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Genetics

A lethal deletion on mouse chromosome 7 affects regulation of liver-cell-specific functions: Posttranscriptional control of serum protein and transcriptional control of aldolase B synthesis

(cell differentiation/gene regulation/hepatic functions/mRNA levels)

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ABSTRACT **Steady-state levels of mRNAs were determined for the serum proteins albumin, α-fetoprotein (AFP), and transferrin, as well as for aldolase B in livers of newborn mice homozygous for a radiation-induced lethal deletion (c^{14CoS}) in chromosome 7. Deficiencies in synthesis and secretion of the serum proteins as well as in activities of certain liver-specific enzymes characterize these homozygotes. The results of RNA dot and gel-blot hybridizations with the respective cloned cDNA probes showed a decrease to one-fourth of aldolase B mRNA levels in homozygous mutant livers compared to normal littermates, in contrast to normal levels of mRNA sequences for the three serum proteins in the mutants. Furthermore, the mRNA sequences were shown to be present as mature mRNA molecules in both mutant and normal littermate livers. We suggest that the deficiencies of liver-specific serum proteins and those of the enzymes caused by the lethal deletions around the albino locus on chromosome 7 of the mouse are due to different causes. In the case of the liver-specific enzyme examined here—i.e., aldolase B—control at the level of transcription or of message stability is affected in the homozygous deletion mutants, whereas the deficiencies of serum proteins are not reflected on the mRNA level and owe their origin to an effect on a posttranscriptional or translational level. These results lend further support to the assumption that the deleted portion of the genome includes genes concerned with the control and regulation of liver cell differentiation.**

Mechanisms involving the genetic control of development and differentiation are as yet largely unknown and are being investigated with the help of a variety of experimental approaches in various model systems and different organisms. One of these makes use of developmental mutants as tools in the analysis of mammalian differentiation and its genetic control.

An experimental system that has been developed in one of our laboratories consists of a series of radiation-induced overlapping deletions in chromosome 7 of the mouse at and around the albino (*c*) locus, all of them lethal when homozygous (1). The smallest deletion, c^{14CoS}, used in these studies measures approximately 1.5 centimorgans. Previous investigations have identified specific effects by the deletions on the differentiation of particular cell types—i.e., the parenchymal liver and certain kidney cells. The parenchymal liver cells of newborn mice homozygous for the perinatally lethal deletions show extensive abnormalities of ultrastructure of specific subcellular organelles (2). Associated with these are deficiencies of several liver-specific enzymes, including glucose-6-phosphatase, tyrosine aminotransferase, serine dehydratase, and UDP-glucoronosyltransferase, which normally increase in the liver around the time of birth (3). In addition, a reduction of the rate of synthesis of the plasma proteins albumin, α-fetoprotein (AFP), and transferrin to approximately 20% of normal was shown in newborn mutant livers by experiments of incorporation with [^{14}C]leucine, whereas incorporation into total liver proteins was decreased only slightly in the mutant animals (4). None of the biochemical deficiencies can be ascribed to deletions of the respective structural genes. In the case of the albumin, AFP, and transferrin genes in the mouse, these are known to map on chromosomes other than that carrying the albino locus (5, 6). As to the enzymes, somatic cell hybridization experiments demonstrated the structural genes for glucose-6-phosphatase and tyrosine aminotransferase to have remained intact in the deletion mutants and to map on chromosomes other than number 7 (7, 8). This suggested that the various tissue-specific biochemical lesions might be due to the deletion of genes concerned with the regulation of other unlinked structural genes normally expressing the specific differentiated state of the hepatocyte. The radiation-induced deletion mutants therefore seemed to offer a potential for the analysis of structure, organization, expression, and function of mammalian regulatory genes.

As a first step towards the identification of the mechanisms by which these regulatory genes exert their control, an analysis was undertaken of level and nature of the mRNA sequences coding for albumin, AFP, and transferrin in livers of newborn c^{14CoS} mutants. In addition, the availability of an aldolase B cDNA probe (9) made it possible to carry out similar studies for the mRNA of this developmentally regulated liver enzyme in the mutants.

