MOLECULAR AND CELLULAR BIOLOGY, Nov. 1982, p. 1427–1435
0270-7306/82/111427-09$02.00/0

Vol. 2, No. 11

Genetic Analysis of α-Fetoprotein Synthesis in Mice

ALEXANDRA BELAYEW AND SHIRLEY M. TILGHMAN*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Received 11 May 1982/Accepted 23 August 1982

The differential induction of α-fetoprotein (AFP) mRNA during liver regeneration in three inbred strains of mice was examined to determine the genetic and molecular bases for the differences in protein production. BALB/cJ, C3H/He, and C57BL/6 mice, previously identified as high, intermediate, and low AFP producers, respectively, were used. Liver AFP mRNA concentrations during normal development and after carbon tetrachloride administration were measured and shown to correlate exactly with the serum protein concentrations. By performing a series of genetic crosses, we identified two unlinked genetic loci that acted independently to affect the inducibility of AFP mRNA. The *raf* gene, previously identified by Olsson et al. (J. Exp. Med. **145**:819–827, 1977), determines the adult basal level of AFP mRNA, and the *Rif* gene affects its inducibility during regeneration. By using a polymorphic restriction endonuclease site within the albumin-AFP structural gene region, we show that neither regulatory gene is closely linked to the structural genes. In addition, neither gene affects the concentration of albumin mRNA during development or liver regeneration.

α-Fetoprotein (AFP) is a 70,000-dalton glycoprotein which is synthesized and secreted by the liver, the yolk sac, and, to a much lesser extent, the gastrointestinal tract of all mammalian fetuses. Its concentration in the serum decreases 10^4- to 10^5-fold within a few weeks after birth in mice and remains at a low basal level in normal adult mice. The synthesis of AFP can resume in hepatocellular carcinomas and teratocarcinomas or in untransformed liver cells during liver regeneration induced by a variety of conditions, such as acute hepatitis, partial hepatectomy, and chemical poisoning (reviewed in references 1 and 19).

The degree of inducibility of AFP in the adult mouse liver during regeneration is strain dependent. Abelev (1) reported that C57BL/6 mice produce 10-fold less AFP after CCl_4 treatment than do C3H mice, whose induced levels are representative of most mouse strains. Olsson et al. (17) and Jalanko (11) described in the BALB/cJ strain an autosomal recessive trait, termed *raf* (regulation of AFP), which in the homozygous adult resulted in an elevated basal serum AFP concentration as well as a 10- to 15-fold overproduction during liver regeneration.

We wished to examine the molecular basis of AFP overproduction in BALB/cJ mice and its underproduction in C57BL/6 mice by assaying the extent of induction of AFP mRNA in these strains. The results showed that the differences in the serum AFP concentrations in these animals can be attributed to differences in steady-state AFP mRNA. Secondly, appropriate genetic crosses between BALB/cJ, C57BL/6, and C3H mice were generated to determine whether the low production of AFP mRNA in C57BL/6 mice was the result of a different allele of the *raf* gene and whether either phenotype segregated with the albumin-AFP structural gene region, previously identified on chromosome 5 (6, 10). By these experiments a second genetic locus, which we term *Rif* (regulation of induction of AFP) is defined. *Rif* and *raf* are unlinked, and neither is closely linked to the structural gene region.

MATERIALS AND METHODS

Animals. BALB/cJ mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. C3H/HeIcr and C57BL/6Icr mice were obtained from the Laboratory Animal Facility of the Institute for Cancer Research, where all the breeding experiments were conducted. All mice were fed a standard diet of Old Guilford O.G.96W and acidified tap water and were housed in pine shavings. Between 5 and 7 p.m., 4-week-old male mice were injected once intraperitoneally with 50 μl of 10% CCl_4 in mineral oil. Control animals received 50 μl of mineral oil at the same time. The mice were killed by cervical dislocation at various times after the injection. For each animal, the liver, kidneys, spleen, heart, and lungs were removed, washed in cold saline, frozen in dry ice, and kept at −70°C.

RNA extraction and analysis. Total RNA was extracted from frozen livers by the guanidinium thiocyanate-guanidinium hydrochloride method described by Chirgwin et al. (5). For the analysis of the backcross

mice, liver cytoplasmic RNA from each animal was prepared by phenol extraction by the method of Berridge and Lane (3). Enrichment of polyadenylated [poly(A)$^+$] RNA was obtained by oligodeoxythymidylate [oligo(dT)]-cellulose chromatography (2).

(i) RNA dot blots. Serial dilutions of each RNA sample in distilled water were spotted onto moist nitrocellulose paper (0.45 μm; Millipore Corp.) previously equilibrated in 3 M NaCl–0.3 M sodium citrate as described by Thomas (23). After being baked for 2 h at 80°C, the paper was prehybridized, hybridized, and washed by the method of Wahl et al. (26).

The probes used were the chimeric plasmids pmAFP3 (7), pmalb2 (12), and pDHFR11 (kindly provided by J. Schilling and R. T. Schimke, Stanford University) containing, respectively, 910, 620, and 1,600 base pairs of AFP, albumin, and dihydrofolate reductase (DHFR) cDNA sequences. They were labeled by nick translation (15) in the presence of [α-^{32}P]dCTP to a specific activity of 4×10^7 to 10×10^7 cpm/μg. Two RNA samples, non-polyadenylated [poly(A)$^-$] RNAs from fetal yolk sacs or adult mouse livers, were used as standards for AFP and albumin mRNA, respectively. Their precise concentration was determined by DNA-RNA hybridization in solution. For accurate quantitation, individual filter "dots" of six serial dilutions were cut out, and the bound radioactivity was determined by liquid scintillation counting. The slope of the plot of micrograms of RNA versus counts per minute was calculated and compared with that for the standard RNAs.

