Reports

Raf, a *Trans*-Acting Locus, Regulates the α-Fetoprotein Gene in a Cell-Autonomous Manner

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Genetic analysis provides an approach for identifying regulatory loci that govern the expression of specific genes within the context of the entire organism. Such analyses have defined two unlinked regulatory loci, termed *raf* and *Rif*, that modulate the levels of α-fetoprotein in liver. Of primary importance for the isolation and characterization of the *raf* product is to determine whether it is produced by the hepatocyte or whether it is produced by a different cell type. By means of analysis of α-fetoprotein expression in livers of embryo aggregation chimeras derived from mice of different *raf* genotypes it was possible to conclude that the product of the *raf* locus is expressed as a hepatocyte autonomous function that acts in *trans* to regulate the level of α-fetoprotein messenger RNA.

THE α-FETOPROTEIN (AFP) IS THE major serum protein of the mammalian fetus where it is synthesized primarily by the visceral endoderm of the yolk sac, fetal liver, and (to a lesser extent) fetal gastrointestinal tract (*1*). The levels of AFP messenger RNA (mRNA) in mouse liver, which decline dramatically after birth (*2*), are under the control of at least two genetically identified *trans*-acting loci, termed *raf* (for regulation of AFP) and *Rif* (for regulation of induction of AFP). The *raf* locus determines the basal level of AFP mRNA in the adult liver and the *Rif* locus determines the magnitude of the transient induction of AFP mRNA in response to liver damage (*3, 4*). *Raf* and *Rif* are linked neither to the AFP structural locus nor to each other (*4*).

Two alleles have been identified for the *raf* locus. The rare raf^b allele present in the BALB/cJ strain is recessive to the raf^a allele found in all other inbred strains tested (*3*). The raf^b allele is responsible for the hereditary persistence of elevated levels of AFP mRNA in the liver of the adult BALB/cJ mouse. It was demonstrated by immunohistochemistry that the persistence of AFP protein in BALB/cJ mice was the consequence of an elevated level of AFP in all hepatocytes, and was not attributable to a greater number of AFP-producing cells (*5*). Furthermore the effects of the *raf* gene are selective in that *raf* does not affect the mRNA levels of the evolutionarily related and closely linked albumin gene (*4*), nor the levels of another onco-fetal protein, γ-glutamyl transpeptidase (*6*). However *raf* does regulate the levels of at least one other structural gene, termed H19, which is unlinked to both *raf* and AFP (*7*).

To address the issue of the site of synthesis of the *raf* gene product, chimeric mice were generated by aggregating BALB/cJ (raf^b/raf^b) and C57BL/6J (raf^a/raf^a) eight cell–stage embryos (*8*). Such animals will be unique combinations of homozygous BALB/cJ and C57BL/6J cells interspersed in most, if not all, tissues of the body (*9*). If the *raf* product is autonomous for the liver cell, then the level of AFP mRNA in any given mosaic liver will fall between the homozygous raf^a (low) and raf^b (high) levels, the exact level determined by the contribution of each strain (genotype) to the hepatocyte population. Thus a 50:50 chimera will have an AFP mRNA level midway between the two homozygous values. If the *raf* product is not cell-autonomous, that is, it acts intercellularly, then the level of AFP mRNA in the liver of a 50:50 chimera should be equal to that of the low-expressing C57BL/6J strain. This argument is based on the observation that (C57BL/6J × BALB/cJ)F_1 heterozygotes, which would have the equivalent 50:50 mixture of raf^a and raf^b gene products in their circulation, exhibit low AFP mRNA levels (*4*).

Such an analysis is only possible if the AFP mRNA difference between raf^a/raf^a and raf^b/raf^b animals is sufficiently great that intermediate levels are distinguishable. Previous studies on pooled animals had shown that there was a 10- to 15-fold difference in both protein and mRNA levels between the BALB/cJ and all other strains tested (*3, 4*). To confirm this on individual animals, 21-day-old mice from both the BALB/cJ and C57BL/6J strains were killed, and hepatic polyadenylated [poly(A)$^+$] RNA was prepared. AFP mRNA was analyzed by agarose gel electrophoresis and, after blotting onto nitrocellulose paper, was quantitated by hybridization to an AFP-specific probe. The mean AFP mRNA value from seven C57BL/6J livers was 1.79 optical density units with a range between 0.85 and 2.70, while the mean value for eight BALB/cJ livers was 36.45 with a range between 17.7 and 56.32 optical density units. Thus there were two distinct nonoverlapping distributions of AFP mRNA for the two strains at this age, with the mean difference being about 20-fold. This difference is large enough to allow us to confidently identify intermediate level animals.