MATERIALS AND METHODS

Normal and Mutant Mice. Mice carrying the lethal albino deletion c^{14CoS} are maintained and bred at the Albert Einstein College of Medicine as heterozygotes with c^{ch} (chinchilla) as the normal allele. Matings of mice heterozygous for c^{ch} and the c^{14CoS} deletion are used to produce deletion homozygous newborn (c^{14CoS}/c^{14CoS}) identified as albinos with unpigmented eyes that die a few hours after birth. Heterozygous and homozygous pigmented normal littermates serve as controls. To obtain livers, newborn animals were sacrified within a few hours after spontaneous birth. Livers were removed, frozen in liquid N_2, stored at −80°C, and subsequently shipped on dry ice to the Laboratoire

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Abbreviations: AFP, α-fetoprotein; kb, kilobase(s).

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For some experiments liver and yolk sac tissues were obtained from adult males and from 15- and 19-day-old fetuses of the C57BL/6J strain bred at Gif-sur-Yvette. These tissues were similarly stored at −80°C before use.

Preparation of Whole Cell RNA. Total RNA was extracted directly from frozen tissues with guanidinium thiocyanate, essentially as described by Chirgwin *et al.* (10). No significant differences were found in the yield of total RNA per mg of wet tissue between the livers of mutant and normal littermate newborn mice.

Recombinant Plasmids Containing cDNA Sequences for Albumin, AFP, Transferrin, and Aldolase B. Mouse albumin and AFP cDNA clones were obtained from adult mouse liver and yolk sac cDNA libraries as described elsewhere (11). The albumin cDNA probes used in this work are a mixture of two recombinant plasmids, pMSA433 and pMSA962 (11); similarly, two AFP-cDNA containing plasmids, pMAFP189 and pMAFP191 (11), are used as specific AFP cDNA probes. The transferrin cDNA clone was isolated from a rat liver cDNA library by *in situ* colony hybridization (12), using the human transferrin cDNA probe Tf66 G2 as reported previously (13). The $A4C_9$ recombinant plasmid containing rat aldolase B cDNA sequences has also been described previously (9).

Labeling of the Recombinant Plasmids by Nick-Translation. The cloned cDNAs were labeled *in vitro* with [^{32}P]dCTP and [^{32}P]dATP (800 Ci/mmol; 1 Ci = 37 GBq) by making use of the nick-translation reaction catalyzed by *Escherichia coli* DNA polymerase I as described by Maniatis *et al.* (14). The nick-translated probe was separated from unincorporated nucleotides by Sephadex G-50 column chromatography. The specific activity of the resulting recombinant [^{32}P]DNA samples was approximately 2–6 × 10^8 cpm/μg.

Dot Hybridization Analysis of mRNA Sequences. Serial dilutions of total cellular RNA (2-μl aliquots in 10 mM phosphate buffer, pH 7.0, containing 0.1% $NaDodSO_4$) were dotted on a sheet of nitrocellulose soaked in a high-salt buffer (3 M NaCl/0.3 M sodium citrate, pH 7.0) according to the procedure of Thomas (15). Alternatively, the RNA samples were diluted 1:10 in the high-salt buffer and 100-μl aliquots were spotted onto nitrocellulose filters by using a Schleicher & Schuell Minifold template. After baking at 80°C for 2 hr, the filters were prehybridized and incubated with the nick-translated cloned cDNA probes at 42°C, essentially as described by Thomas (15). After hybridization the filters were washed and autoradiographed as described elsewhere (16). The resulting autoradiograms were quantitatively scanned with a Joyce–Loebl double-beam recording microdensitometer.

Gel Electrophoresis and RNA Blot Hybridization. Gel electrophoresis of total RNA preparations was carried out on 1.2% agarose vertical gels in 20 mM Na/Na_2 phosphate buffer, pH 7.0, and 2.2 M formaldehyde. The RNA samples were adjusted to 50% (vol/vol) formamide/2.2 M formaldehyde/10 mM phosphate buffer, incubated for 5 min at 60°C, cooled fast on ice, and applied to the agarose gel. After electrophoresis the RNA was transferred from the gels to nitrocellulose filters as reported elsewhere (16). The bound RNA was then hybridized to the ^{32}P-labeled recombinant cDNA probes, extensively washed, and autoradiographed as described by Gal *et al.* (16).

