 7.6 kb of DNA upstream from the 5′ end of the AFP gene linked to a five-exon AFP minigene were correctly regulated; the transgenes were expressed in a tissue-restricted manner, shut off at birth, and reactivated during liver regeneration (24). However, the AFP promoter alone, which is functionally active when transfected into cultured hepatoma cells, was inactive in transgenic mice (18), demonstrating that the upstream enhancers are required for the developmental activation of AFP. Also, each of the three AFP enhancers individually were active in the three AFP-permissive tissues, the yolk sac, fetal liver, and fetal gut, although to different levels (18). Transgenic experiments have also demonstrated that the postnatal repression of AFP is not due to a decrease in enhancer activity (8) but, rather, is controlled by a repressor region located between the AFP promoter and upstream enhancers (39).

Previous studies of AFP in transgenic mice were performed

* Mailing address: Department of Microbiology & Immunology, University of Kentucky College of Medicine, 800 Rose St., Lexington, KY 40536-0084. Phone: (606) 257-5167. Fax: (606) 257-8994. Electronic mail address: spearb@uklans.uky.edu.

with various fragments of AFP 5′-flanking DNA linked to a 5-exon minigene consisting of the first 3 and last 2 exons of the 15-exon AFP gene (8, 18, 24, 38, 39). These studies cannot eliminate the possibility that regions within the AFP minigene contribute to AFP regulation. In fact, nuclear run-on studies suggested that *raf* functions at the posttranscriptional level and would thus imply that AFP structural gene sequences are involved (38). We wished to explore further the role of regulatory elements flanking the 5′ end of the AFP gene in the absence of the five-exon AFP minigene, particularly in regard to postnatal *raf*- and *Rif*-mediated control. To accomplish this, we have linked a portion of the AFP 5′ region containing enhancer element I, the repressor, and the promoter, to the mouse major histocompatibility complex (MHC) class I *H-2D*^{d} structural gene. Linking AFP regulatory domains to a heterologous gene body will distinguish regulation mediated through the 5′ control region from regulation via elements within the five-exon AFP minigene. Regulation through 5′ control elements should be seen with the AFP-D^d hybrid gene, whereas regulation mediated by regions within the AFP minigene, either transcriptional via the interaction of *trans*-acting factors with intragenic DNA sequences or posttranscriptional via RNA elongation, processing, or stability, should not be observed in the AFP-D^d hybrid gene. We demonstrate that the AFP-D^d transgene is expressed in the appropriate tissues and is postnatally repressed and reactivated during liver regeneration in parallel with the endogenous AFP gene. Therefore, proper transcriptional control does not require the AFP structural gene. In addition, the AFP 5′ control region is sufficient to confer *raf* and *Rif* responsiveness to the linked *H-2D*^{d} structural gene. These results are most consistent with the conclusion that *raf* and *Rif* act at the level of transcriptional initiation. We propose a model of *raf* regulation that couples transcriptional to posttranscriptional control, thus incorporating our results and those of Vacher et al. (38).

MATERIALS AND METHODS

Plasmids. The plasmid AFP(3.8)-D^d was derived from AFP(7.6)-D^d, which contains the three distinct AFP enhancer elements, the repressor, and the promoter (32). A 5.5-kb *Bam*HI-*Eco*RI fragment of AFP(7.6)-D^d, extending from a *Bam*HI site at −1.0 kb of the AFP gene (relative to the AFP cap site) to an *Eco*RI site at the 3′ end of the *H-2D*^{d} structural gene, was inserted into a modified pUC9 vector cut with *Bgl*II and *Eco*RI. This produced AFP(1.0)-D^d, which contains the AFP repressor and promoter. A 2.8-kb *Bam*HI fragment containing AFP enhancer element I (15) was inserted in its normal orientation into the *Bam*HI site 5′ of the AFP promoter in AFP(1.0)-D^d. The resulting vector, AFP(3.8)-D^d, contains the AFP enhancer element I, repressor, and promoter. This hybrid gene contains 25 bp of AFP sequence downstream of the AFP transcription start site; therefore, the AFP-D^d transcript contains a small segment of AFP sequence.

Production and identification of transgenic animals. To prepare DNA for microinjection, the AFP(3.8)-D^d hybrid gene was released from vector sequences by digestion with *Bst*EII (the *Bst*EII site is at the 5′ end of enhancer I) and *Eco*RI. The resulting 8.0-kb fragment was purified by agarose gel electrophoresis and CsCl ultracentrifugation as described previously (20). The quality and quantity of DNA were monitored by agarose gel electrophoresis, and the purified AFP(3.8)-D^d fragment was diluted to a final concentration of 5 ng/μl in phosphate-buffered saline. F_2 hybrid embryos from C57BL/6 × C3H parents were given microinjections as described by Hogan et al. (20) and transferred to pseudopregnant ICR/HSD females. All procedures were performed at the University of Kentucky Transgenic Mouse Facility. Two weeks after birth, progeny were screened for the presence of the transgene in tail DNA by using Southern blot analysis (31). The DNA was digested with *Hin*dIII and probed with an enhancer I fragment that could distinguish the transgene from the endogenous AFP gene. Three founder mice, termed E.1, E.2, and E.3, were identified.

Analysis of mRNA. Total RNA was prepared from mouse tissues by the lithium chloride procedure as described previously (2) or by using RNazol B (Tel-Test, Inc., Friendswood, Tex.) as specified by the manufacturer. The quality of the RNA samples was monitored by formaldehyde gel electrophoresis and ethidium bromide staining.

The RNAs were analyzed by RNase protection assays essentially as described previously (25). Briefly, 50 to 100 μg of total RNA and 10^5 cpm of radiolabeled RNA probe 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 amount of 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 (AFP-D^d probe). Digestions with RNase A (18 μg per sample; Sigma Biochemicals) and RNase T_1 (270 U per sample; GIBCO/BRL) were performed with 330-μl volumes 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 per sample; GIBCO/BRL) and sodium dodecyl sulfate (20 μl of a 10% solution) were added to each sample, and the samples were given a 30-min incubation at 37°C. The samples were extracted with phenol-chloroform-isoamyl alcohol (50:48:2) and ethanol precipitated. Dried pellets were suspended in 80% formamide–Tris-borate-EDTA (TBE) buffer–0.1% bromophenol blue–xylene cylanol, 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. To quantitate the radioactivity in a particular band, we also analyzed dried gels by using a radioanalytic imaging system (Ambis, San Diego, Calif.).

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− (Strategene). The AFP-D^d RNase probe was prepared by inserting a 695-bp fragment of the AFP(3.8)-D^d hybrid gene (from a *Pvu*II site at −610 of AFP 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.

