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raf Regulates the Postnatal Repression of the Mouse α-Fetoprotein Gene at the Posttranscriptional Level

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The mouse α-fetoprotein (AFP) gene is transcribed at a high rate in liver during the second half of gestation. Its steady-state mRNA levels decrease 10^4-fold shortly after birth, at least in part as the consequence of a dramatic decrease in its transcription rate. The final basal level of AFP mRNA in adult liver is influenced by a *trans*-acting locus on chromosome 15 termed *raf*. Two strategies were used to demonstrate that the *raf* gene acts posttranscriptionally to affect the processing and/or stability of AFP transcripts. Transgenic mouse studies demonstrated that *raf* gene action is independent of both positive and negative transcription control elements of the AFP gene. Nuclear run-on analysis was used to confirm that transcriptions of both AFP transgenes and another endogenous *raf*-responsive gene, H19, are invariant with respect to the *raf* genotype. Thus, the postnatal repression of the AFP gene is mediated by both transcriptional and posttranscriptional mechanisms.

Genetic analysis in lower eucaryotes has proved a powerful tool for revealing the complexity of the regulatory circuits that govern gene expression. In some instances, such as the *SIR* genes which regulate the silencer of the yeast mating-type switch, this analysis has identified gene products that would have been overlooked by the biochemical approach of isolating DNA-binding proteins (36). Likewise, the role of Gal80 in the negative regulation of transcription of the genes in the galactose regulon was first suggested from genetic analyses (28). This genetic approach is more difficult in multicellular organisms, because there is rarely a selection for the genes of interest. Several years ago, two unlinked *trans*-acting loci which affect the expression of the mouse α-fetoprotein (AFP) gene in the liver were identified by screening inbred strains of mice (3, 27). The *raf* locus determines, at least in part, the adult basal level of AFP in the liver, while *Rif* affects the degree to which the mRNA can be induced during liver regeneration. Both genes are pleiotropic, in that they affect at least one other gene in the liver, the H19 gene (29).

The *raf* gene has been mapped to chromosome 15 in the mouse, 2 to 3 centimorgans proximal to the c-*myc* gene (4). Two alleles of *raf* have been defined; raf^b is found in the BALB/cJ strain and is recessive to the raf^a allele in all other inbred mouse strains tested (27). There is no phenotypic difference between strains before birth when transcription of the AFP gene is high. However, shortly after birth, AFP mRNA in BALB/cJ mice declines more slowly and achieves a 15- to 20-fold-higher adult basal level than in other strains of mice (3). This is the consequence of elevated mRNA levels per cell rather than a difference in the number of AFP-producing cells in the liver (24). The action of *raf* is cell autonomous (42) and is apparently restricted to the liver, as the repressions of AFP and H19 in the gut and of H19 in muscle are not under the control of *raf* (29).

The mechanism underlying the response of the AFP gene to *raf* has not been elucidated. Nuclear run-on assays, which are commonly used to distinguish transcriptional and post-transcriptional events, are not sensitive enough to detect the very low levels of transcription of the AFP gene in adult liver (38). As an alternative approach to elucidating the *raf*-dependent control point, we have determined the *cis*-acting sequences required by the AFP gene to respond to *raf*. For this, we have taken advantage of transgenic mouse lines which harbor different amounts of the AFP locus (6, 18, 23, 41). These animals, which were generated in the raf^a genetic background, were backcrossed to BALB/cJ mice, and the adult basal levels of the transgene mRNAs were analyzed. These studies indicate that the action of the *raf* gene is independent of both the positive and negative transcriptional control elements of the AFP gene. Rather, *raf* acts posttranscriptionally to affect the processing and/or stability of both AFP and H19 transcripts.

MATERIALS AND METHODS

Animals. BALB/cJ, C57BL/6, and SJL/J mice were purchased from the Jackson Laboratory. The transgenic lines used, derived from F_2 zygotes of C57BL/6 × SJL/J parents, have been described previously (6, 18, 23, 41). They were crossed twice to BALB/cJ, and backcross progeny were analyzed at 3 to 6 weeks after birth.

RNA extraction and analysis. Total RNAs were prepared from adult livers by the phenol (18), guanidinium thiocyanate-guanidinium hydrochloride (7), or LiCl-urea extraction procedure (1). Enrichment for poly(A)$^+$ RNA was obtained by oligo(dT)-cellulose chromatography (2).

Poly(A)$^+$ RNA samples were electrophoresed in 1.5% agarose gels in the presence of formaldehyde and transferred onto a nitrocellulose membrane as described previously (37). The blots were baked for 2 h at 80°C and hybridized to DNA probes labeled by nick translation (25) or to RNA probes prepared by transcription, using SP6 polymerase, of fragments cloned into pGEM (26). The probe used to selectively

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detect the endogenous AFP mRNA was pAFP1, a cDNA which spans exons 4 through 9 (40). The probe used to detect both the endogenous AFP gene and minigene transcripts was a 440-bp *Hin*cII fragment which spans exon 1. The probe used to detect the endogenous albumin gene and minigene transcripts was an *Ava*I-*Hin*dIII fragment which spans exon 1 (6). High-stringency washes were done four times for 20 min at 70°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) (43). Each blot was also hybridized to a DNA probe specific for ribosomal protein L32 (10) to quantify the amount of RNA loaded for each sample.