(ii) DNA-RNA hybridizations. RNA excess hybridizations were performed in a final volume of 50 μl in sealed capillary tubes containing 400 mM NaCl, 10 mM Tris-hydrochloride (pH 7.0), 2 mM EDTA, 0.004 to 5 mg of RNA per ml, and 10 to 20 pg of the [^{32}P]cDNA probe. After being held for various times at 68°C, the solutions were quickly cooled on ice and transferred into 1.5 ml of 300 mM NaCl–30 mM sodium acetate (pH 4.5)–3 mM $ZnCl_2$–10 μg of denatured calf thymus DNA per ml. A 1-ml amount of this solution was digested with 300 U of S1 nuclease (*Aspergillus oryzae*; Miles Laboratories) for 1 h at 42°C. The hybrids resistant to S1 digestion and the nondigested control (0.5 ml) were precipitated in cold 5% trichloroacetic acid (TCA) and filtered onto nitrocellulose. The percentage of S1-resistant hybrids was calculated after determination of the radioactivity on the dried filters by liquid scintillation counting.

The probes used were the cDNA inserts of either chimeric plasmid pmAFP1 (25) or pmalb2 (12) labeled selectively on the anticoding strand by T4 DNA polymerase in the presence of [α-^{32}P]dCTP by the method of O'Farrell et al. (16). *Eco*RI linearized plasmid (0.1 μg) was incubated at 37°C in 20 μl of 30 mM Tris-acetate (pH 8.0)–10 mM magnesium acetate–60 mM potassium acetate–0.5 mM dithiothreitol–0.1 mg of bovine serum albumin per ml–5 U of T4 DNA polymerase (Bethesda Research Laboratories) until the cDNA insert anticoding strand was completely digested by the 3′→ 5′ exonuclease activity of the enzyme. Polymerase activity was allowed to proceed in 40 μl of the same solution containing 150 mM cold dATP, dGTP, and dTTP and 150 μCi of [α-^{32}P]dCTP by incubation at 37°C. The reaction was stopped by addition of 50 mM EDTA and heating at 65°C for 5 min. After ethanol precipitation in the presence of tRNA, the plasmid was digested with *Hin*dIII to release the insert, which was then purified by electrophoresis on an agarose gel, electroelution, and ethanol precipitation with carrier tRNA.

The probes were boiled for 10 min and quenched on ice just before use. The S1 resistance of the denatured probes was 10 and 20% for the albumin and AFP probes, respectively. These values have not been subtracted from the data presented.

C_rt values were standardized for a salt concentration of 0.18 M NaCl (4), and a $C_rt_{1/2}$ of 3.6×10^{-3} was used for both pure mRNAs to calculate absolute values for mRNA concentrations. This value is derived by using a corrected $C_rt_{1/2}$ value for α and β globin mRNA (9), whose complexity is 1,200 nucleotides or 55% that of AFP or albumin mRNA, which is 2,200 nucleotides (7).

Genomic DNA extraction. The nuclei were purified from the pooled kidneys, spleen, heart, and lungs from each mouse by the method of Wu et al. (27). The nuclei were suspended in 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl–25 mM EDTA and lysed in 1% sodium dodecyl sulfate. The DNA was digested for 2 h at 37°C in 1 mg of proteinase K (Beckman) per ml, and then the solution was adjusted to 0.5 M NaCl and extracted with phenol-chloroform-isoamyl alcohol (25:25:1). After ethanol precipitation, the DNA was dissolved in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), digested for 30 min at 37°C with 20 μg of RNase A (previously boiled for 20 min in 20 mM sodium acetate, pH 5.5) per ml, and then extracted with phenol.

Genomic DNA (10 μg) was digested overnight at 37°C by 2.5 U of *Eco*RI, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose by the procedure of Southern (21). The paper was baked for 2 h at 80°C, prehybridized, hybridized, and washed as described by Wahl et al. (26).

The probe used was a 0.7-kilobase (kb) *Eco*RI-*Pvu*II fragment purified from pAFP14Z, a subcloned *Eco*RI genomic DNA fragment of λ AFP14 (12).

[*methyl*-^{3}H]thymidine incorporation. Mice were injected intraperitoneally with 100 μCi of [*methyl*-^{3}H]thymidine (specific activity, 75 Ci/mmol; New England Nuclear Corp.) between 12 p.m. and 1 p.m. and killed 4 h later. The livers were removed, washed in cold 0.9% NaCl, and frozen on dry ice. For each liver, a 1:10 (wt/vol) homogenate was made with a Potter-Elvejhem homogenizer in 60 mM Tris-hydrochloride (pH 7.5)–0.25 M sucrose. A 0.1-ml amount of the homogenate was precipitated in 1 ml of 10% TCA and centrifuged, the pellet was washed two times with 10% TCA and dissolved overnight at 52°C in 400 μl of NCS (Amersham Corp.), and the radioactivity was determined by liquid scintillation counting.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was measured by the method of Taketa and Watanabe (22) by following the rate of NADPH formation. The activity is expressed in standard units (micromoles of NADPH formed per minute).