***raf* regulation.** For *raf* studies, transgenic male E.1, E.2, and E.3 mice (*raf*^{a}) were crossed to BALB/cJ females (*raf*^{b}; obtained from Jackson Laboratory, Bar Harbor, Maine). The resulting male offspring were screened for the presence of the transgene. These males (*raf*$^{a/b}$) were backcrossed to BALB/cJ females. Transgenic animals from this mating were identified by Southern analysis and sacrificed at 4 weeks of age, and the liver and gut RNA was analyzed by RNase protection. The *raf* genotype of these animals was determined by analyzing AFP mRNA levels in the liver. Theoretically, 50% of these offspring should be transgenic; half of the transgenic mice should be *raf*$^{a/b}$, and half should be *raf*^{b}.

***Rif* regulation.** For liver regeneration studies, adult (2 to 3 months old) transgenic animals were given intraperitoneal injections of 50 μl of mineral oil containing 10% carbon

tetrachloride (CCl_4). Control animals were given injections of mineral oil alone. Three days after treatment, animals were sacrificed and liver and gut RNA was prepared. For *Rif* studies, $Rif^{a/b}$ E.2 male mice were identified. These mice were crossed to nontransgenic C57BL/6 × C3H ($Rif^{a/b}$) females. The resulting transgenic offspring were detected by Southern analysis. At 6 weeks of age, animals were treated with CCl_4 as described above. The animals were sacrificed 3 days after treatment, and liver mRNA was prepared. Levels of AFP and AFP-D^d were determined by RNase protection. Theoretically, 25% of the transgenic animals should have high AFP levels (Rif^a) and 75% should have low AFP levels ($Rif^{a/b}$ or Rif^b) during liver regeneration.

RESULTS

Production of transgenic animals. Original efforts to produce transgenic mice were performed with AFP(7.6)-D^d. This hybrid gene contains the entire AFP 5′ control region, including all three AFP enhancer elements, fused to a 4.5-kb genomic DNA fragment encoding the mouse MHC *H-2D*^{d} class I structural gene (9, 32). Despite repeated attempts, we were unable to obtain founder animals harboring this construct. We speculate that inappropriate D^d protein levels early in development, as a result of the combined activity of the three AFP enhancers, may result in embryo death. This notion is supported by studies showing that ES cell-derived embryos expressing D^d from the β-actin promoter die when differentiated in vivo (21). We reasoned that limiting the AFP control region to a single enhancer would reduce transgene expression early in development and allow normal embryonic maturation. To accomplish this, the hybrid gene AFP(3.8)-D^d was generated by linking a 3.8-kb fragment of DNA that flanks the 5′ end of the mouse AFP gene body to the *H-2D*^{d} structural gene. The 3.8-kb fragment contains enhancer element I, repressor, and promoter of the AFP gene. Previous studies have shown that this 3.8-kb fragment is sufficient to direct the appropriate expression of a linked five-exon AFP minigene, including postnatal repression and reactivation during liver regeneration (18). The tissue specificity of AFP(3.8)-D^d was initially tested by transient transfections into cultured cell lines. The hybrid gene was expressed at high levels and correctly initiated in AFP-permissive HepG2 human hepatoma cells but was not active in HeLa cells (data not shown). Immunofluorescence staining with an H-2D^d-specific monoclonal antibody (32) demonstrated high D^d protein levels on the surface of transfected cells, indicating that the reporter gene mRNA is efficiently translated (data not shown).

Purified AFP(3.8)-D^d DNA was microinjected into the F_2 hybrid embryos from C57BL/6 × C3H parents. Three founder animals, E.1, E.2, and E.3, were identified by Southern analysis of tail DNA obtained from biopsy specimens. Each founder contained 5 to 10 copies of the injected transgene, and all three founder mice transmitted the transgene to offspring in a Mendelian manner, suggesting that the transgenes integrated into a single chromosomal site.

Transgene expression in fetal and neonatal animals. AFP is normally expressed at high levels in the yolk sac and fetal liver and at low levels in the fetal gut. To determine whether AFP(3.8)-D^d showed the same pattern of expression as the endogenous AFP gene, RNA was prepared from the yolk sac, liver, and gut of embryonic day 18 animals and a variety of tissues from 1-day-old pups. An RNase protection assay was used to measure steady-state transcript levels. As shown in Fig. 1, the AFP(3.8)-D^d transgene was expressed at high levels in all three fetal tissues in E.1, E.2, and E.3 mice. Transgene

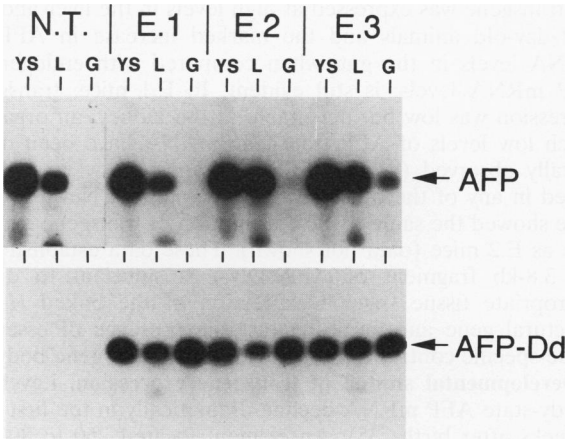

FIG. 1. Expression of AFP-D^d in the three AFP-permissive fetal tissues. Transgenic E.1, E.2, and E.3 mice were mated, and the fetuses and yolk sacs were collected at embryonic day 18. Transgenic fetuses were identified by analyzing tail DNA. Total RNA was prepared from the yolk sacs (YS), livers (L), and guts (G). RNase protection analysis was performed with 5 μg of RNA from nontransgenic (N.T.), E.1, E.2, and E.3 mice. The arrow in the top panel indicates the protected AFP probe (AFP; 55 nucleotides), whereas the arrow in the bottom panel indicates the protected transgene probe (AFP-D^d; 90 nucleotides).

expression (lower panel) in the yolk sac and liver was similar to that of the endogenous AFP gene (upper panel). However, levels of transgene mRNA in the gut were significantly higher than those of the endogenous gene; this phenomenon also has been observed with AFP minigene constructs (8, 18). The size of the protected RNA probe was 90 nucleotides, indicating that hybrid gene transcripts correctly initiated at the AFP cap site. That the AFP-D^d RNase probe specifically hybridizes to the transgene mRNA is confirmed by the lack of hybridization to RNA from nontransgenic tissues.