Quantitation of RNA by S1 nuclease protection. To quantitate the levels of AFP and transgene RNA, a *Pvu*II-*Sau*3A1 fragment spanning exon 1 from bp −604 to +56 was used. The fragment was labelled with [γ-^{32}P]ATP and polynucleotide kinase at the *Sau*3A1 site at bp +56 in the AFP gene. Poly(A)$^+$ RNA (1 to 2 μg) and probe (30,000 cpm) were ethanol precipitated in the presence of tRNA and resuspended in 50 μl of hybridization buffer containing 80% formamide, 10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.5], 400 mM NaCl, and 1 mM EDTA. Samples were heated at 80°C for 10 min and transferred to 42°C for 12 to 16 h. Samples were digested for 1 h at 37°C by addition of S1 nuclease (*Aspergillus oryzae*; Boehringer Mannheim Biochemicals) in 450 μl of buffer containing 250 mM NaCl, 30 mM sodium acetate (pH 4.5), and 3 mM zinc acetate. Reactions were stopped by the addition of EDTA to 6 mM, and samples were extracted with phenol-chloroform(1:1) and precipitated with ethanol. Pellets were resuspended in 80% formamide–TBE buffer (90 mM Tris-borate [pH 8.3], 4 mM EDTA)–0.1% bromophenol blue–xylene cyanol, denatured at 90°C for 2 min, and electrophoresed on a 7 M urea–7.5% acrylamide gel in TBE buffer. Autoradiography of dried gels was done at −80°C for 2 to 5 h.

Nuclear run-on assay. Assays were performed as described by Konieczny and Emerson (22), using livers from 3- to 4-week-old transgenic animals. A small part was saved to prepare poly(A)$^+$ RNA. The rest of the liver was resuspended in 4 ml of reticulocyte standard buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 2.75 mM dithiothreitol, 20 U of RNAsin per ml)–0.5% Nonidet P-40. Tissue was homogenized in a pestle tissue grinder, centrifuged at 8,000 rpm, resuspended, and rehomogenized. Nuclei were obtained by centrifugation at 27,000 rpm for 45 min in an SW40 rotor at 4°C. The nuclear pellet was resuspended in 200 μl of 5 mM $MgCl_2$–10 mM Tris-HCl (pH 7.5)–0.5 M sorbitol–2.5% Ficoll–0.008% spermidine–1 mM dithiothreitol–50% glycerol. For each run-on reaction, to one 200-μl nuclear aliquot was added 100 μl of 160 mM Tris-HCl–600 mM NH_4Cl–30 mM $MgCl_2$ and 100 μl of a ribonucleotide mix (0.625 mM ATP, 0.312 mM GTP, 0.312 mM CTP, and 0.3 mM [α-^{32}P]UTP at 800 Ci/mmol). The reaction mixture was incubated at 27°C for 10 min, then 16 μl of a 1-mg/ml DNase I solution was added, and the mixture was incubated for 10 min. One-third volume of 10 mM Tris-HCl (pH 7.5)–15 mM EDTA–3% SDS–1 mg of proteinase K per ml–3 mg of heparin per ml was added, and the reaction mixture was incubated at 42°C for 2 h.

Each reaction mixture was extracted four times with phenol-chloroform (1:1) and precipitated with 400 μl of 4 M ammonium acetate and 750 μl of ethanol. The pellet was resuspended in 100 μl of H_2O and added to 3 ml of 4× SET (600 mM NaCl, 200 mM Tris-HCl [pH 8.0], 20 mM EDTA)–0.02% bovine serum albumin–0.02% polyvinylpyrrolidone–0.03% Ficoll–100 μg of denaturated salmon sperm DNA per ml–100 μg of yeast tRNA per ml–50% formamide.

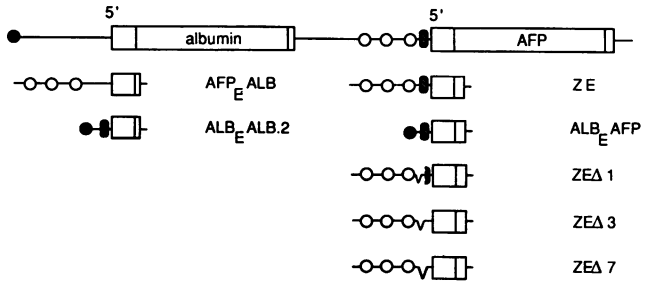


FIG. 1. Structures of transgenes introduced into the mouse germ line. The upper line represents the configuration of the albumin (ALB) and AFP genes in the mouse genome. The albumin gene enhancer (black circle) maps between −9 and −12 kb relative to the albumin gene transcriptional start site, and the three AFP gene enhancers (open circles) are at −6.5, −5, and −2.5 kb relative to the AFP gene cap site. Constructs ZE (23) and ALB$_E$AFP and AFP$_E$ALB (6) have been previously described. Constructs ZEΔ1, ZEΔ3, and ZEΔ7 are composed of the three AFP enhancers and the AFP minigene (open box) and are deleted for nucleotides between −838 and −604 (ZEΔ1), −838 and −250 (ZEΔ3), and −838 and −118 (ZEΔ7) upstream of the AFP gene (41). Construct ALB$_E$ALB.2 has the AFP dominant negative repressor sequence (−250 bp to −1 kb; hatched oval) introduced between the albumin gene enhancer and the albumin minigene (open box) (41).

Nitrocellulose filters were prepared by binding 10 μg of each linearized plasmid with a slot blot apparatus. After restriction enzyme digestion, each DNA was denatured for 30 min in 0.1 M NaOH and neutralized by the addition of 10 volumes of 6× SSC. Filters were baked for 2 h at 80°C and prehybridized in 5 ml of hybridization buffer at 42°C for 4 h.

Hybridization was performed in a final volume of 3 ml for 50 to 60 h at 42°C. Filters were washed in 1× SET–0.1% SDS at room temperature for 30 min and twice at 60°C in 0.1× SET–0.1% SDS. Autoradiography was done for 2 to 4 days at −80°C.