Eight chimeric mice were obtained by aggregating C57BL/6J and BALB/cJ eight cell–stage embryos. Visual inspection of coat color showed one entirely black animal and seven that were obviously chimeric. The animals were killed at approximately day 21 postnatally and various organs were removed for analysis. The degree of mosaicism was determined for each organ by the use of DNA restriction fragment length polymor-

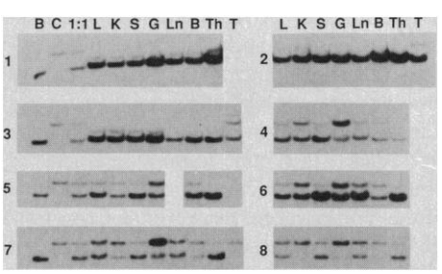

Fig. 1. The degree of genotypic mosaicism in tissues from eight chimeric mice. Chimeras were generated by the aggregation of BALB/cJ and C57BL/6J eight cell–stage embryos. Aggregates were cultured overnight and reimplanted into the uteri of pseudopregnant female recipients. Eight chimeric animals (numbered 1 to 8 in order of increasing contribution of BALB/cJ to coat color) were obtained and killed at 21 days postnatally. Genomic DNA was isolated (*20*) from the livers (L), kidneys (K), spleens (S), guts (G), lungs (Ln), brains (B), thymuses (Th), and testes (T) of the eight chimeric animals. Liver DNA from homozygous C57BL/6J (B), BALB/cJ (C), and an artificial 1:1 (by weight) mixture of the two livers (1:1) were treated in a similar way and served as controls. DNA (10 μg) was digested with Eco RI, separated by electrophoresis through a 1% agarose gel, blotted onto nitrocellulose, and probed with a ^{32}P-labeled fragment spanning the first exon of the AFP gene (*20, 21*). The RFLP observed with this probe is the result of an extra Eco RI site at −5.4 kb in the AFP 5′ flanking region in C57BL/6J as compared to BALB/cJ (*4*). Essentially the same results were obtained by analysis of the H19 gene Msp I RFLP (*7*). Animals 1, 4, 6, and 8 were females.

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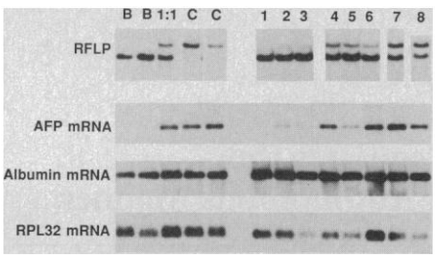

Fig. 2. Quantitation of AFP mRNA in the livers of eight chimeric mice. Polyadenylated RNA and DNA were isolated from the same individual liver homogenates as in Fig. 1 (numbered 1 to 8) as described (*20*). Liver poly(A)$^+$ RNA from homozygous BALB/cJ (C) and C57BL/6J (B) mice of similar age, along with an artificial mixture (1:1 by weight) of the two livers served as controls. The RNA (7 μg) was separated by electrophoresis in a 1.5% formaldehyde agarose gel, blotted onto nitrocellulose, and probed with a ^{32}P-labeled fragment derived from the first exon of the AFP gene. As controls, several identical sets of blots were hybridized, the AFP label washed off, and the blot reprobed with labeled complementary DNA clones for either albumin mRNA (*22*) or ribosomal protein L32 (RPL32) mRNA (*23*) or both. The RFLPs are Eco RI digests of the liver DNA samples as described in Fig. 1.

phism (RFLPs) (*10*) as autonomous cell markers. Genomic DNA was assayed for the presence of RFLPs previously identified at the AFP/albumin gene (*4*) and H19 gene loci (*7*) (Fig. 1). The animals were numbered in the order of increasing BALB/cJ contribution to coat color. In general, this order held for the other organs we tested, in that our results show a reasonable concordance in the degree of mosaicism between liver and other tissues analyzed from an individual animal.

We did note one prevalent skewing in our survey. As shown in Fig. 1 for animals 4 to 8, there was a tendency of thymus, and to a lesser extent spleen, to be composed predominantly of C57BL/6J cells and therefore to depart from the uniformity of mosaicism demonstrated for the other tissues. It may be that this is the consequence of a more rapid acquisition of competent T cells during the early postnatal period in C57BL/6 mice (*11*).

To ask whether there was a linear correlation between the degree of mosaicism in liver and AFP mRNA, which would argue in favor of the cell-autonomous model, poly(A)$^+$ RNA and DNA were isolated from the same liver homogenates. By isolating total nucleic acids from the liver both genotype and phenotype may be assayed from the same sample, thus avoiding the problems of uneven mosaicism within a tissue (*12*). One potential drawback of assaying tissue homogenates is that cells other than hepatic parenchymal cells will be represented in the DNA mosaicism evaluation. This should not be a severe constraint because the hepatic parenchymal cells represent approximately 70% of the liver (*13*), and the likelihood of an extreme and consistent discrepancy between hepatocytes and stromal cells is probably small.