RESULTS

AFP mRNA during postnatal development. The persistence of an elevated level of serum AFP after birth in BALB/cJ mice was first used to identify the *raf* trait (17). To test whether this

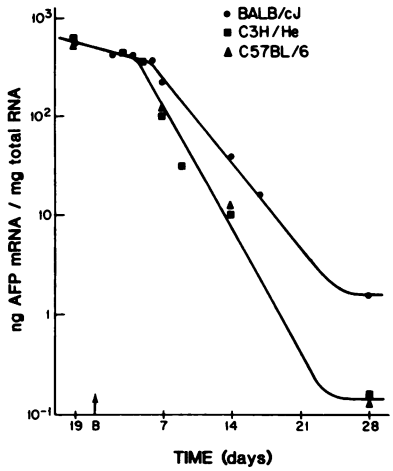

FIG. 1. Decrease in total hepatic AFP mRNA after birth. The concentration of AFP mRNA in total liver RNA from BALB/cJ (●), C3H/He (■), and C57BL/6 (▲) at 19 days of gestation and at the indicated times after birth (B) were determined by dot blot hybridization of total RNA for all points except those at 28 days, which were determined by using poly(A)$^+$ RNA.

was the result of a persistence of AFP mRNA, we extracted total RNA (5) from the livers of C57BL/6 (low AFP producers), C3H/He (intermediate AFP producers), and BALB/cJ (high AFP producers) mice at different times during the late prenatal and early postnatal periods. AFP mRNA levels were determined by RNA dot blot hybridization (23) with a labeled probe consisting of cloned AFP mRNA sequences (25). The AFP mRNA steady-state concentration decreased to the same extent in each strain during the first week after birth (Fig. 1). From the first to the third week, there was an approximately fivefold difference in the rate of decrease in the BALB/cJ strain compared with that in the other strains. This difference was significantly less than the 100-fold difference between BALB/cJ and C3H/He mice in the serum protein concentrations observed by Olsson et al. (17) at 3 weeks. On the other hand, Jalanko (11) observed differences in serum AFP after birth between BALB/cJ and C57BL/6 mice that are more in keeping with the results shown in Fig. 1. In all three strains the concentration of liver AFP mRNA fell below the lower limits of detectability in total RNA by 3 to 4 weeks after birth. When total RNA from 4-week-old BALB/cJ mice, enriched 25-fold in poly(A)$^+$ RNA by chromatography on oligo(dT)-cellulose, was assayed by RNA dot blot hybridization, a basal AFP mRNA level of about 1 ng/mg of total RNA was detected. The basal levels in both C3H and C57BL/6 mice were approximately 10-fold less, or 0.1 ng/mg of total RNA. Thus, the 10-fold-elevated serum AFP levels in BALB/cJ mice, attributable to the *raf* gene, is regulated at the level of steady-state mRNA.

AFP induction during liver regeneration. Differences in the extent of induction of AFP mRNA in 4-week-old animals after CCl_4 injection was next examined. CCl_4 injection results in extensive liver necrosis and regeneration, during which time there is a transient increase in AFP serum concentration which peaks 4 days after treatment (reviewed in references 1 and 19). Animals were taken 3 days after injection, and total hepatic RNA was prepared. An accurate measure of AFP mRNA was obtained by hybridizing a large excess of total RNA in solution to a denatured double-stranded cDNA insert of a chimeric AFP cDNA plasmid, pmAFP1 (25), in which the strand complementary to the mRNA was labeled by T4 DNA polymerase (16). The degree of hybridization was assessed after S1 nuclease digestion.

The $C_rt_{1/2}$ values (Fig. 2) correspond, respectively, to 180 ng of AFP mRNA/mg of total RNA for BALB/cJ mice and 12 ng/mg for C3H/He mice. In C57BL/6 mice, the levels were so low they could not be determined in total RNA. With poly(A)$^+$ RNA, the concentration of AFP mRNA was estimated by RNA dot blot analysis to be 1 ng/mg of total RNA. The limited extent of AFP mRNA induction in C57BL/6 mice was not caused by suboptimal doses of CCl_4, as doses up to 10-fold higher than that used to obtain the results shown in Fig. 2 did not result in increased levels of AFP mRNA.

The 15-fold difference between the induced levels of AFP mRNA in BALB/cJ and C3H/He mice and the additional 10-fold difference between the levels in C3H/He and C57BL/6 mice mirror very well the differences described by Jalanko (11) for the serum protein concentra-

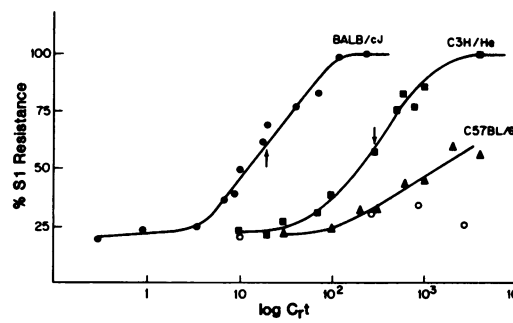

FIG. 2. Quantitation of AFP mRNA in total hepatic RNA during liver regeneration. The concentration of AFP mRNA was determined with total hepatic RNA from BALB/cJ (●), C3H/He (■), and C57BL/6 (▲) 4-week-old mice 3 days after CCl_4 treatment by solution hybridization to cloned, labeled AFP cDNA. (○) Background of the ^{32}P-labeled AFP cDNA reassociation.

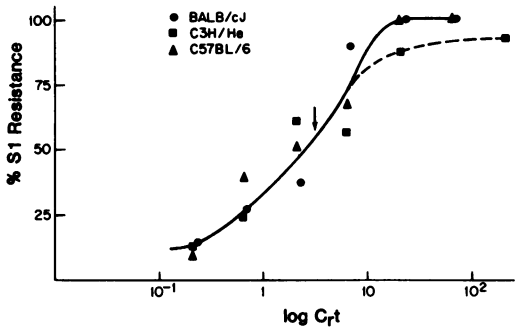

FIG. 3. Quantitation of albumin mRNA in total hepatic RNA during regeneration. The concentration of albumin mRNA in total RNA from BALB/cJ (●), C3H/He (■), and C57BL/6 (▲) mice 3 days after CCl_4 treatment was determined by hybridization in solution to ^{32}P-labeled cloned albumin cDNA.

tions. These differences could not be attributed to the quality of the RNA preparations, as no variation in albumin mRNA levels was seen in any of these strains (Fig. 3). A steady-state level of 1.2 μg of albumin mRNA per mg of total RNA was measured in all of the strains and did not change during regeneration.