RESULTS

Strategy for the genetic analysis of *raf* regulation. The feasibility of using transgenic mice for mapping the *raf* response element was tested by using a transgenic mouse line carrying the transgene AFP ZE (line 47-3 in reference 23; Fig. 1). This construct consists of 7.6 kb of AFP 5′ flanking DNA and includes the three distal enhancers (open circles), which are absolutely required for activation of the gene (13, 14, 18), a dominant negative element between bp −800 and −250 (closed oval), which is required for the postnatal repression of the gene (41), and the proximal promoter. The flanking sequences are linked to an internally deleted AFP structural gene containing the first three and last two exons of the gene, fused within a chimeric intron (34). The minigene transcript can be distinguished from the endogenous transcript by virtue of its smaller size.

The founder transgenic animals were (C57BL/6J × SJL/J)F_2 hybrids and therefore homozygous for the dominant *raf*^{a} allele that directs a low level of AFP mRNA in the adult liver (27). To study the response of the transgene to the *raf*^{b} allele, founders were crossed twice to BALB/cJ mice, so that the progeny in the second generation exhibited an equal proportion of high (*raf*^{b}) and low (*raf*$^{a/b}$) expression of the endogenous AFP gene. For each transgenic line, the respon-

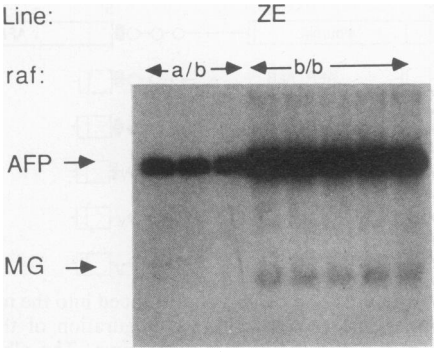


FIG. 2. *raf* regulation in (BALB/cJ × founder) × BALB/cJ progeny of a ZE founder. Progeny derived from a backcross between the ZE founder 47-3 (23) and BALB/cJ were sacrificed at 3 weeks of age. Poly(A)$^+$ liver RNAs were separated on denaturing gels, blotted, and hybridized with an AFP-specific probe. The two transcripts corresponding to the endogenous gene (AFP) and the transgene (MG) are indicated by arrows. The levels of endogenous AFP transcripts were used to define the *raf* genotype, with a low level corresponding to the $raf^{a/b}$ heterozygotes and a high level corresponding to the homozygous raf^{b} animals.

siveness of the transgene was determined and compared with that for the endogenous gene.

As shown in Fig. 2 for a subset of the animals tested, when eight transgenic backcross progeny were analyzed with an AFP gene probe that detects both the endogenous and minigene mRNAs, three exhibited the low levels of endogenous AFP mRNA characteristic of the $raf^{a/b}$ heterozygote and five displayed the significantly higher levels of the raf^{b} homozygote. Importantly, the minigene mRNA behaved identically, indicating that the transgene was responding to the *raf* gene in the same manner as the endogenous gene. Similar results were obtained with two other independent ZE lines (data not shown). The ZE line in Fig. 2 was generated with plasmid sequences attached to the ZE construct, unlike all other lines in this report. This explains the low level of transgene RNA.

Role of the AFP enhancers in *raf* regulation. The albumin gene, which lies 32 kb upstream of the AFP gene in the mouse genome (19), is activated during embryogenesis at the same time as the AFP gene. Unlike the AFP gene, it maintains its transcription rate after birth at levels that are independent of the *raf* gene (38). We had previously shown that the temporal difference in expression between these two related genes lies not in their enhancers (6) but in the negative element of the AFP gene, in that insertion of the AFP negative element in front of the albumin gene confers upon it negative regulation after birth (41).

To determine whether any of the AFP enhancers was required for the response of the gene to the *raf* gene product, transgenic mice carrying a chimeric gene in which the AFP enhancers were used to drive transcription of an internally deleted albumin minigene from the albumin promoter (AFP_EALB; Fig. 1) were backcrossed to BALB/cJ. This construct had previously been shown to behave identically to the albumin gene, in that high-level transcription was achieved early in development and maintained throughout the life of the animal (6). Although the appropriate segregation of *raf* occurred in these animals, which is evident from the response of the endogenous AFP gene, the transgene levels were invariant in *raf* heterozygotes and homozygotes in two independent lines, N-331 and E-645 (Fig. 3A). Thus,