A broader range of tissues from newborn pups was examined for AFP-D^d expression (Fig. 2). In the E.1 and E.2 lines,

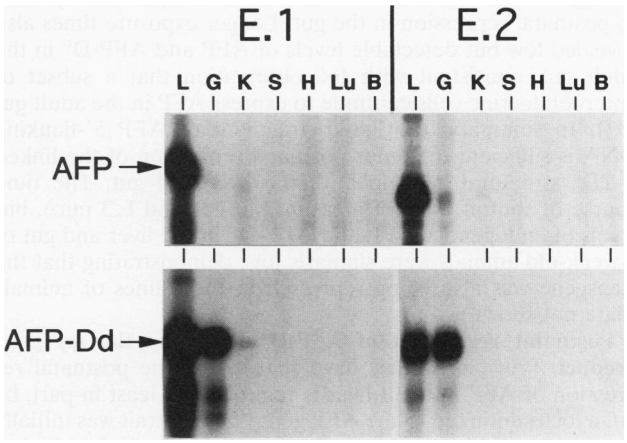

FIG. 2. Expression of AFP-D^d in newborn mice. Transgenic E.1 and E.2 mice were mated, and the resulting pups were sacrificed 1 day after birth. Total RNA was prepared from the liver (L), gut (G), kidney (K), spleen (S), heart (H), lung (Lu), and brain (B) of transgenic pups. RNase protection analysis was performed with 5 μg of RNA. The arrow in the top panel indicates the protected AFP probe (AFP), whereas the arrow in the bottom indicates the transgene (AFP-D^d) probe.

the transgene was expressed at high levels in the liver and gut of 1-day-old animals and the marked increase in AFP-D^d mRNA levels in the gut, when compared with endogenous AFP mRNA levels, is still evident. In E.1 mice, transgene expression was low but detectable in the kidney, an organ in which low levels of AFP transgene mRNA have been occasionally observed (8, 18). AFP-D^d transcripts were not detected in any of the other day 1 tissues tested. Newborn E.3 mice showed the same tissue distribution of transgene expression as E.2 mice (data not shown). These data establish that the 3.8-kb fragment of AFP DNA is sufficient to direct appropriate tissue specific expression of the linked *H-2D^d* structural gene and argue against the presence of essential tissue-specific control elements within the AFP gene body.

Developmental shutoff of transgene expression. Levels of steady-state AFP mRNA decline dramatically in the first 2 to 4 weeks after birth (35). An element located 250 to 800 bp upstream of the AFP transcription start site is required for this repression (39). To ask whether the AFP(3.8)-D^d transgene was also developmentally repressed, we prepared RNA from the liver and gut of E.2 animals at several perinatal time points and assayed it by RNase protection (Fig. 3). Shut-off of AFP(3.8)-D^d in the liver parallels that seen for the endogenous AFP gene (Fig. 3A). Reduced levels of AFP and AFP-D^d mRNAs were first seen in the liver of 3-day-old pups (lane 3), indicating that the repression of both genes initiated at roughly the same time. There was a lag in the rate of silencing of the transgene when compared with AFP, which is most evident at postnatal day 14, but repression of both genes was complete by 3 weeks of age (lane 6). Longer exposure times showed low but detectable levels of both AFP and AFP-D^d transcripts in the livers of adult mice (data not shown), supporting studies demonstrating that repression in the liver was not absolute (6).

The AFP(3.8)-D^d transgene was also postnatally repressed in the gut of E.2 animals (Fig. 3B). In contrast to what was seen in the liver, the decline in transgene mRNA levels begins several days after repression of the endogenous AFP gene is first observed. Repression of AFP was first seen in 1-day old animals, whereas transgene repression was first evident in 7-day-old pups. It is not known whether the delay in transgene repression is related to the high levels of its expression in the fetal gut or whether it reflects different gene-specific responses to postnatal repression in the gut. Longer exposure times also revealed low but detectable levels of AFP and AFP-D^d in the adult gut, consistent with the observation that a subset of enteroendocrine cells continue to express AFP in the adult gut (37). In summary, the 3.8-kb fragment of AFP 5′-flanking DNA is sufficient to confer postnatal repression of the linked *H-2D^d* structural gene in both the liver and gut. The time course of shutoff was not measured in E.1 and E.3 mice, but levels of endogenous AFP and AFP-D^d in the liver and gut of 4-week-old animals were similarly low, demonstrating that the transgene was also being repressed in these lines of animals (data not shown).

Postnatal regulation of AFP-D^d levels by the *raf* gene product. Previous studies have shown that the postnatal repression of AFP in adult liver is regulated, at least in part, by *raf*, a locus unlinked to the AFP gene. The *raf* trait was initially observed in BALB/cJ mice, which have 15- to 20-fold-higher levels of adult liver AFP mRNA than other mouse strains do (22, 28). To further understand how *raf* controls AFP levels, we sought to determine whether the AFP(3.8)-D^d transgene is responsive to *raf* regulation. Transgenic mice from all three lines, which are homozygous for the *raf^a* allele, were crossed to BALB/cJ mice (*raf^b*). Transgene-positive pups were crossed back to BALB/cJ mice. Fifty percent of the offspring from this mating should contain the transgene; half of these transgenic mice should carry *raf^b* and have high adult AFP levels, and half should carry *raf$^{a/b}$* and have low adult AFP levels. The transgenic progeny of this second cross were sacrificed at 28 days of age, RNA was prepared, and levels of AFP and AFP-D^d mRNA were compared (Fig. 4). The endogenous AFP mRNA levels in adult liver allowed us to identify the *raf* genotype of each mouse. As expected, two patterns were seen: low AFP, which is barely detectable (Fig. 4A, lanes 1, 7, 10, 11, and 14), and high AFP (lanes 2 to 6, 8, 9, 12, and 13). These are labeled L (low) and H (high), respectively, below Fig. 4A. Expression of AFP(3.8)-D^d also showed a high and low pattern which correlated exactly with the assigned *raf* genotype (Fig. 4B). Cross-hybridization of the AFP-D^d RNase probe to endogenous MHC class I transcripts (MHC arrow in Fig. 4B) provided an internal standard to confirm that equal amounts of RNA have been used in each lane. Since transgene levels in livers of offspring from all three founder mice were modulated by the *raf* genotype, the *cis*-acting elements required for *raf* must be located within the AFP(3.8)-D^d hybrid gene.

Previous studies demonstrated that *raf* activity is restricted

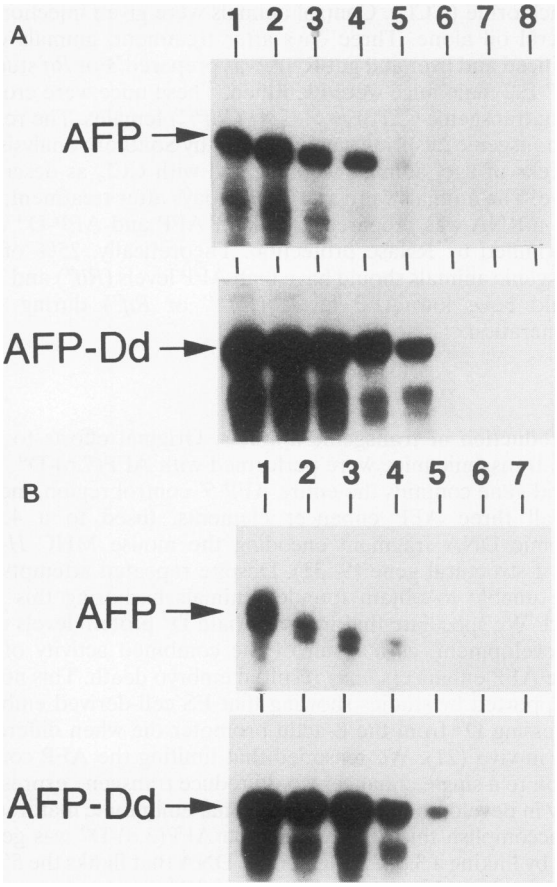

FIG. 3. Developmental shutoff of AFP-D^d in transgenic mice. Total RNA was prepared from the liver and gut of transgenic E.2 mice at various perinatal times. RNase protection analysis was performed with 5 μg of tissue RNA. (A) Time points with liver RNA: fetal (embryonic day 18); postnatal days 1, 3, 7, 14, 21, and 28; and 3 months (lanes 1 to 8, respectively). Top panel, AFP; bottom panel, AFP-D^d. (B) Time points with gut RNA: fetal (embryonic day 18); postnatal days 1, 3, 7, 14, and 21; and 3 months (lanes 1 to 7, respectively). Top panel, AFP; bottom panel, AFP-D^d.