The differences in AFP mRNA induction (Fig. 2) could be caused by various degrees of liver necrosis and subsequent regeneration. To assess this possibility, we measured three markers of liver necrosis and DNA replication. Glucose-6-phosphate dehydrogenase activity, which is enhanced by liver injury (22), was assayed in the soluble fraction of liver homogenates prepared from animals 2 days after CCl_4 injection and from control animals. In the C3H/He and C57BL/6 mice, the activity increased from 15 mU/mg of protein in the controls to 40 mU/mg of protein. In BALB/cJ mice, the induction was less than twofold.

DHFR mRNA levels, which are stabilized during cellular division (14), were assayed in liver poly(A)$^+$ RNA by RNA dot blot hybridization, with ^{32}P-labeled DHFR cDNA plasmid used as a probe. A two- to threefold increase in its concentration was found 2 days after CCl_4 treatment in all three mouse strains (data not shown).

Finally, DNA synthesis, as measured by [*methyl*-^{3}H]thymidine incorporation into the TCA-precipitable fraction of a liver homogenate during a 4-h pulse, reached a maximum 2 days after CCl_4 injection (Fig. 4B) and returned to normal 8 days later, in agreement with the data obtained by Schultze et al. (20). No significant difference in either the time course or the extent of DNA synthesis was observed among the three mouse strains. When histological slides of liver slices from pulse-labeled animals 3 days after CCl_4 administration were examined by autoradiography to determine the number of labeled nuclei, 16% of the cells were labeled in each strain. Thus, the difference in the inducibility of AFP mRNA observed cannot be attributed to different responses to CCl_4-induced liver injury. This conclusion is supported by Jalanko (11), who showed that when regeneration is induced by alternative means, such as partial hepatectomy, AFP serum levels are elevated in BALB/cJ mice relative to another BALB/c subline, BALB/cDUB mice.

Figure 4A also shows the close temporal relationship between AFP mRNA induction and DNA synthesis. As illustrated for BALB/cJ mice, an increase in AFP mRNA was observed by 1 day after CCl_4 injection, peaked at 3 days, and returned to basal levels by 10 days. The time

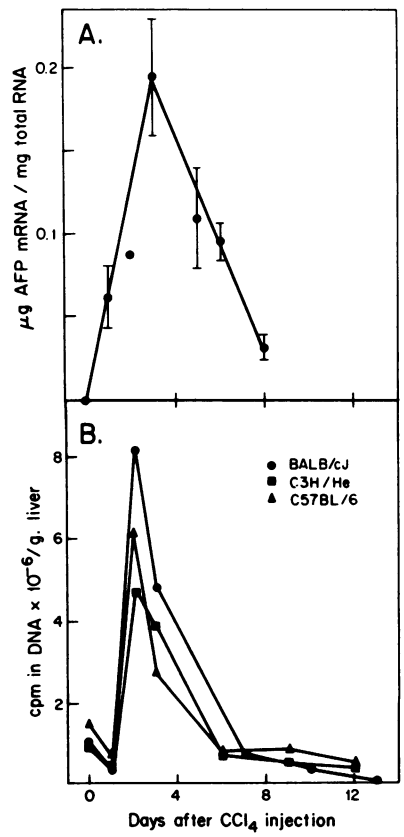

FIG. 4. Time course of AFP mRNA induction and DNA synthesis during liver regeneration. (A) Concentration of AFP mRNA in total hepatic RNA from BALB/cJ mice was assayed by dot blot hybridization at the indicated times after CCl_4 administration. (B) Incorporation of [*methyl*-^{3}H]thymidine into TCA-precipitable DNA was determined in BALB/cJ (●), C3H/He (■), and C57BL/6 (▲) mice during the same time periods after CCl_4 administration. The values are the mean results from four to eight animals per point.

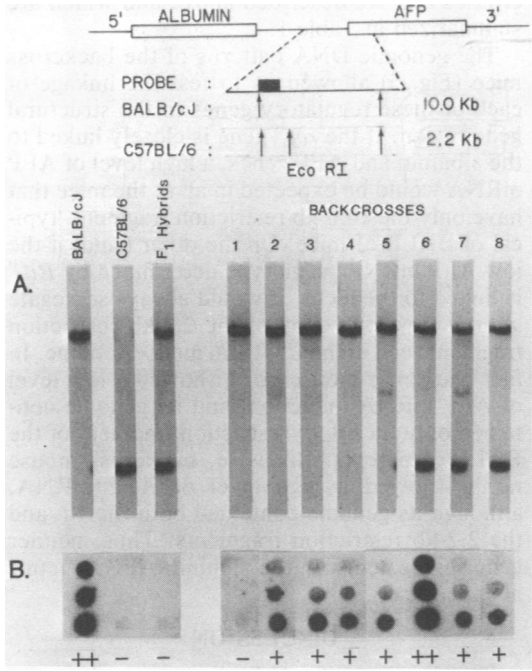

FIG. 5. Genetic analysis of AFP mRNA inducibility during liver regeneration. (Top) Albumin-AFP structural gene region, drawn with the positions of the two genes indicated by the rectangles. Beneath these is shown the relative position of a polymorphic *Eco*RI site, located between the genes, that generates a 10-kb *Eco*RI fragment in BALB/cJ DNA and a 2.2-kb band in C57BL/6 DNA when either is hybridized to a 0.65-kb *Eco*RI-*Pvu*II restriction probe, as indicated. (A) Genomic DNA from the parental strains, the F_1 hybrids, and eight representative (C57BL/6 × BALB/cJ)F_1 × BALB/cJ backcross mice were digested with *Eco*RI, analyzed on a 1% agarose gel, transferred to nitrocellulose, and hybridized to the *Eco*RI-*Pvu*II probe. (B) Total hepatic RNA from the same animals was blotted onto nitrocellulose (0.4, 0.8, and 1.6 μg, top to bottom) and hybridized to pmAFP3. The symbols refer to high (++), medium (+), and low (−) AFP mRNA levels, Each column in panels A and B represents a single mouse.

courses in C3H/He and C57BL/6 mice were identical (data not shown).