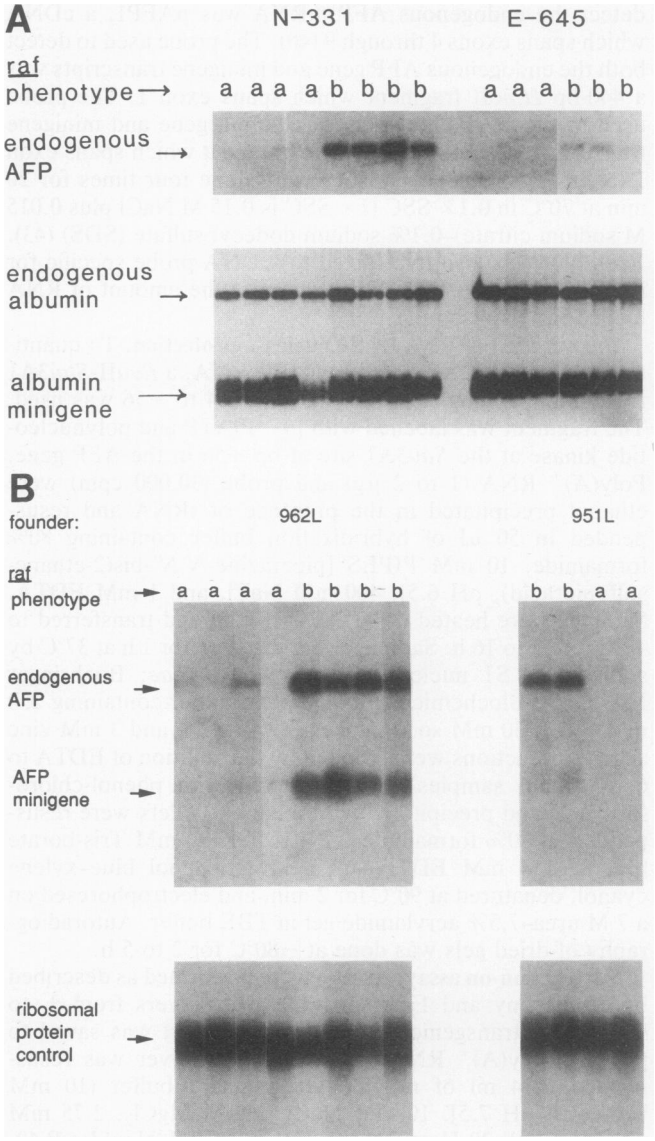


FIG. 3. *raf* regulation in (BALB/cJ × founder) × BALB/cJ progeny of AFP_EALB and ALB_EAFP transgenic lines. (A) Lines N-331 and E-645 are two examples of AFP_EALB founders in which the three AFP enhancers have been linked to the albumin minigene. After detection of the albumin transgene (minigene) and its endogenous counterpart (albumin) by hybridization to a specific albumin exon 1 probe, the blot was stripped and reprobed with an AFP-specific probe to determine the *raf* genotype as described for Fig. 2. (B) Lines 962L and 951L harbor the ALB_EAFP construct in which the albumin enhancer was cloned upstream of the AFP minigene. Poly(A)$^+$ RNAs of backcross progeny were hybridized to an AFP probe which detects exon 1. The integrity and loading of the RNA were confirmed by using the ribosomal protein L32 probe (10).

the AFP enhancers are not sufficient to confer a response to *raf* upon a heterologous gene. It should be noted, however, that the levels of expression of the albumin minigene in both lines are high, and it might not have been possible to observe a 10- to 15-fold-higher level due to the effects of *raf*.

Conversely, when the albumin enhancer, located at approximately 10 kb upstream of the albumin gene (31), was linked to an AFP minigene with the negative element and promoter intact (ALB_EAFP; Fig. 1), down-regulation (6) and

raf responsiveness of the transgene after birth were demonstrated in two independent lines (Fig. 3B). These two experiments suggest that the AFP enhancers are neither necessary nor sufficient for regulation of the AFP gene by *raf*.

Role of the AFP negative element in *raf* regulation. The dominant negative element in the AFP gene had been identified by generating a series of deletions (ZEΔ; Fig. 1) in the 5′ flanking domain between the promoter and the most proximal enhancer. Deletion of 200 bp between bp −604 and −838 (ZEΔ1) resulted in postnatal repression of the transgene that was at least 100-fold less efficient than that observed with the intact ZE construct. A larger deletion between bp −250 and −838 (ZEΔ3) completely abolished down regulation (41). The implication that a negative element had been identified was confirmed by inserting a segment of AFP 5′ flanking DNA corresponding to bp −250 to −1010 between the promoter and enhancer of the albumin minigene (ALB_EALB.2; Fig. 1). This resulted in its postnatal repression and clearly indicated that the negative regulation of the AFP gene was due, at least in part, to the action of a separable negative element in the 5′ proximal segment of the gene (41).

Animals carrying these nested deletions of the ZE construct were examined for their ability to respond to the *raf* gene. Figure 4A shows the results from backcrosses to BALB/cJ of two lines carrying construct ZEΔ1, which harbors the smallest deletion between bp −604 and −838 (Fig. 1). In both cases, the adult basal AFP minigene mRNA level responded appropriately to the two *raf* alleles, despite the fact that the down-regulation had been impaired. An S1 nuclease assay that detects both the endogenous and minigene RNA was used to accurately quantitate the differences in RNA levels in heterozygous and homozygous animals. Although this assay does not discriminate between the two RNAs, it is evident from Fig. 4 that the minigene represents the great majority of the AFP-specific RNA in these adult animals. As indicated in Table 1, there was a 5.5-fold difference in the levels of AFP and minigene transcripts in *raf* heterozygotes and homozygotes in line G2 and a larger 10- to 20-fold difference in line G1 (data not shown). One animal in line G1 was typed as a heterozygote, on the basis of the endogenous AFP mRNA level, but displayed an elevated minigene mRNA level and is probably a raf^b homozygote. Individual variation of this kind has been observed previously (42).

With the larger deletion, ZEΔ3, which removes bases between bp −250 and −838 and eliminates all postnatal repression, there was little if any difference between mature minigene mRNA levels in *raf* heterozygotes and homozygotes (Fig. 4B and Table 1). This result suggested that the ability of the transgene to respond to *raf* had been lost by removal of the repressor. However, two additional observations were difficult to reconcile with this conclusion. First, it was striking, especially in line F1, that the liver RNAs of raf^b homozygotes contained a new set of bands, indicated by the stars in Fig. 4B, that were distinct from the endogenous AFP gene (closed squares) and minigene mRNAs. These transcripts are detected only with probes derived from the minigene itself, as indicated by their absence in the AFP panel, for which an internal downstream probe absent from the minigene was used. These bands correspond to full-length and partially spliced precursors of the minigene mRNA, as determined by using probes to the introns of the minigene (data not shown), and are preferentially accumulating in the raf^b homozygotes.