The data for both the hepatic RFLPs and AFP mRNA levels are shown in Fig. 2. The values for AFP mRNA, which ranged between 3 and 70 optical density units, were somewhat higher than those for the control animals because the chimeric animals were 12 to 24 hours younger. As illustrated in Fig. 3, where the data are represented graphically, there was an excellent correlation between the degree of BALB/cJ genotypic contribution in liver and the level of AFP mRNA. Animals 4 to 8, which contained from 25 to 70% BALB/cJ-derived cells, exhibited significantly higher mRNA levels than the first three animals, which were predominantly derived from C57BL/6J. These data are consistent with the *raf* gene product acting in a cell-autonomous manner in liver to affect AFP gene transcription.

The straightforward interpretation of the data in Fig. 3 is that the *raf* gene product is not produced in a distal organ. However, it is possible to devise unlikely scenarios in which *raf* is not cell-autonomous. For example, if there is a nonhepatic site of synthesis of *raf*, it would have to have a mosaic profile that is grossly different from that in liver, composed predominantly of BALB/cJ cells in animals 4 through 8. This is highly unlikely, as there is a general tendency in C57BL/6 ↔ BALB/cJ chimeras to favor C57BL/6 contribution, as previously reported for C57BL/10 ↔ BALB/c chimeras (*14, 15*). In addition no such skewing was observed in our analysis of other tissues in Fig. 1.

Our interpretation of the experimental results depends upon the assumption that the two alleles of *raf* in a heterozygous cell act independently of one another. If, as a second unlikely scenario requires, *raf* is dosage-compensated such that in a heterozygous cell the absence of any functional *raf* product from the raf^b allele results in a twofold overproduction of *raf* product from the raf^a allele, then one cannot predict the phenotype of chimeras from the phenotype of the heterozygote. However, for this possibility to complicate the interpretation of our data, several conditions, in addition to dosage compensation, must hold. First, the raf^b mutation must result in either the absence of the *raf* gene product or a substantially altered one; second, the concentration of raf^a must be limiting in both homozygotes and heterozygotes; and finally the degree of chimerism of the distal organ must be exactly the same as the chimerism in the liver. We view these coincidences as unlikely.

Thus, our results argue that the *raf* gene product is expressed by the individual hepatocyte and functions in a cell-autonomous manner to affect AFP gene transcription. This conclusion is consistent with recent observations that H19 mRNA in muscle and gut as well as AFP mRNA in gut are repressed after birth, but in neither tissue is the repression under *raf* control (*7, 16*). This apparent liver specificity of the *raf* gene product, together with the recessive nature of the variant raf^b protein, and the observation that the *raf* phenotype is first detected when AFP transcription ceases shortly after birth, are consistent with a model whereby the *raf* gene product is a cell-specific repressor of transcription (*4*). Two recent observations suggest that the target site for such a protein lies very near the AFP structural gene. Krumlauf, Fergussen-Smith, and Tilghman (*17*) have shown that human hereditary persistence of AFP in a Scottish kindred segregates as an autosomal dominant trait linked to the AFP structural gene. One could envisage that these individuals harbor a mutation in a DNA-binding sequence for the *raf* product. This sequence

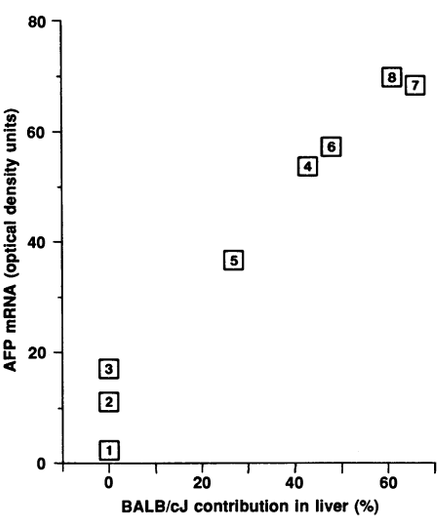

Fig. 3. The correlation between the genotype and *raf* phenotype in the livers of eight chimeric mice. Densitometric scans of the DNA and the mRNA blots in Fig. 2 were made on appropriately exposed films. The BALB/cJ contribution was calculated by determining the ratio of the 10.0-kb BALB/cJ to 7.8-kb C57BL/6J bands in each lane and comparing it to the ratio in the 1:1 mixture, as well as (BALB/cJ × C57BL/6J)F_1 DNA samples. AFP mRNA levels were determined by correcting the optical density of the hybridizing band in each lane with the values for the albumin mRNA and RPL32 mRNA from the same blot. The values obtained were the means of three separate gel analyses. The numbers on each point correspond to the numbering of the chimeric animals in Figs. 1 and 2.

must be contained within the proximal 7.6 kb of DNA 5′ to the AFP gene, as demonstrated recently in transgenic mouse strains where integrated AFP gene constructs exhibited *raf* regulation (*18*). The generation of transgenic mouse strains carrying different segments of the AFP gene regulatory domain (*19, 20*) should allow the fine mapping of the nucleotide sequences responsible for *raf* regulation, which will aid in the identification and eventual characterization of the *raf* gene product.