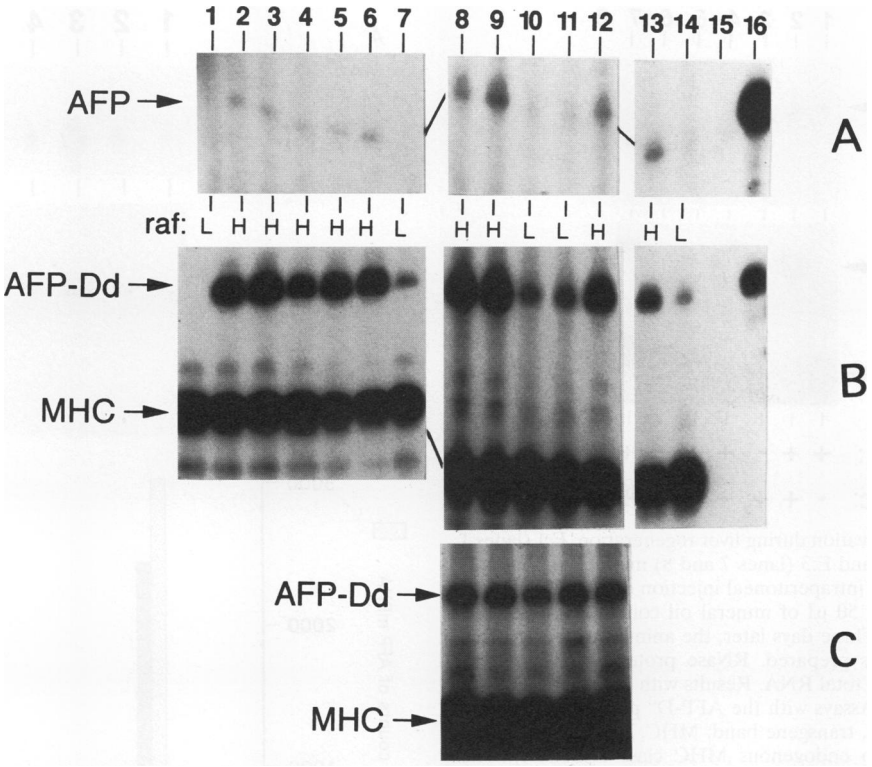

FIG. 4. Regulation of AFP-D^d levels by *raf*. Transgenic mice were crossed twice to BALB/cJ animals, and the transgenic (lanes 2 to 14) and nontransgenic (lane 1) offspring of the second mating were sacrificed at 4 weeks of age. Each lane (1 to 14) corresponds to a different animal; lanes 1 to 7 are from E.1, lanes 8 to 12 are from E.2, and lanes 13 and 14 are from E.3. RNase protection assays were performed with 100 μg of total RNA prepared from the liver or gut. (A) Lanes 1 to 14 show AFP levels in the liver. Lane 15 and 16 contain negative (tRNA) and positive (5 μg of fetal liver mRNA) controls, respectively. The *raf* genotype of each animal, high (H) or low (L) as judged by steady-state AFP mRNA levels, is designated below each lane. (B) Lanes 1 to 14 show AFP-D^d levels in the liver. The 49 nucleotides of the D^d portion of the AFP-D^d riboprobe cross-hybridizes to endogenous MHC class I mRNA (MHC arrow) and serves as an internal control for RNA loading. Lane 15 contains tRNA. Lane 16 contains RNA from AFP(3.8)-D^d-transfected human HepG2 cells. (C) AFP-D^d levels in the gut of the five E.2 animals (lanes 8 to 12).

to the liver since AFP levels in the gut (37) and H19 levels in the gut and muscle (29) were not influenced by the *raf* genotype. We wished to determine whether AFP-D^d levels in the gut were also independent of *raf*. Therefore, we analyzed transgene levels in the gut of E.2 animals whose *raf* phenotype in the liver had been previously determined (Fig. 4C, lanes 8 to 12). Although three of these animals had high AFP-D^d levels (lanes 8, 9, and 12) and two had low AFP-D^d levels (lanes 10 and 11) in the liver, no difference in AFP-D^d mRNA levels was observed in the gut RNA from these mice.

Induction of transgene expression during liver regeneration. The AFP gene is transiently reactivated in adult mice during liver regeneration. To determine whether this was due to an increase in transcription or a change in mRNA stability, we tested whether AFP-D^d levels would be increased during liver regeneration. Transgenic mice were given injections of mineral oil containing CCl_4, an acute hepatotoxin that leads to cell death and subsequent cellular regeneration; control mice were injected with mineral oil alone. The animals were sacrificed 3 days after treatment, a time when AFP mRNA levels are maximal (4), and RNA was prepared and assayed by RNase protection. As a control for the efficacy of CCl_4 treatment, we measured endogenous AFP gene activation. As seen in Fig. 5 (top panel), AFP levels increased in the five CCl_4-treated animals (lanes 1, 2, 4, 5, and 7) compared with those treated only with mineral oil (lanes 3, 6, and 8). Levels of AFP-D^d mRNA also increased in animals that were treated with CCl_4 compared with untreated animals, thus demonstrating activation of the transgene. Cross-hybridization of the AFP-D^d probe to endogenous MHC class I transcripts (MHC arrows in Fig. 5), which are not altered by CCl_4 treatment, confirmed that equal amounts of RNA had been analyzed in each lane. The basal levels of AFP and AFP-D^d mRNA in the gut of CCl_4-treated and control animals remained the same, indicating that reactivation is restricted to the liver during regeneration (data not shown).

Regulation of AFP-D^d induction by the *Rif* gene product. AFP reactivation is controlled by the *Rif* locus, which was originally defined by strain-specific differences in the extent of AFP induction during liver regeneration (1, 4). C57BL/6 mice produce roughly 10-fold less AFP mRNA after CCl_4 treatment than do C3H mice. The *Rif^b* allele found in C57BL/6 mice is dominant over the "normal" *Rif^a* allele present in C3H and other strains of mice. We set up the appropriate genetic crosses to determine whether AFP(3.8)-D^d was *Rif* responsive.