Genetic analysis of AFP induction during regeneration. We first asked whether any major rearrangement of DNA sequences could be found in the albumin-AFP gene region which might account for the differences among the three strains as shown in Fig. 2. Approximately 60 kb of contiguous DNA containing the mouse albumin and AFP genes have been isolated from a BALB/cNIH genomic library and characterized in detail (8, 10, 12). With six different single-copy DNA fragments scattered along the region used as probes, and 20 different restriction enzymes, no differences among the genomic DNA from the three strains were found except for an extra *Eco*RI site in C57BL/6 mouse DNA, located about 5 kb upstream from the 5′ end of the AFP gene (Fig. 5). This polymorphic site was then used to follow the segregation of the structural genes in the genetic crosses described below.

Olsson et al. (17) demonstrated by appropriate genetic crosses that the *raf* trait which controls the persistence of AFP production in BALB/cJ mice behaves as an autosomal recessive gene. We confirmed this result by crossing female C3H/He mice (intermediate or wild-type producers) with BALB/cJ males and backcrossing the F_1 hybrid females with BALB/cJ males. The male offspring of both generations were injected with CCl_4 at 4 weeks of age and killed 3 days later. For each mouse, total or poly(A)$^+$ cytoplasmic RNA was prepared from the liver (2, 3), and the RNA was assayed for both AFP and albumin mRNA content by RNA dot blot hybridization. The albumin mRNA served as an internal standard, because it was not altered by CCl_4 treatment in any of the strains considered (Fig. 3).

The F_1 hybrids (C3H/He × BALB/cJ) expressed AFP mRNA at a level comparable to that of the C3H/He parent, and their backcrosses to BALB/cJ mice were equally distributed between intermediate and high AFP producers (Table 1), which was consistent with high AFP production behaving like a single recessive Mendelian trait.

To investigate the genetic basis of the low AFP production in C57BL/6 mice, we crossed females of this strain with C3H/He males, and the F_1 hybrid females were then backcrossed to C3H/He males. When hepatic AFP mRNA was assayed in animals treated with CCl_4, it was observed that the F_1 hybrids showed the AFP mRNA levels of the C57BL/6 parent and that the backcross animals were equally distributed between low and intermediate producers (Table 1).

TABLE 1. Distribution of AFP mRNA inducibility in genetic crosses

No.	Cross	No. (%) of mice with AFP mRNA level[a]:		
		++	+	−
1	(C3H/He × BALB/cJ)F_1 × BALB/cJ	8	9	
2	(C57BL/6 × C3H/He)F_1 × C3H/He		5	5
3	(C57BL/6 × BALB/cJ)F_1 × BALB/cJ	6 (22)	13 (48)	8 (30)

[a] High (++), intermediate (+), and low (−) levels of hepatic AFP mRNA were determined 3 days after CCl_4 injection.

Thus, the low production observed in C57BL/6 mice behaves as a single autosomal dominant trait.

Two questions arise from the results described above. Is the limited AFP mRNA induction found in the C57BL/6 mice caused by an allele of the *raf* gene or a separate genetic locus? Are these BALB/cJ and C57BL/6 genetic traits linked to the albumin-AFP genes?

To answer these questions, we crossed C57BL/6 females (low AFP producers) with BALB/cJ males (high AFP producers) and backcrossed the F_1 females with BALB/cJ males. For each mouse, the CCl_4 induction of AFP mRNA in the liver was examined by RNA dot blot hybridization as described above. Moreover, high-molecular-weight genomic DNA was extracted from the pooled kidneys, spleen, heart, and lungs of each mouse, digested with *Eco*RI, fractionated by electrophoresis on an agarose gel, and transferred to nitrocellulose paper (21). The papers were hybridized to an intergenic genomic DNA probe, which hybridized to a 10.0-kb fragment in BALB/cJ DNA and a 2.2-kb fragment in C57BL/6 DNA due to the extra *Eco*RI site found in this strain (Fig. 5).

The parental BALB/cJ strain showed a high level of AFP mRNA along with the 10.0-kb genomic fragment, whereas the parental C57BL/6 strain had a low AFP mRNA level and the 2.2-kb genomic fragment (Fig. 5). The genomic DNA pattern of a (C57BL/6 × BALB/cJ)F_1 hybrid contained the restriction fragments typical of both parents. The F_1 RNA phenotype was identical to that of the C57BL/6 parent, indicating that low inducibility is dominant over high inducibility, as was expected from the previous crosses.

The degree of inducibility of AFP mRNA in (C57BL/6 × BALB/cJ)F_1 × BALB/cJ backcross mice did not partition in the 50:50 distribution that would be expected for two different alleles of the same Mendelian trait, *raf*. Instead, as suggested by the data shown in Fig. 5 and Table 1, 22% of the backcross mice were high producers, 30% were low producers, and 48% exhibited an intermediate level similar to that seen in C3H/He mice. These results are consistent with the presence of two separate unlinked genetic loci; the recessive *raf* trait in BALB/cJ mice, which we will now designate raf^b to distinguish it from the "wild-type" raf^a allele in C3H/He and C57BL/6 mice, and a separate trait, which we term *Rif* (regulation of induction of AFP), which is dominant in C57BL/6 mice and will be referred to as Rif^b, with respect to the "wild-type" Rif^a allele in C3H/He and BALB/cJ mice. That there are only two and not more genetic loci affecting AFP mRNA inducibility in these mice is supported by the two other series of crosses that we described above and which are summarized in Table 1.