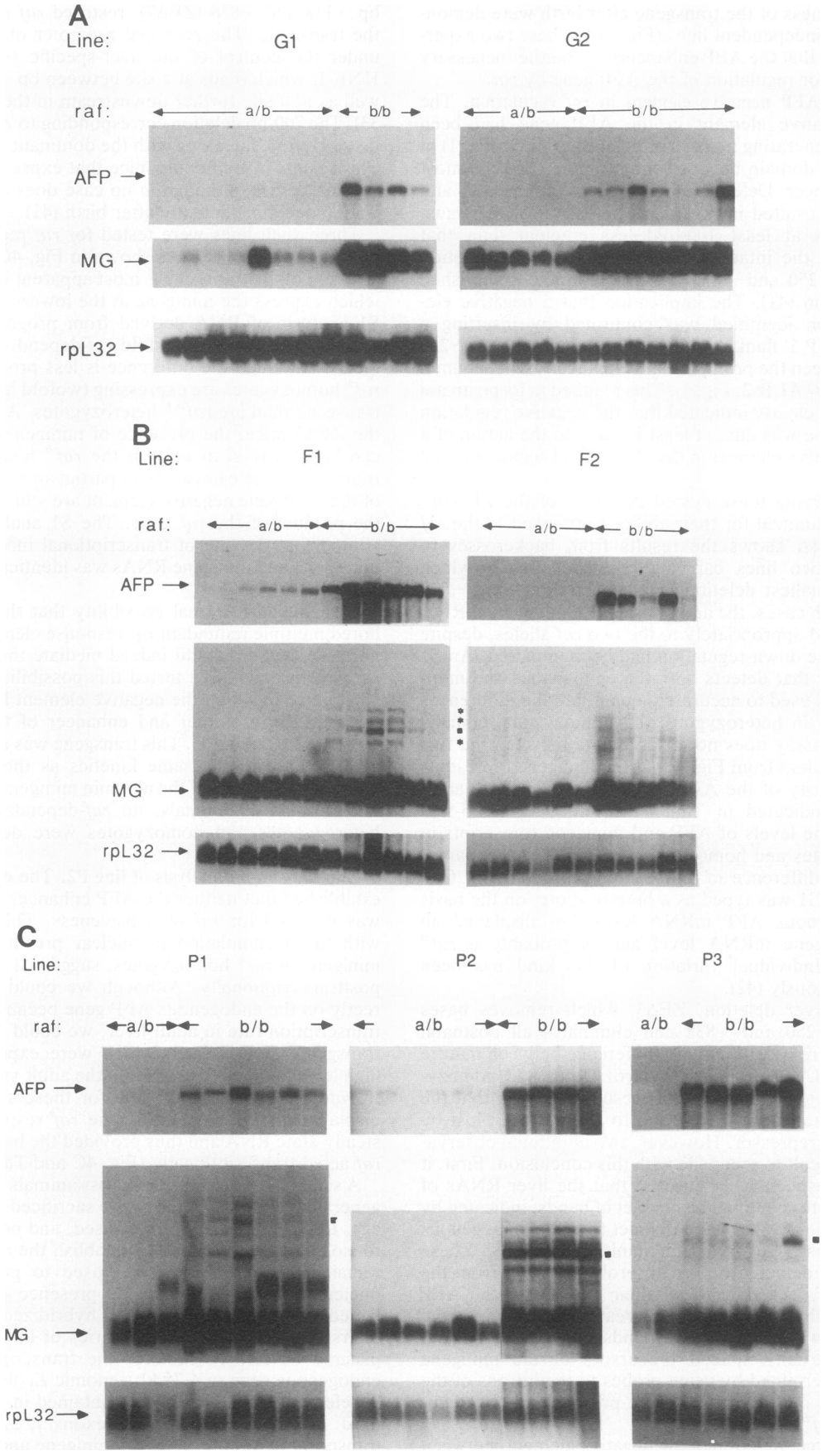
Second, a larger deletion of the negative element, between bp −118 and −838 (ZEΔ7), restored *raf* responsiveness to the transgene. The proximal promoter of the AFP gene is under the control of the liver-specific *trans*-acting factor HNF-1, which binds at a site between bp −125 and −100 as well as at a site further downstream in the promoter (9, 11, 33). The 700-bp deletion corresponding to ZEΔ7 removes the distal HNF-1 site along with the dominant negative element. This results in transgenic mice that express the minigene at variable levels, although in no case does the RNA concentration decline efficiently after birth (41).

Three such lines were tested for *raf* regulation by backcrossing to BALB/cJ. As shown in Fig. 4C, the *raf* responsiveness of the transgene is most apparent in lines P1 and P2, which express the minigene at the lowest level in the adult. S1 analysis of RNA derived from progeny of P1 and P2 exhibited a five- to sevenfold *raf*-dependent difference (Table 1). Although the difference is less pronounced with P3, raf^b homozygotes are expressing twofold higher levels of the transgene than are $raf^{a/b}$ heterozygotes. As in the case with the ZEΔ3 mice, the presence of minigene precursor bands can be clearly seen only in the raf^b homozygotes. Thus, transgenic mice carrying both partial and complete deletions of the AFP gene negative element are still able to respond to the product of the *raf* gene. The S1 analysis also demonstrated that the site of transcriptional initiation of endogenous gene and minigene RNAs was identical in raf^a and raf^b animals.

It remained a formal possibility that the AFP gene harbored multiple redundant *raf* response elements and that the negative element could indeed mediate the response to the *raf* gene product. We tested this possibility by using transgenic mice in which the negative element had been inserted between the promoter and enhancer of the albumin gene (ALB_EALB.2; Fig. 1). This transgene was down-regulated in adult liver with the same kinetics as the AFP gene (41). Although the levels of the albumin minigene transcripts were very low in all animals, no *raf*-dependent differences in heterozygotes and homozygotes were detected (data not shown).

Nuclear run-on analysis of line P2. The data in Fig. 2 to 4 established that neither the AFP enhancer nor the repressor was required for *raf* responsiveness. This result, coupled with the accumulation of nuclear precursors of the AFP minigene in raf^b homozygotes, suggested that *raf* might act posttranscriptionally. Although we could not test this directly on the endogenous AFP gene because of its very low transcription rate in adult liver, we could exploit the ZEΔ7 transgenic mouse lines, which were expressing relatively high levels of minigene RNA in the adult yet still responding to *raf*. We chose the P2 line for these studies because it displayed the greatest transgene *raf* response in terms of steady-state RNA and thus provided the best test of whether *raf* acted transcriptionally (Fig. 4C and Table 1).