To accomplish this, male E.2 *Rif$^{a/b}$* mice were bred to nontransgenic C57BL/6 × C3H (*Rif$^{a/b}$*) females and nine transgenic offspring were identified. At 6 weeks of age, eight of these mice were treated with CCl_4 and sacrificed after 3 days; liver RNA was prepared for RNase protection. AFP mRNA was induced in all eight CCl_4-treated mice (Fig. 6A, lanes 1 to 8) when compared with the control littermate treated only with mineral oil (lane 9). Two of these eight mice (Fig. 6A, lanes 3 and 7) showed AFP levels that were three- to fourfold higher

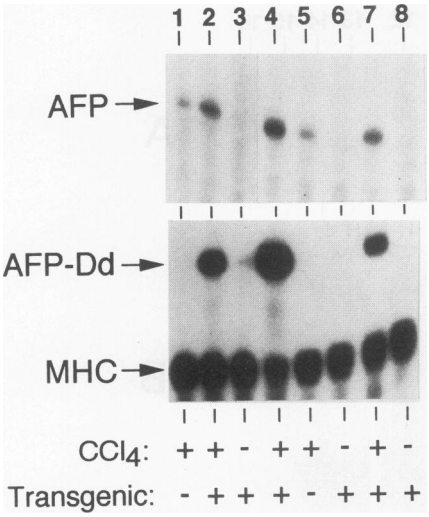

FIG. 5. AFP-D^d reactivation during liver regeneration. E.1 (lanes 1 to 3), E.2 (lanes 4 to 6), and E.3 (lanes 7 and 8) mice (2 to 3 months old) were treated with an intraperitoneal injection of 50 μl of mineral oil (lanes 3, 6, and 8) or 50 μl of mineral oil containing 10% CCl_4 (lanes 1, 2, 4, 5, and 7). Three days later, the animals were sacrificed and total liver RNA was prepared. RNase protection assays were performed with 100 μg of total RNA. Results with the AFP probe are shown in the top panel. Assays with the AFP-D^d probe are shown in the lower panel (AFP-D^d, transgene band; MHC, cross-hybridization of the AFP-D^d probe to endogenous MHC class I mRNA). The transgenic and treatment status of each mouse is denoted at the bottom of the figure.

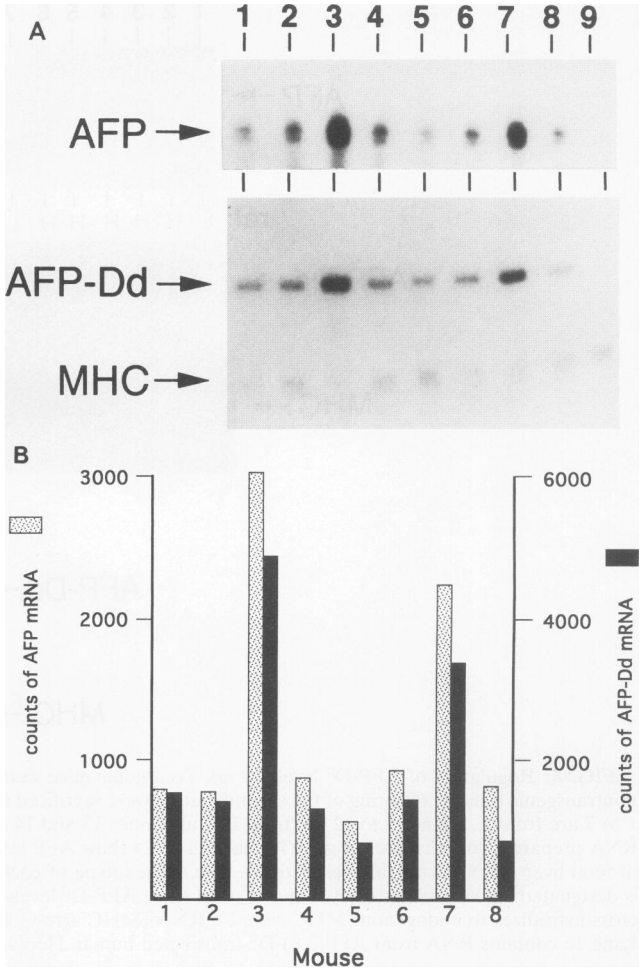

FIG. 6. Regulation of AFP-D^d by *Rif* during liver regeneration. Transgenic *Rif*$^{a/b}$ male E.2 mice were mated to nontransgenic *Rif*$^{a/b}$ female mice. At 6 weeks of age, eight of the nine transgenic offspring mice (lanes 1 to 8) were treated with CCl_4 in mineral oil; the ninth animal (lane 9) was treated with mineral oil alone. Three days after treatment, animals were sacrificed and liver RNA was prepared. (A) RNase protection assays were performed with 100 μg of total RNA. The top panel shows AFP levels in the nine animals. The lower panel shows levels of the transgene mRNA (AFP-D^d) and cross-hybridizing class I mRNA (MHC). The lower panel shows a shorter autoradiographic exposure than in previous figures; this enables the difference in AFP-D^d levels to be more easily visualized. (B) Quantitation of the RNase protection data in panel A. Acrylamide gels shown in panel A were analyzed by using an Ambis radioanalytic imaging system. Total counts per minute in the different bands were determined. The AFP and AFP-D^d levels were normalized to MHC levels, and the relative levels of protected probe are shown. Numbers 1 to 8 represent mice 1 to 8 in panel A.

than those in the other six CCl_4-treated mice, thus suggesting these two to be *Rif*^{a} offspring. The AFP(3.8)-D^d transgene was also activated at different levels in the CCl_4-treated animals; mice 3 and 7 showed higher levels of induction than the other six CCl_4-treated mice did (Fig. 6B, lower panel, compare lanes 3 and 7 with lanes 1, 2, 4 to 6, and 8). The cross-hybridizing endogenous MHC mRNA, which is not activated during regeneration, was present at roughly equivalent levels in all nine transgenic animals (Fig. 6A, lower panel). The levels of radiolabeled probe protected by AFP and AFP-D^d mRNA were quantitated and normalized to MHC levels (Fig. 6B). The two patterns of expression, high and low, were evident for both genes. AFP and AFP-D^d levels were quite uniform in the six low-level-expression-inducing mice (lanes 1, 2, 4 to 6, and 8). The two high-level-expression-inducing mice (lanes 3 and 7) had three- to fourfold-higher levels of AFP and AFP-D^d than the low-level-expression-inducing mice. Although these differences were not as great as the 10-fold difference previously observed with *Rif* (4), these results clearly demonstrate that AFP(3.8)-D^d is *Rif* regulated like the endogenous AFP gene.