RESULTS

Levels of Albumin, AFP, and Transferrin mRNA Sequences in Livers from Newborn Deletion Homozygotes and Normal Littermates. Since c^{14CoS} deletion homozygous mice have been shown to be deficient in synthesis of albumin, AFP, and transferrin (4), it was of particular interest to analyze the mRNAs coding for these proteins in the newborn homozygous mutants. Levels of albumin, AFP, and transferrin mRNA sequences in total RNA preparations from livers of newborn mutant mice and normal littermates were determined by dot hybridization analysis (Fig. 1, rows 2 and 3). For comparative purposes whole cell RNA preparations from adult (lane 1) normal C57BL mouse livers were included in the analysis. The differences in levels of albumin and AFP mRNA sequences in the RNA preparations between normal adult and newborn mice (Fig. 1, rows 1 and 3) are consistent with the results of previous studies concerning the differential expression of albumin and AFP genes during mouse development (17, 18). Whereas the level of albumin mRNA in the liver of newborn mutants does not differ significantly from that of normal littermates (Fig. 1*A*, rows 2 and 3), the relative proportion of AFP mRNA sequences appears to be somewhat higher in the livers of homozygous mutants than in those of normal littermates (Fig. 1*B*, rows 2 and 3). A densitometric analysis of the autoradiographs (Fig. 1 *A* and *B*, *Left*) quantitates these observations and indicates

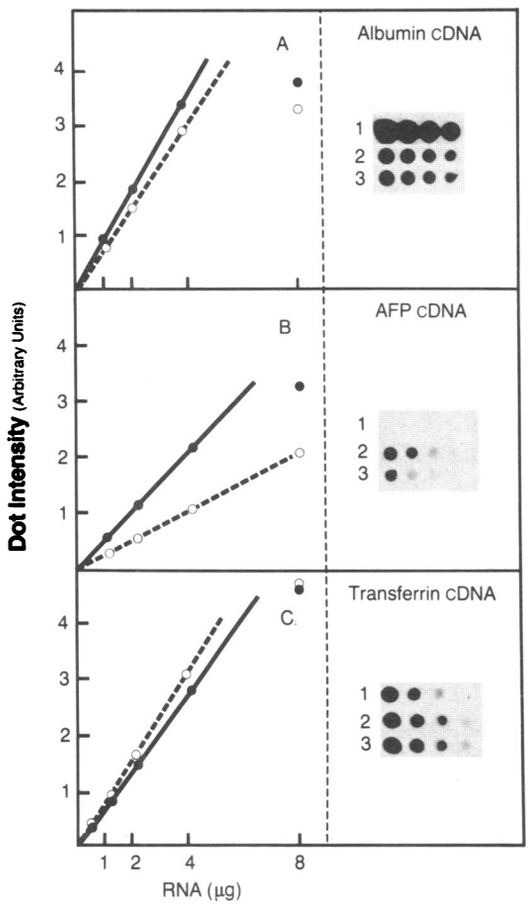

FIG. 1. Quantitation of albumin, AFP, and transferrin mRNA levels in total RNA preparations from normal and mutant mouse livers. Twofold serial dilutions of total RNA from normal adult mouse liver (row 1), and livers from newborn deletion mutant (row 2) and normal littermates (row 3) were spotted onto nitrocellulose filters. The dot blots were incubated with the nick-translated mouse albumin cDNA (*A*), AFP cDNA (*B*), and transferrin cDNA (*C*) probes. The intensity of each dot was quantitatively evaluated by density scanning of appropriate autoradiograms such as those shown on the right. On the left are plots of dot intensity versus RNA mass for newborn mutants and normal littermates. Each point represents the average of several independent determinations. Dot intensities are given in arbitrary units, which are different for *A*, *B*, and *C*. ●, Deletion mutants; ○, normal littermates.

that AFP mRNA levels are increased 2-fold in mutant livers. Densitometric analysis of the autoradiographic data of dot hybridization with the transferrin cDNA probe (Fig. 1*C*) also failed to detect any differences in the relative proportions of transferrin mRNA between the homozygous deletion mutants and normal littermates (Fig. 1*C*, *Left*).