A series of transgenic backcross animals of the P2 line was generated, and the animals were sacrificed at 3 to 4 weeks of age. Individual livers were excised, and portions were used to isolate poly(A)$^+$ RNA to establish the *raf* genotype. The remainder of each liver was used to prepare nuclei for nuclear run-on analysis in the presence of [^{32}P]UTP. Labelled RNA was recovered and hybridized to nitrocellulose filters on which was fixed a series of linearized denatured plasmid DNAs. To monitor the transcription rate of the endogenous gene, a 4.75-kb genomic *Eco*RI fragment ("B" in reference 15) which is not contained in the minigene was used. A cDNA encompassing exons 1, 2, and 15 detected transcription of both the AFP minigene and the endogenous


A
Line:
G1
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raf:
a/b
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a/b
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TABLE 1. AFP gene and transgene mRNA levels in liver RNAs[a]

Construct	Line	Avg cpm ± SD		Fold difference, $raf^b/raf^{a/b}$
		$raf^{a/b}$	raf^b	
ZEΔ1	G2	2.5 ± 0.4 (5)	13.7 ± 4.0 (7)	5.5
ZEΔ3	F1	9.0 ± 0.8 (6)	10.7 ± 0.7 (6)	1.2
	F2	8.5 ± 1.5 (7)	11.3 ± 0.5 (5)	1.3
ZEΔ7	P1	1.1 ± 3.2 (3)	5.5 ± 1.5 (5)	5.0
	P2	8.4 ± 3.0 (7)	56.9 ± 5.0 (3)	6.8
	P3	21.8 ± 13.0 (4)	48.2 ± 6.4 (7)	2.3

[a] An S1 nuclease protection assay was used to quantitate the concentration of AFP gene and transgene mRNAs in the liver RNAs depicted in Fig. 4. The dried gels were analyzed in a Betascope, and the average values are presented for the number of animals indicated in parentheses. As the analyses for each line were performed separately, the values are not directly comparable between lines.

gene, and a plasmid containing an internal segment of the albumin gene was used as a non-*raf*-responsive control.

As expected from previous results (38), no exclusive endogenous AFP gene signal could be detected in either $raf^{a/b}$ or raf^b nuclei (Fig. 5). However, a signal corresponding to the endogenous AFP gene plus the minigene transcript could be readily detected in all animals. Furthermore, the ratio of that signal to the albumin gene signal was approximately equivalent in animals derived from the same litter, irrespective of their *raf* genotype. For animals 1 and 2, which were derived from one litter, the ratios of AFP/albumin gene transcription were 0.16 in a raf^b homozygote and 0.06 in the heterozygote, a 2.5-fold difference. This cannot account for the much larger 7- to 10-fold difference in steady-state RNAs in the same animals (Fig. 5B). The discrepancy between transcription rate and steady-state RNA was even greater in animals 3 and 4, for which a 25% difference was obtained for transcription rates in a heterozygote and homozygote which displayed a 10-fold difference in steady-state RNAs. Note that animals can be compared only within litters, as AFP gene transcription is still declining at this time, and differences in timing as small as 1 day produce significant differences in transcription rates. However, within a litter, the animals are directly comparable.

From this experiment, we conclude that the transgene was being transcribed at a rate independent of the *raf* genotype. This conclusion was reinforced by examining the relative transcription rates of the endogenous mouse H19 gene in $raf^{a/b}$ and raf^b animals from the two litters. This gene had been previously shown to be *raf* responsive (29), as illustrated by the difference in the steady-state H19 RNA levels in Fig. 5. However, relative to the albumin gene transcription rate, the H19 gene transcription rate was identical in $raf^{a/b}$ and raf^b animals and could not account for the 10- to 50-fold difference in steady-state RNAs. This finding demonstrates the posttranscriptional effects of *raf* on an endogenous single-copy gene and reinforces our conclusions drawn from the multicopy AFP transgene.

DISCUSSION

Previous genetic analysis of the effects of the *raf* gene product on AFP were consistent with its behaving as a cell-autonomous negative regulator (42). However, these experiments could not distinguish the level of action of the *raf* gene product. This study was initiated to identify what element or elements in the AFP gene were required for *raf* responsiveness as the first step to elucidating its mode of action.

The action of the *raf* gene is not evident until after birth, coincident with the dramatic change in AFP transcription rate (3). For this reason, we initially focused our attention on the dominant negative transcriptional element in the AFP gene. In addition, the recessive behavior of the variant raf^b allele was consistent with its encoding a negative regulator. However, the retention of *raf* responsiveness in a series of lines carrying partial and complete deletions in the repressor demonstrated that *raf* achieves its effect independent of the dominant negative element (Fig. 4). In keeping with this conclusion, the repressor element alone could not confer upon the albumin minigene responsiveness to *raf*.

Nuclear run-on analysis was then used to demonstrate that the effects of the *raf* gene were exerted at a posttranscriptional level on both the AFP transgene and the endogenous H19 gene. This assay has been widely used to measure the loading of RNA polymerase II on target genes in intact nuclei. The underlying assumption is that by pulsing the nuclei for very short periods of time in vitro, incorporation of radioactivity is confined to short segments of nascent chains, and any contribution from RNA processing or turnover is minimized (17). The most compelling evidence for a disparity between transcription rate and steady-state RNA between *raf* genotypes was obtained for the endogenous H19 gene. The difference between H19 RNA levels in raf^a and raf^b is 15- to 50-fold, whereas there was at most a 1.5-fold increase in transcription rate (Fig. 5). The differences in steady-state AFP minigene RNA levels are less dramatic; nevertheless, the 0.25- to 2.5-fold differences in transcription rates could not account for the 7- to 10-fold difference in steady-state RNA levels.