DISCUSSION

The developmental control of gene regulation can be studied by introducing DNA into the mouse germ line. In the transgenic studies described here, we linked a portion of the mouse AFP 5′ regulatory DNA to a heterologous gene body to further characterize the role of this region in postnatal AFP regulation. The 3.8-kb AFP fragment that was used contains enhancer I, the repressor, and the promoter regions. Previous studies have shown enhancer I to be the strongest of the three AFP enhancers (18). The structural gene for the mouse MHC class I D^d protein was used as a reporter of AFP control element activity. Class I D^d expression can be monitored at the mRNA level with specific RNA probes and at the protein level with D^d-specific monoclonal antibodies (32).

Previous AFP transgenic studies examined AFP 5′ control regions linked to an AFP minigene and thus could not eliminate the possibility that the five-exon minigene was contributing to proper tissue-specific and developmental control, at either the transcriptional or posttranscriptional level (8, 18, 24, 38, 39). For example, posttranscriptional regulation at the level of mRNA processing and/or stability could modulate

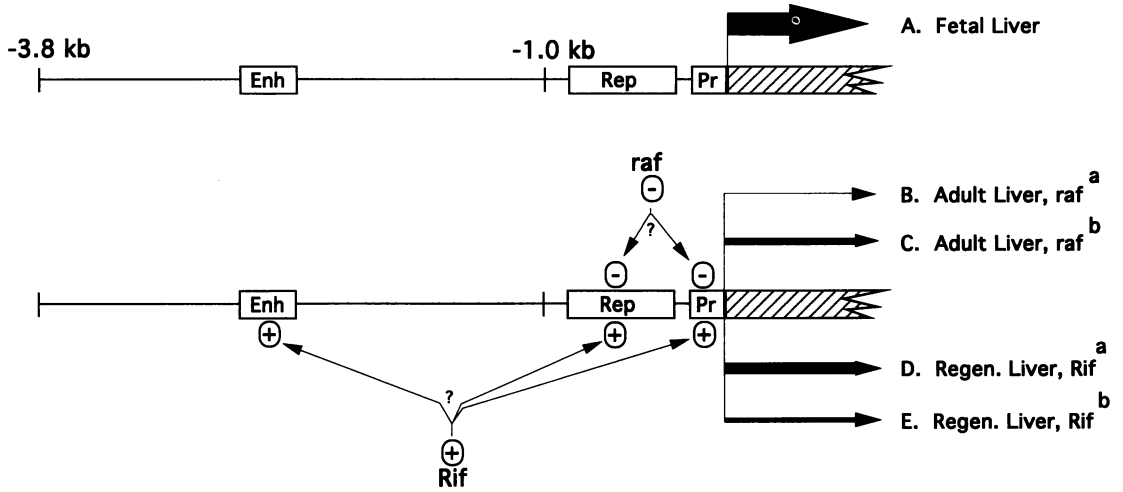

FIG. 7. Model of postnatal AFP control by *raf* and *Rif*. The AFP(3.8)-D^d gene, which has 3.8 kb of the AFP upstream control region sequences linked to the *H-2D^d* structural gene (cross-hatched region), is shown. AFP control regions contained in the hybrid gene are boxed and include the 300-bp minimal enhancer I region (Enh), 550-bp repressor region (Rep), and 200-bp promoter (Pr). The *raf* product is designated as a negative (−) regulator. *Rif* activity is induced during liver regeneration in response to liver injury and is presumably a positive-acting (+) factor. Potential sites of action for *raf* and *Rif* are shown; question marks indicate that the exact sites are not yet known. The levels of transgene expression under different conditions are designated by the horizontal arrows toward the right of the figure; the thicker the arrow, the more RNA is made. (A) In the absence of *raf* in the fetal liver, AFP-D^d is expressed at maximal levels. (B) In adult liver, *raf^a* represses AFP-D^d expression to low baseline levels. (C) In the presence of *raf^b*, AFP-D^d levels in adult liver are significantly lower than in fetal liver but higher than in *raf^a* mice, suggesting that *raf^b* is a less effective repressor than *raf^a*. (D and E) During liver regeneration, AFP-D^d levels are induced above basal levels in adult liver (B). Induction in the presence of *Rif^a* (D) is roughly threefold higher than in the presence of *Rif^b* (E), suggesting that *Rif^a* is a stronger inducer of gene expression. Whether *raf* and *Rif* physically interact with each other or bind to the same region cannot be determined in the present study.

steady-state AFP mRNA levels, or sequence motifs within exons or introns could modulate the rate of transcription. Numerous examples of control regions within or 3′ of structural genes can be found; the existence of an intragenic negative element within the mouse AFP structural gene has in fact been suggested (27).

Our findings that the AFP(3.8)-D^d gene is expressed in the proper tissues, is turned off after birth, and is reactivated during liver regeneration all demonstrate that the AFP sequences present in this hybrid gene are sufficient to confer tissue-specific and developmental control of the linked *D^d* gene body. Therefore, we conclude that sequences within the AFP minigene are not required for any of these regulatory events and that specificity is regulated solely by the 5′ control elements. Two notable differences between AFP(3.8)-D^d and endogenous AFP expression were observed. First, transgene mRNA levels are significantly higher in the fetal gut. In previous studies, AFP minigenes linked to enhancer I but not to enhancer II or III were expressed at significantly higher levels in the gut than is the endogenous AFP gene (18). Our data support the notion proposed by Hammer et al. (18) that elevated transgene levels in the gut are due to an increase in transcription. One explanation is that the transgenes are simply more accessible to positive *trans*-acting factors. A more intriguing possibility is that the AFP gene contains a gut-specific negative element that is outside the sequences included in the enhancer I AFP minigene constructs and AFP(3.8)-D^d. The second difference is the presence of low but detectable AFP-D^d levels in the kidneys, an observation also made by Tilghman and coworkers with other AFP transgenes (8, 18). These results suggest that AFP transgenes are more accessible to transcription factors in the kidneys than is the endogenous AFP gene. Interestingly, hepatocyte nuclear factor 1 (HNF-1) and HNF-4 are expressed at high levels in the liver, gut, and kidneys (3, 30). HNF-1 has been shown to regulate AFP through at least one binding site in the AFP promoter (11); a direct role for HNF-4 in AFP control has not been established. Whether low AFP transgene expression in the kidneys is due to HNF-1 and/or HNF-4 will require further studies.

Since the AFP(3.8)-D^d hybrid gene was postnatally repressed, we could determine whether liver AFP-D^d mRNA levels are modulated by the *raf* product. AFP-D^d mRNA levels do parallel the levels of endogenous AFP mRNA; mice have either high AFP and AFP-D^d mRNA levels or low AFP and AFP-D^d mRNA levels. This correlation was seen in offspring of all three transgenic founder mice. These data indicate that the AFP component of the AFP(3.8)-D^d hybrid gene is sufficient to confer *raf* regulation on the heterologous gene body and suggest that *raf* acts at the transcriptional level. Since the AFP enhancers are not required for *raf* control (38), *raf* must act via the repressor or promoter (Fig. 7). The behavior of *raf* as a negative regulator is consistent with its interacting with the repressor region. However, further dissection of these elements will be required to address this question. Although Fig. 7 shows *raf* as the sole repressor of AFP expression, our studies cannot rule out the involvement of additional postnatal negative regulators. Additionally, we cannot determine whether the *raf^b* allele represents a null mutation or encodes a protein with reduced repressor activity. Resolution of these issues must await identification of the *raf* product. The observation that AFP-D^d levels in the gut are not *raf* responsive is consistent with previous evidence that *raf* activity is restricted to the liver (29, 37).