The genomic DNA patterns of the backcross mice (Fig. 5) allowed us to test the linkage of each of these regulatory genes to the structural gene region. If the raf^b gene is closely linked to the albumin and AFP genes, a high level of AFP mRNA would be expected in all of the mice that have only the 10.0-kb restriction fragment, typical of BALB/cJ mice. On the other hand, if the low AFP mRNA phenotype determined by Rif^b is linked to the locus, it would always segregate with at least one copy of the 2.2-kb restriction fragment seen in the C57BL/6 mouse genome. In fact backcross mouse no. 1 showed a low level of AFP mRNA induction, and its genome contained only the 10-kb restriction fragment of the BALB/cJ parents. Likewise, backcross mouse no. 6 showed a high level of AFP mRNA, although its genome contained both the 10- and the 2.2-kb restriction fragments. Thus, neither gene segregated with the albumin-AFP structural gene region.

DISCUSSION

By using specific hybridization probes, we have shown that the raf^b allele, which controls the elevated basal levels of AFP in BALB/cJ mice and its subsequent overproduction during regeneration, acts by increasing the level of AFP mRNA. Differences in mRNA levels in each liver could result from regulatory mechanisms acting either on the dynamics of cell populations in the liver, i.e., by varying the number of AFP-producing cells, or at the intracellular level on the rates of synthesis or catabolism of AFP mRNA per cell. Strong support for the latter was obtained by Kuhlman (13), who used immunofluorescence staining of liver slices to demonstrate an equivalent number and distribution of AFP-containing hepatocytes after CCl_4 treatment in BALB/cJ and C3H/He mice. The difference in AFP production was solely accounted for by the relative intensities of fluorescence staining per cell.

Our studies with C57BL/6 mice revealed the existence of a second genetic locus in the mouse that affects AFP gene expression during liver regeneration. This locus, which we termed *Rif*, affects the inducibility of AFP mRNA during regeneration induced by either CCl_4 (Fig. 2) or partial hepatectomy (11). The degree of liver damage or DNA synthesis cannot account for the strain-specific differences, as a similar degree of inducibility of glucose-6-phosphate dehydrogenase, DHFR mRNA, and [*methyl*-^{3}H]thymidine incorporation into DNA in the liver was found in all three mouse strains (Fig. 4).

The genetic crosses (Fig. 5) allowed us to conclude that *Rif* and *raf* were separate, un-

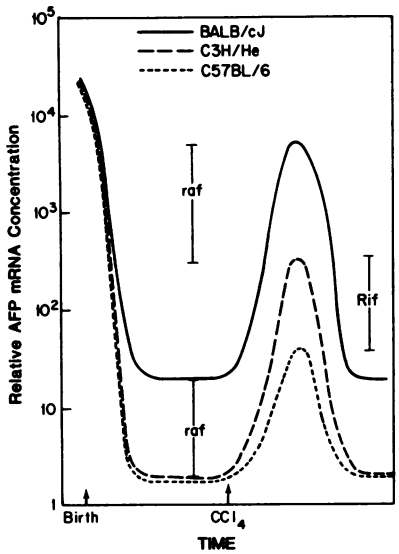

FIG. 6. Model to explain the action of the *Rif* and *raf* genes. The data from Fig. 1 and 2 have been redrawn to emphasize the roles of *raf* and *Rif* in liver development and regeneration. The difference between the basal levels of AFP mRNA in BALB/cJ (——) and both C3H/He (– – –) and C57BL/6 (. . . . .) mice and the difference in the induced levels during regeneration between BALB/cJ and C3H/He mice are ascribed to *raf* gene action. The difference between C3H/He and C57BL/6 mouse AFP mRNA during regeneration is ascribed to *Rif* gene action.

linked loci, neither of which segregated with the structural albumin-AFP gene region on chromosome 5 (6, 10). We would propose the following model to explain the independent actions of *Rif* and *raf*. In mice which are homozygous for the Rif^a allele, a 200-fold induction of AFP mRNA was observed over the basal level determined by raf^b or raf^a in BALB/cJ and C3H/He mice, respectively (Fig. 6). The dominant allele, Rif^b, in C57BL/6 mice results in only a 10-fold induction over the low basal level controlled by raf^a; i.e., the inducibility is entirely under the influence of the *Rif* gene, which acts upon a constant basal level of AFP mRNA that is determined by *raf*. This allows us to understand the 1:2:1 distribution of high, medium, and low producers of AFP in the (C57BL/6 × BALB/cJ)F_1 × BALB/cJ backcrosses (Fig. 5 and 7). The 25% high-producer phenotypes are generated from genotypes indistinguishable from the BALB/cJ parents (raf^b/raf^b Rif^a/Rif^a), and the 25% low-producer phenotypes are generated from (raf^a/raf^b Rif^a/Rif^b) mice that are equivalent to the F_1 hybrids. The intermediate producers are equally distributed between (raf^a/raf^b Rif^a/Rif^a) mice, which have a low basal level determined by raf^a but a 200-fold induction of mRNA determined by Rif^a and thus resemble wild-type C3H/He mice [(raf^a/raf^a Rif^a/Rif^a)], and (raf^b/raf^b Rif^a/Rif^b) mice, which have a high basal level coupled to a low 10-fold induction of mRNA. This effectively explains why, in the last group, the presence of the dominant Rif^b allele did not result in a low-producer phenotype.