That *raf* affects its target genes posttranscriptionally was first suggested by the accumulation of RNA processing intermediates of the AFP transgene in raf^b homozygotes. This was most apparent in those lines which exhibited high levels of postnatal transgene expression, the ZEΔ3 and ZEΔ7 lines (Fig. 4). In the case of ZEΔ3 lines F1 and F2, the presence of nuclear precursors was the only phenotypic consequence of the raf^b mutation (Fig. 4B). raf^a mice already express the ZEΔ3 construct in adult liver at very high levels, approximately 5% of total mRNA, as determined

FIG. 4. *raf* responsiveness in (BALB/cJ × founder) × BALB/cJ progeny of animals bearing deletions of the AFP repressor. (A) Lines G1 and G2 carry ZEΔ1, in which nucleotides between −838 and −604 upstream of the AFP gene were deleted. Each sample was first hybridized with an AFP probe which recognizes exon 1 in both the AFP endogenous gene and minigene (MG) and then hybridized with an AFP-specific probe which recognizes exons 4 to 9, which are present only in the endogenous RNA. Lastly, the same blot was probed with a ribosomal protein L32 (rpL32)-specific probe as described for Fig. 3. (B) Lines F1 and F2 contain ZEΔ3, in which nucleotides between −838 and −250 have been deleted. The protocol described for panel A was used to detect AFP endogenous and minigene RNAs. Transcripts corresponding to the nuclear precursors of the minigene are marked by stars, while the mature endogenous AFP gene is indicated by the square. (C) Lines P1, P2, and P3 contain ZEΔ7, in which nucleotides between −838 and −118 upstream of the AFP gene have been removed. The squares denote the mature endogenous AFP mRNA.

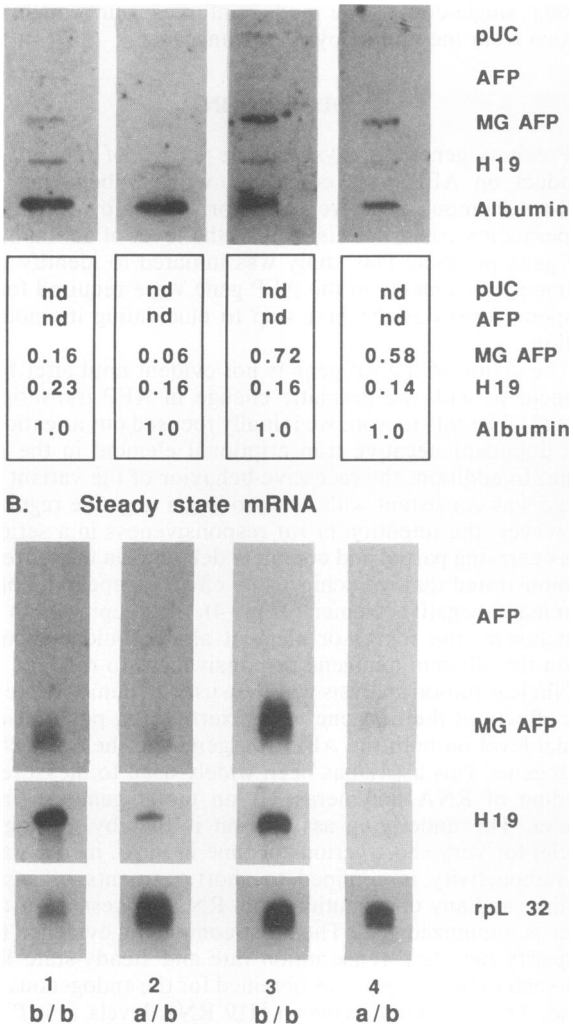

FIG. 5. Transcriptional analysis of the AFP minigene in adult liver. (A) Liver nuclei were isolated from 3 to 4-week-old $raf^{a/b}$ and raf^{b} littermates of a (founder P2 × BALB/cJ) × BALBcJ backcross. The [^{32}P]UTP-labelled transcripts were hybridized to filters on which had been bound denatured linearized plasmid DNA as indicated on the right. Animals 1 and 2 were derived from one litter, and animals 3 and 4 were derived from another. Appropriate exposures of the X-ray films were scanned by densitometry, and the areas under the peaks were quantitated. The values for each of the peaks were expressed relative to that for albumin, which was assigned an arbitrary value of 1.0. MG, minigene; nd, not detectable. (B) Poly(A)$^+$ RNA was isolated from the identical livers, analyzed by agarose gel electrophoresis, and hybridized sequentially to labelled DNA probes that detect both the AFP endogenous gene and minigene (MG), the endogenous H19 gene, and ribosomal protein 32 (rpL 32). The *raf* genotype of each animal was determined from these Northern (RNA) blots and is indicated at the bottom.

by a comparison with AFP mRNA in fetal liver. Thus, it would be impossible to achieve a further 10- to 15-fold increase when the transgene is crossed into a raf^{b} genetic background. The lack of a *raf*-dependent difference in minigene RNA levels in ZEΔ3 mice is best explained if the rate of nuclear to cytoplasmic transport of minigene transcripts becomes the rate-limited step in the synthesis and processing of the minigene in the high-level-expressing strains. Normally in raf^{a} adult mice, the *raf* gene product accelerates the turnover of the nuclear RNAs, leading to a smaller pool of RNAs to be transported to the cytoplasm. In adult raf^{b} mice, the turnover of the primary transcripts is retarded, leading to larger pool of cytoplasmic RNAs. However, in the high-level-expressing ZEΔ3 mice, the transport mechanism itself becomes rate limiting, leading to the accumulation of processing intermediates in raf^{b} mice. Consistent with this model, the ability to detect a *raf*-dependent difference in mature minigene RNA was inversely proportional to the level of expression of the transgene, with the greatest response occurring in the lowest expressors. There is no indication that the minigene transcripts titrate a limiting factor in raf^{a} mice, as the *raf* responsiveness of the endogenous AFP and H19 genes is unaffected by the high levels of the transgene.