Vacher et al. (38) recently reported that *raf* acts at the posttranscriptional level. Nuclear run-on experiments indicated that the transcription rates of AFP transgenes and the endogenous H19 gene, which is also *raf* regulated, were not sufficiently different between *raf^b* mice (high mRNA levels)

and $raf^{a/b}$ mice (low mRNA levels) to account for the 15- to 20-fold difference in steady-state mRNA levels. Any model of posttranscriptional regulation implies that mRNAs from the AFP minigene and H19 gene share a common motif that would interact with the *raf* product. Sequence analysis of mouse and human AFP and H19 genes revealed one possible motif, a potential stem-loop structure adjacent to the polyadenylation signal of these mRNAs (38). A prediction of this model is that a heterologous gene linked to AFP 5′ control elements should not be *raf* regulated. Therefore, we were surprised to find that AFP-D^d mRNA levels were indeed responsive to *raf*. The putative stem-loop structure at the 3′ end of the AFP and H19 mRNAs is not present in the *H-2D*^{d} mRNA and is therefore not likely to be involved in *raf* control. The first 25 nucleotides of the AFP-D^d hybrid mRNA, however, does come from the AFP gene, and we cannot rule out the possibility that this region confers posttranscriptional control. However, we believe that this is unlikely since no sequence or potential secondary structure homologies between this small region of AFP and H19 mRNA are found. With the exception of this 25-bp region, the rest of the 1.8-kb AFP-D^d mRNA is identical to the *raf*-nonresponsive endogenous *H-2D*^{d} mRNA. The homology between the AFP(3.8)-D^d transgene and endogenous MHC class I genes is useful, because it provides an internal control for RNase protection assays but precludes the use of nuclear run-on assays to measure transcription rate of the AFP(3.8)-D^d hybrid gene.

Taken together, our results and those of Vacher et al. (38) may indicate a complex regulatory mechanism that couples transcriptional to posttranscriptional events. The following model could account for such a situation. *raf*, acting as a negative transcription factor activated soon after birth, would interact directly or indirectly with a *cis*-acting element within the AFP promoter or repressor region (Fig. 7). The *raf* product, physically associated with the AFP gene, could then interact with a component of the RNA polymerase II (RNAP II) preinitiation complex. It is clear that upstream factors can interact with basal factors that are components of the RNAP II preinitiation complex (10, 13, 14, 17, 33). We would predict that this interaction would not alter transcription initiation, since initiation is the same in raf^a and raf^b mice but could affect other aspects of RNAP II activity. For example, elongation by RNAP II through pause sites or mRNA 3′-end formation could be affected. Changes at either of these points could alter steady-state mRNA levels. A potentially interesting factor, in regard to this model, is TFIIF. This protein, a basal factor of the RNAP II preinitiation complex, has been shown to affect mRNA elongation (5, 12). Thus, TFIIF could serve as a link between upstream binding factors and posttranscriptional control. Any structural gene, when linked to AFP control regions, would be subject to such control. Direct evidence for this model will require the characterization of *raf*. A precedent for regulation in which transcription and mRNA processing are coupled exists in the expression of small nuclear RNA genes U1 and U2; studies indicate that upstream control elements are important for small nuclear RNA 3′-end formation (19).

Finally, AFP(3.8)-D^d expression in all three lines increased during liver regeneration. Using the appropriate genetic crosses, we have established that the transgene is regulated during regeneration by *Rif*. The simplest explanation is that AFP reactivation during renewed cell proliferation, including *Rif*-mediated control, is due to an increase in transcription. We cannot, however, rule out more complicated models of regulation. Further dissection of the elements within the 3.8 kb of AFP DNA is required to localize the *cis*-acting region that is necessary for reactivation (Fig. 7). In addition, characterization of the *Rif* product is needed to further elucidate the role of this factor on AFP reactivation. For example, it is not clear whether *Rif* is solely responsible for AFP reactivation or, rather, is a single component of a more complex event. Also, we cannot determine whether *Rif* might act to displace *raf* during liver regeneration, thus leading to increased AFP expression, or if these two regulators function independently. H19, originally identified in a selection strategy devised to identify *raf*-regulated genes, is also controlled by *Rif* (29). This supports the notion that these regulators interact with each other or act through a common *cis*-acting region to modulate AFP expression.

Regardless of *Rif* action, the AFP(3.8)-D^d transgenic mice should be useful tools with which to explore the basis for reactivating AFP transcription. Previous studies with mice indicate that only a subset of hepatocytes express AFP during liver regeneration (26, 36). If the AFP(3.8)-D^d transgene follows a similarly restricted pattern, only that subset of hepatocytes will express D^d molecules on their cell surface. By using D^d-specific monoclonal antibodies, flow cytometry could be used to purify viable D^d-positive cells from D^d-negative cells. Sensitive assays, such as reverse transcriptase-PCR, could be used to identify any changes in transcription factors between these two cell populations. This possibility suggests a potentially broader role for the *H2-D*^{d} gene as a means of enriching for viable populations of transgene-positive cells in transgenic mice.

ACKNOWLEDGMENTS

I thank Michael Green and the University of Kentucky Transgenic Mouse Facility for generating the transgenic mice used in these studies, Amy Wilson-Ellis for technical assistance, and Martha Peterson for critically reading the manuscript.

This work was supported by Public Health Service grant GM-45253 and Council for Tobacco Research grant 3512.

REFERENCES

1. **Abelev, G. I.** 1971. Alpha-fetoprotein in oncogenesis and its association with malignant tumors. Adv. Cancer Res. **14:**295–358.
2. **Auffray, C., and F. Rougeon.** 1980. Purification of mouse immunoglobulin heavy-chain mRNAs from total myeloma tumor RNA. Eur. J. Biochem. **107:**303–314.
3. **Baumhueter, S., D. B. Mendel, P. B. Conley, C. J. Kuo, C. Turk, M. K. Graves, C. A. Edwards, G. Courtois, and G. R. Crabtree.** 1990. HNF-1 shares three sequence motifs with the POU domain proteins and is identical to LF-B1 and APF. Genes Dev. **4:**372–379.
4. **Belayew, A., and S. M. Tilghman.** 1982. Genetic analysis of α-fetoprotein synthesis in mice. Mol. Cell. Biol. **2:**1427–1435.
5. **Bengal, E., O. Flores, A. Krauskopf, D. Reinberg, and J. Aloni.** 1991. Role of the mammalian transcription factors IIf, IIs, and IIX during elongation by RNA polymerase II. Mol. Cell. Biol. **11:** 1195–1206.
6. **Bernuau, D., A. Poliard, and G. Feldmann.** 1988. *In situ* cellular analysis of α-fetoprotein gene expression in regenerating rat liver after partial hepatectomy. Hepatology **8:**997–1005.
7. **Blankenhorn, E. P., R. Duncan, C. Huppi, and M. Potter.** 1988. Chromosomal location of the regulator of mouse α-fetoprotein, afr-1. Genetics **119:**687–691.
8. **Camper, S. A., and S. M. Tilghman.** 1989. Postnatal repression of the α-fetoprotein gene is enhancer independent. Genes Dev. **3:**537–546.
9. **Evans, G. A., D. H. Margulies, B. Shykind, J. G. Seidman, and K. Ozato.** 1892. Exon shuffling: mapping polymorphic determinants on hybrid mouse transplantation antigens. Nature (London) **300:** 755–757.
10. **Ferreri, K., G. Gill, and M. Montminy.** 1994. The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex. Proc. Natl. Acad. Sci. USA **91:**1210–1213.