Preliminary evidence that both *Rif* and *raf* act at the level of transcription of the AFP gene has been obtained by nuclear transcription assays (S. M. Tilghman, unpublished data). When hepatic nuclei from both CCl_4-treated and control BALB/cJ mice were incubated for 5 min in the

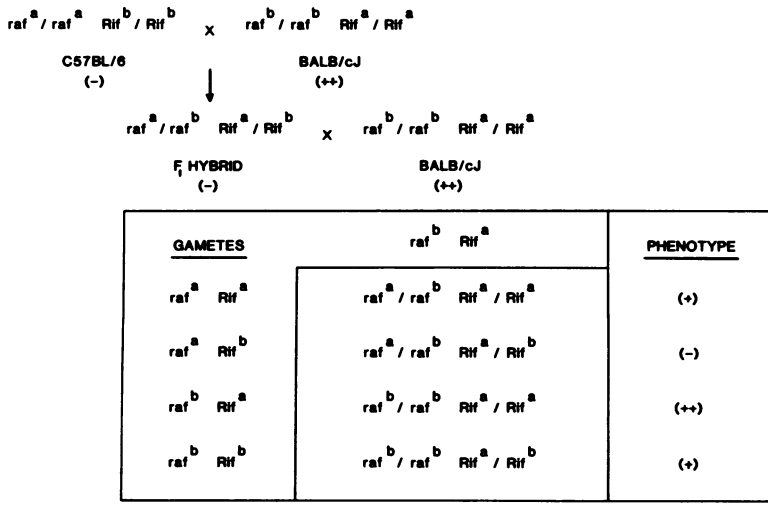

FIG. 7. Genotypic and phenotypic analysis of (C57BL/6 × BALB/cJ)F_1 × BALB/cJ mice. The data shown in Fig. 5 are interpreted in terms of two genetic loci, *Rif* and *raf*, to explain the 1:2:1 distribution of high (++), medium (+), and low (–) AFP mRNA levels in CCl_4-treated animals.

presence of [^{32}P]UTP, a significant level of nascent AFP transcripts relative to albumin transcripts was observed only with the nuclei from treated animals, suggesting that the induction, under the control of *Rif*, acts at transcription. The technique was not sensitive enough to detect the 15-fold-lower AFP mRNA induction seen in the C3H/He mice, which implies that the elevated basal AFP mRNA level in BALB/cJ mice, under the control of *raf*, must also be controlled at least in part at the level of transcription. If *raf* influenced only the half-life of AFP mRNA, then a similar in vitro AFP transcription rate for BALB/cJ and C3H/He mouse nuclei, which are both *Rif*^{a}/*Rif*^{a}, would have been seen.

The *raf*^{b} allele is probably rare in inbred mice. Of the 27 strains tested by Olsson et al. (17), only the BALB/c substrain BALB/cJ showed an elevated basal AFP level. Potter and Wax (18) recently reviewed the genetic history of the BALB/cJ line and were able to identify in the literature only four other references to genetic differences between BALB/cJ mice and other BALB/c sublines: a difference in Qa-2 and Qa-3 lymphocyte alloantigens, which maps to chromosome 17; a decreased susceptibility to plasmacytomas; a twofold difference in three adrenal medullary enzymes in catecholamine biosynthesis; and extremely aggressive behavior. The latter three traits have not been mapped. A higher incidence of different *Rif* alleles is likely, as Jalanko (11) found a low AFP mRNA induction in both C57BL/6 and C57BL/10 mice, and we have found AKR mice to be low producers as well (data not shown).

No differences in AFP mRNA concentrations were observed among the three strains in fetal liver RNA (Fig. 1). The effects of the recessive *raf*^{b} allele were first observed at 1 week postpartum, when the concentration of AFP mRNA is rapidly declining as the result of a decrease in the rate of transcription of the AFP gene (24). The *Rif*-controlled phenotype has only been observed during reinduction of AFP mRNA in regenerating livers. Thus, it must be concluded that neither of these regulatory loci are acting early in development to affect AFP mRNA levels. It is possible that other regulatory loci, as yet unidentified, fulfill this function in development. Alternatively, it may be that regulation of the AFP gene in the liver requires only the postnatal repression of a constitutive, unmodulated rate of expression of the gene before birth. The appearance of the *raf*-mediated phenotype early in the decline of AFP mRNA and the recessive nature of the *raf*^{b} allele are consistent with the *raf* gene product's being a *trans*-acting negative regulator, or repressor, although other more complicated possibilities cannot be excluded. Whatever the mode of action of the *raf* gene product, the maintenance of the 15-fold difference in both basal and induced levels of AFP mRNA between BALB/cJ and C3H/He mice suggests that it is unaffected by liver injury or regeneration. In contrast, the action of the *Rif* gene product can only be observed during this time. If *Rif* encodes an inducer, than the dominance of the less biologically active *Rif*^{b} gene product in C57BL/6 mice could only be explained by assuming that it blocks the action of *Rif*^{a}, possibly by having a greater affinity for a receptor site. On the other hand, if *Rif* codes for a second repressor that is inactivated during regeneration, then the inefficient inactivation of the variant *Rif*^{b} in *Rif*^{b}/*Rif*^{b} homozygotes or *Rif*^{a}/*Rif*^{b} heterozygotes could explain the dominance of *Rif*^{b}.

At present we do not know whether *raf* and *Rif* act on the AFP gene alone or whether their effects are exerted on other genes as well. Jalanko (11) observed no differences in the basal or induced level of another hepatic oncofetal protein, γ-glutamyltranspeptidase, in BALB/cJ or C57BL/6 mice. What is clear is that neither locus affects the level of albumin mRNA during development or liver regeneration. This result underscores the independent genetic regulation of these two tightly linked (10) and evolutionarily related genes (7, 12) and clearly establishes that an obligate reciprocal switch in their expression does not occur in the adult mouse liver. This is also likely to be true during development, as we have recently examined their expression in early fetal livers and observed a coordinate and parallel increase in both mRNAs until birth (24).