The accumulation of the nuclear precursors, while consistent with an effect of *raf* at the level of nuclear processing and/or destabilization of the primary transcript, does not rule out other models. In most instances in which regulation of RNA stability has been documented, the mechanism is thought to act via the stabilization of a mature cytoplasmic mRNA, for example, the stabilization of vitellogenin mRNA by estrogen in *Xenopus laevis* oocytes (5). In several well-documented cases of regulation of cytoplasmic mRNA stability, the cell cycle-dependent histone mRNA destabilization (16) and tubulin mRNA destabilization (12), the mechanism is thought to occur cotranslationally. There are fewer examples in which regulation is imposed in the nucleus. Prendergast and Cole (32) identified two genes whose expression was affected by c-*myc* at a step between transcription and export into the cytoplasm. Kiledjian and Kadesch (21) have recently shown that the differential expression of the human liver/bone/kidney alkaline phosphatase gene in osteoblasts versus liver cannot be explained by differences in either transcription or cytoplasmic RNA stability. They argue, on the basis of the buildup of nuclear forms in the high-level-expressing tissue, that the control point may be the stability of the primary transcript. Their detection of splicing intermediates in the high-level-expressing osteoblasts is consistent with this hypothesis and is reminiscent of the precursor accumulation that we observe in raf^{b} homozygotes (Fig. 4).

Whatever the mechanism, *raf* utilizes elements in the AFP gene that are contained within the minigene, that is, the first 2.4 kb and the last 1.0 kb of the 22-kb gene. This would include the entire 5′ and 3′ untranslated regions, which have been shown to harbor control elements for mRNA stability (8). Signals that confer differential stability of RNAs include secondary structure, such as the stem-loop at the 3′ end of histone mRNA required for its degradation after S phase (30), and specific primary sequence, such as the A+U-rich motif in the 3′ untranslated region of a variety of growth-regulating genes (20, 35). A survey of sequences in these regions of the AFP gene and the entire H19 gene revealed few primary sequence similarities that could account for the common *raf*-dependent posttranscriptional regulation. However, in both RNAs we noted that the polyadenylation signal was preceded by a putative stem-loop whose structure was conserved in the human cognates as well (Fig. 6). No comparable predicted structure occurs at this position in the serum albumin gene, which is not *raf* regulated (3), in several species examined. The H19 RNA product, which is not thought to be translated, displays extensive secondary structure throughout its length (39). Thus, the stem-loop at its polyadenylation site is not unique. The pattern of compensatory base changes in the human sequence, indicated by the

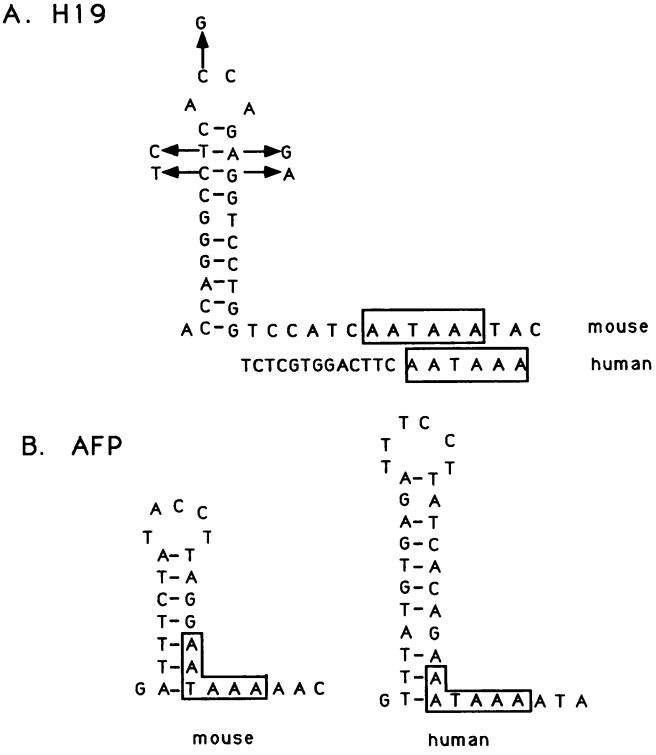

FIG. 6. Stem-loop at the 3′ ends of AFP and H19 RNAs. The sequences immediately surrounding the polyadenylation sites of the human and mouse AFP and H19 RNAs are depicted. The AAUAAA polyadenylation signal is indicated by a box. (A) The mouse H19 RNA sequence, with changes in the human RNA indicated by arrows. The two sequences downstream of the stem loop are indicated separately. (B) The 3′ ends of the human and mouse AFP RNAs.

arrows in Fig. 6, argues strongly that this structure is being conserved in evolution. In contrast, AFP mRNA displays very little predicted secondary structure, and the 3′ distal loop depicted in Fig. 6 is the most prominent example. Whether this putative structure mediates *raf* responsiveness remains to be determined.

The degree to which transcriptional and posttranscriptional mechanisms contribute to the postnatal repression of AFP mRNA cannot be determined at present. Only one variant allele of *raf* has been identified, in the BALB/cJ strain of mice. That variant allele results in a 15- to 50-fold increase in adult basal levels of AFP and H19 RNAs, respectively. If the *raf* mutation in BALB/cJ is a null, it would imply that *raf* plays a relatively minor role in the developmental regulation of AFP, which undergoes a 10^4-fold decrease in mRNA levels, but a more substantial role in the down-regulation of H19, which declines 600-fold. If, on the other hand, the mutation is a subtle one, it leaves open the possibility that *raf* plays a more prominant role for both genes. A molecular analysis of the wild-type and mutant *raf* genes will resolve this issue.

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