11. **Feuerman, M. H., R. Godbout, R. S. Ingram, and S. M. Tilghman.** 1989. Tissue-specific transcription of the mouse α-fetoprotein gene promoter is dependent on HNF-1. Mol. Cell. Biol. **9:**4204–4212.
12. **Flores, O., E. Maldonado, Z. Burton, J. Greenblatt, and D. Reinberg.** 1988. Factors involved with specific transcription by mammalian RNA polymerase II: RNA polymerase II associated protein 30 is an essential component of transcription factor IIF. J. Biol. Chem. **263:**10812–10816.
13. **Gill, G., E. Pascal, Z. H. Tseng, and R. Tjian.** 1994. A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the Drosophila TFIID complex and mediates transcriptional activation. Proc. Natl. Acad. Sci. USA **91:**192–196.
14. **Gill, G., and R. Tjian.** 1992. Eukaryotic coactivators associated with the TATA box binding protein. Curr. Opin. Genet. Dev. **2:**236–242.
15. **Godbout, R., R. S. Ingram, and S. M. Tilghman.** 1986. Multiple regulatory elements in the intergenic region between the α-fetoprotein and albumin genes. Mol. Cell. Biol. **6:**477–487.
16. **Godbout, R., R. S. Ingram, and S. M. Tilghman.** 1988. Fine-structure mapping of the three mouse α-fetoprotein enhancers. Mol. Cell. Biol. **8:**1169–1178.
17. **Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon, and R. Tjian.** 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell **75:**519–530.
18. **Hammer, R. E., R. Krumlauf, S. A. Camper, R. L. Brinster, and S. M. Tilghman.** 1987. Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. Science **235:**53–58.
19. **Hernandez, N., and R. Lucito.** 1988. Elements required for transcription initiation of the human U2 snRNA gene coincide with elements required for snRNA 3′ end formation. EMBO J. **7:**3125–3134.
20. **Hogan, B., F. Constantini, and E. Lacy.** 1986. Manipulating the mouse embryo. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Jaffe, L., E. J. Robertson, and E. K. Bikoff.** 1992. Developmental failure of chimeric embryos expressing high levels of H-2D^d transplantation antigens. Proc. Natl. Acad. Sci. USA **89:**5927–5931.
22. **Jalanko, H.** 1979. Alpha-fetoprotein and γ-glutamyltranspeptidase in mice. Effect of raf gene. Int. J. Cancer **24:**394–397.
23. **Johnson, P. M., and S. L. McKnight.** 1989. Eucaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. **58:**799–839.
24. **Krumlauf, R., R. E. Hammer, S. M. Tilghman, and R. L. Brinster.** 1985. Developmental regulation of α-fetoprotein genes in transgenic mice. Mol. Cell. Biol. **5:**1639–1648.
25. **Melton, D., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage Sp6 promoter. Nucleic Acids Res. **12:** 7035–7056.
26. **Mohanty, M., P. K. Das, A. Mittal, and N. C. Nayak.** 1978. Cellular basis of α-fetoprotein synthesis by hepatocytes of adult mouse after hepatotoxic injury and partial hepatectomy. Int. J. Cancer **22:**181–188.
27. **Molne, M., C. Houart, J. Szpirer, and C. Szpirer.** 1989. Combinatorial control of positive and negative, upstream and intragenic regulatory DNA domains of the mouse alpha-foetoprotein gene. Nucleic Acids Res. **17:**3447–3457.
28. **Olsson, M., G. Lindahl, and E. Ruoslahti.** 1977. Genetic control of alpha-fetoprotein synthesis in the mouse. J. Exp. Med. **145:**819–830.
29. **Pachnis, V., A. Belayew, and S. M. Tilghman.** 1984. Locus unlinked to α-fetoprotein under the control of the murine *raf* and *Rif* genes. Proc. Natl. Acad. Sci. USA **81:**5523–5527.
30. **Sladek, F. M., W. Zhong, E. Lai, and J. E. Darnell.** 1999. Liver-enriched transcription factor AHNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev. **4:**2353–2365.
31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
32. **Spear, B. T., and S. M. Tilghman.** 1990. Role of α-fetoprotein regulatory elements in transcriptional activation in transient heterokaryons. Mol. Cell. Biol. **10:**5047–5054.
33. **Stringer, K. F., C. J. Ingles, and J. Greenblatt.** 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature (London) **345:**783–786.
34. **Tilghman, S. M.** 1985. The structure and regulation of the mouse α-fetoprotein and albumin genes. Oxf. Surv. Eukaryotic Genes **2:**160–206.
35. **Tilghman, S. M., and A. Belayew.** 1982. Transcriptional control of the murine albumin: α-fetoprotein locus during development. Proc. Natl. Acad. Sci. USA **79:**5254–5257.
36. **Tuczek, H.-V., P. Fritz, T. Wagner, U. Braun, A. Grau, and G. Wegner.** 1981. Synthesis of alpha-fetoprotein (AFP) and cell proliferation in regenerating livers of NMRI mice after partial hepatectomy. Virchows Arch. B **38:**229–237.
37. **Tyner, A. L., R. Godbout, R. S. Compton, and S. M. Tilghman.** 1990. The ontogeny of α-fetoprotein gene expression in the mouse gastrointestinal tract. J. Cell Biol. **110:**915–927.
38. **Vacher, J., S. A. Camper, R. Krumlauf, R. S. Compton, and S. M. Tilghman.** 1992. *raf* regulates the postnatal repression of the mouse α-fetoprotein gene at the posttranscriptional level. Mol. Cell. Biol. **12:**856–864.
39. **Vacher, J., and S. M. Tilghman.** 1990. Dominant negative regulation of the mouse α-fetoprotein gene in adult liver. Science **250:**1732–1735.
40. **Wen, P., E. R. Groupp, G. Buzard, N. Crawford, and J. Locker.** 1991. Enhancer, repressor, and promoter specificities combine to regulate the rat α-fetoprotein gene. DNA Cell Biol. **10:**525–536.