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Identification and Localization of Mutations at the Lesch-Nyhan Locus by Ribonuclease A Cleavage

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Many mutations leading to human disease are the result of single DNA base pair changes that cannot be identified by Southern analysis. This has prompted the development of alternative assays for point mutation detection. The recently described ribonuclease A cleavage procedure, with a polyuridylic acid–paper affinity chromatography step, has been used to identify the mutational lesions in the hypoxanthine phosphoribosyltransferase (HPRT) messenger RNAs of patients with Lesch-Nyhan syndrome. Distinctive ribonuclease A cleavage patterns were identified in messenger RNA from 5 of 14 Lesch-Nyhan patients who were chosen because no HPRT Southern or Northern blotting pattern changes had been found. This approach now allows HPRT mutation detection in 50 percent of the cases of Lesch-Nyhan syndrome. The polyuridylic acid–paper affinity procedure provides a general method for analysis of low abundance messenger RNAs.

THE LESCH-NYHAN (LN) SYNdrome is a severe, X-linked, recessive neurological disease that is the consequence of a defect in the hypoxanthine phosphoribosyltransferase (HPRT) gene, leading to HPRT enzyme deficiency (*1*). Analysis with HPRT complementary DNA (cDNA) probes has revealed that, in approximately 85% of LN cases, this gene appears normal by Southern and Northern blotting (*2*). This result suggests that most cases are the result of point mutations, or small DNA deletions or rearrangements. Among the LN cases with abnormal Southern blotting patterns, a heterogeneous collection of HPRT gene alterations has been observed. Together with the predominance of "small" lesions (below the resolution of Southern blotting), the heterogeneity of the mutations makes the characterization of HPRT deficiency at the nucleic acid level a difficult task.

Ribonuclease A (RNase A) cleavage has recently been employed to detect previously characterized β-globin mutations in genomic DNA (*3*) and c-Ki-*ras* variants in RNA from tumor cell lines (*4*). The RNase A cleavage assay is based on the fact that some single base mismatch sites in RNA hybrids with RNA or DNA will be cleaved by RNase A. A single RNA probe can be used to identify the presence of a base substitution, or a pair of overlapping probes can be used to unambiguously locate mutation sites. The precise requirements for susceptibility to RNase A attack are not yet clear, but it seems likely that 30 to 50% of possible single base mispairings will be cleaved (*3, 4*). Mismatches resulting from deletions, insertions, or rearrangements offer greater potential for RNase A cleavage because of more extensive single-stranded regions within the hybrids.

We have analyzed HPRT messenger RNAs (mRNAs) by RNase A cleavage. The mRNA can be examined with a single HPRT, antisense RNA probe generated from HPRT cDNA cloned into in vitro transcription vectors. This circumvents the difficulty of analyzing genomic DNA sequences, as the human HPRT coding sequences are distributed in nine exons spanning 44 kb of DNA (*5*). Preliminary RNase A cleavage experiments showed that HPRT mRNA, which represents 0.01% of the mRNA population (*6*), was difficult to detect in total cellular RNA preparations. To improve the sensitivity of the procedure we have used polyuridylic acid [poly(U)]–affinity paper (*7*) (Fig. 1). This "messenger affinity paper" (mAP) (Amersham) provides a solid support on which to recover RNA probe that is hybridized to polyadenylated HPRT mRNA, while nonpolyadenylated RNA, including unbound probe, is washed free. The HPRT mRNA can then be eluted from the mAP under conditions that will not denature the mRNA:RNA probe hybrids, before RNase A cleavage. This simple modification greatly increases the signal to noise ratio, presumably because it selects the polyadenylated fraction of RNA, containing the HPRT sequences, while simultaneously removing unbound radiolabeled probe that might contribute to background.

Analysis of RNA extracts from HeLa cells (which contain HPRT mRNA) and RJK 853, a human lymphoblastoid cell line with a total HPRT gene deletion (HPRT$^-$) (*2*), revealed that antisense HPRT RNA probes (Fig. 2A) are protected only when HPRT

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