ACKNOWLEDGMENTS

We thank V. Pachnis and R. Riblet for their advice and encouragement during the course of this work and J. Schilling and R. T. Schimke for their gift of pDHFR11.

This work was supported by a Basil O'Connor Starter Grant (to S.M.T.), Public Health Service grants CA-28050 (to S.M.T.), CA-06927, and RR-05539 (to the Institute for Cancer Research) from the National Institutes of Health, and an appropriation from the Commonwealth of Pennsylvania.

LITERATURE CITED

1. **Abelev, G. I.** 1971. Alpha-fetoprotein in oncogenesis and its association with malignant tumors. Adv. Cancer Res. **14**:295–358.
2. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408–1412.
3. **Berridge, M. V., and C. D. Lane.** 1976. Translation of *Xenopus* liver messenger RNA in *Xenopus* oocytes: vitellogenin synthesis and conversion to yolk platelet proteins. Cell **8**:283–297.
4. **Britten, R. J., D. E. Graham, and B. R. Neufeld.** 1974. Analysis of repeating DNA sequences by reassociation. Methods Enzymol. **29**:363–406.
5. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and**

W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.

6. **D'Eustachio, P., R. S. Ingram, S. M. Tilghman, and F. H. Ruddle.** 1981. Murine α-fetoprotein and albumin: two evolutionary linked proteins encoded on the same mouse chromosome. Somat. Cell Genet. **7**:289–294.
7. **Gorin, M. B., D. L. Cooper, F. Eiferman, P. Van de Rijn, and S. M. Tilghman.** 1981. The evolution of α-fetoprotein and albumin. I. A comparison of the primary amino acid sequences of mammalian α-fetoprotein and albumin. J. Biol. Chem. **256**:1954–1959.
8. **Gorin, M. B., and S. M. Tilghman.** 1980. Structure of the α-fetoprotein gene in the mouse. Proc. Natl. Acad. Sci. U.S.A. **77**:1351–1355.
9. **Honjo, T., S. Packman, D. Swan, M. Nau, and P. Leder.** 1974. Organization of immunoglobulin genes: reiteration frequency of the mouse K chain constant region gene. Proc. Natl. Acad. Sci. U.S.A. **71**:3659–3663.
10. **Ingram, R. S., R. W. Scott, and S. M. Tilghman.** 1981. α-Fetoprotein and albumin genes are in tandem in the mouse genome. Proc. Natl. Acad. Sci. U.S.A. **78**:4694–4698.
11. **Jalanko, H.** 1979. Alpha-fetoprotein and γ-glutamyltranspeptidase in mice. Effect of raf gene. Int. J. Cancer **24**:394–397.
12. **Kioussis, D., F. Eiferman, P. Van de Rijn, M. B. Gorin, R. S. Ingram, and S. M. Tilghman.** 1981. The evolution of α-fetoprotein and albumin. II. The structures of the α-fetoprotein and albumin genes in the mouse. J. Biol. Chem. **256**:1960–1967.
13. **Kuhlman, W. D.** 1979. Immunoperoxidase labelling of alpha$_1$-fetoprotein (AFP) in normal and regenerating livers of a low and a high AFP producing mouse strain. Histochemistry **64**:67–75.
14. **Leys, E. J., and R. E. Kellems.** 1981. Control of dihydrofolate reductase messenger ribonucleic acid production. Mol. Cell. Biol. **1**:961–971.
15. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
16. **O'Farrell, P. H., E. Kutter, and M. Nakanishi.** 1980. A restriction map of the bacteriophage T$_4$ genome. Mol. Gen. Genet. **179**:421–435.
17. **Olsson, M., G. Lindahl, and E. Ruoslahti.** 1977. Genetic control of alphafetoprotein synthesis in the mouse. J. Exp. Med. **145**:819–827.
18. **Potter, M., and J. S. Wax.** 1981. Genetics of susceptibility to pristane-induced plasmacytomas in BALB/cAn: reduced susceptibility in BALB/cJ with a brief description of pristane-induced arthritis. J. Immunol. **127**:1591–1595.
19. **Ruoslahti, E., and M. Seppälä.** 1979. Alpha-fetoprotein in cancer and fetal development. Adv. Cancer Res. **29**:275–346.
20. **Schultze, B., H. Gerhard, E. Schump, and W. Maurer.** 1973. Autoradiographische Untersuchung über die Proliferation der Hepatocyten bei der Regeneration der CCl_4—Leber der Maus. Virchows Arch. Abt. B. Zellpath. **14**:329–343.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by electrophoresis. J. Mol. Biol. **98**:503–517.
22. **Taketa, K., and A. Watanabe.** 1971. Interconvertible microheterogeneity of glucose-6-phosphate dehydrogenase in rat liver. Biochim. Biophys. Acta **235**:19–26.
23. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
24. **Tilghman, S. M., and A. Belayew.** 1982. Transcriptional control of the murine albumin/α-fetoprotein locus during development. Proc. Natl. Acad. Sci. U.S.A. **79**:5254–5257.
25. **Tilghman, S. M., D. Kioussis, M. B. Gorin, P. J. Garcia Ruiz, and R. S. Ingram.** 1979. The presence of intervening sequences in the α-fetoprotein gene of the mouse. J. Biol. Chem. **254**:7393–7399.
26. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687.
27. **Wu, C., P. M. Bingham, K. J. Livak, R. Holmgren, and S. C. R. Elgin.** 1979. The chromatin structure of specific genes. I. Evidence for higher order domains of defined DNA sequence. Cell **16**:797–806.