The results of studies of aldolase B mRNA levels are shown in Fig. 2, which presents an autoradiogram of the hybridization of the dot blots to a rat aldolase B cDNA probe (9). Obviously this rat cDNA probe is able to cross-hybridize efficiently to the mouse aldolase B mRNA even under the highly stringent conditions used in these experiments. The level of aldolase B mRNA is higher in the adult (Fig. 2, row 1) than in the normal newborn littermate liver (Fig. 2, row 3). This is not unexpected, since aldolase B has been shown to progressively replace the fetal isozymes, aldolases A and C, during rat liver development (19). Although the aldolase B cDNA probe is able, apparently, to cross-hybridize with rat aldolase A mRNA, it must be pointed out that the poor thermal stability of the hybrids (20) is likely to prevent the detection of mouse aldolase A mRNA sequences under the highly stringent hybridization conditions used here. Most interestingly, the results of a quantitative analysis of the autoradiographic data (see Fig. 2 *Left*) demonstrate that the levels of aldolase B mRNA sequences in the livers isolated from mutant mice are one-fourth of those in livers from their normal littermates.

Altogether, the results reported here demonstrate that the steady-state levels of mRNAs for the plasma proteins albumin, AFP, and transferrin are not significantly diminished in the livers of homozygous deletion mutants; in fact, levels of AFP mRNA sequences appear to be increased in the mutants. In contrast, the amount of aldolase B mRNA is considerably decreased in the homozygous mutant mice.

Size Analysis of Albumin, AFP, Transferrin, and Aldolase B mRNAs in RNA Preparations from Normal and Mutant Mouse Livers. To identify the nature of the mRNAs for albumin, AFP, and transferrin, as well as aldolase B, detected in the dot hybridization experiments, and to make certain that measurements of mRNA levels in mutant and normal livers were not affected by possible degradation of particular RNA preparations, RNA samples from livers of mutant and normal littermates were subjected to electrophoresis in denaturing formaldehyde/agarose gels. The RNA was transferred from the gel onto nitrocellulose paper and then hybridized to one of the four [^{32}P]cDNA probes. The autoradiographs of the blots are shown in Fig. 3 [(lanes 8 (mutant) and 9 (normal littermate)]. RNA samples from normal mouse yolk sac (lanes 4, 5, and 7) and fetal (lanes 3 and 6) and adult mouse liver (lane 2), as well as adult rat liver (lane 10), were also included for comparative purposes. All

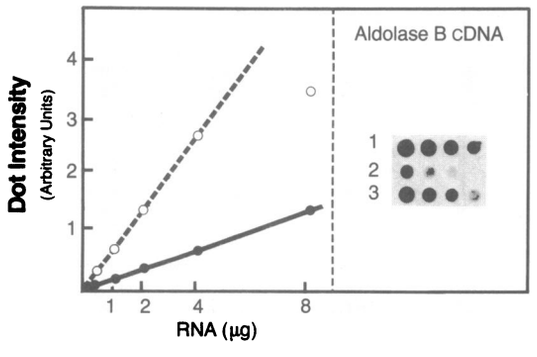

FIG. 2. Dot hybridization analysis of aldolase B mRNA sequences in total RNA preparations from normal and mutant mouse livers. Dot blots of the different RNA preparations were prepared exactly as described in the legend of Fig. 1. The RNA bound to the nitrocellulose filters was hybridized to the ^{32}P-labeled aldolase B cDNA probe. (*Right*) Autoradiogram of a single dot hybridization experiment (row 1, adult mouse liver; row 2, newborn mutant; row 3, normal littermate). The intensity of each dot was quantitated with a recording microdensitometer. The area under the absorbance curves was integrated and plotted as a function of μg of RNA applied. (*Left*) Plot of intensity versus RNA mass for deletion mutants (●) and normal littermates (○). Each point represents the average of several independent determinations.

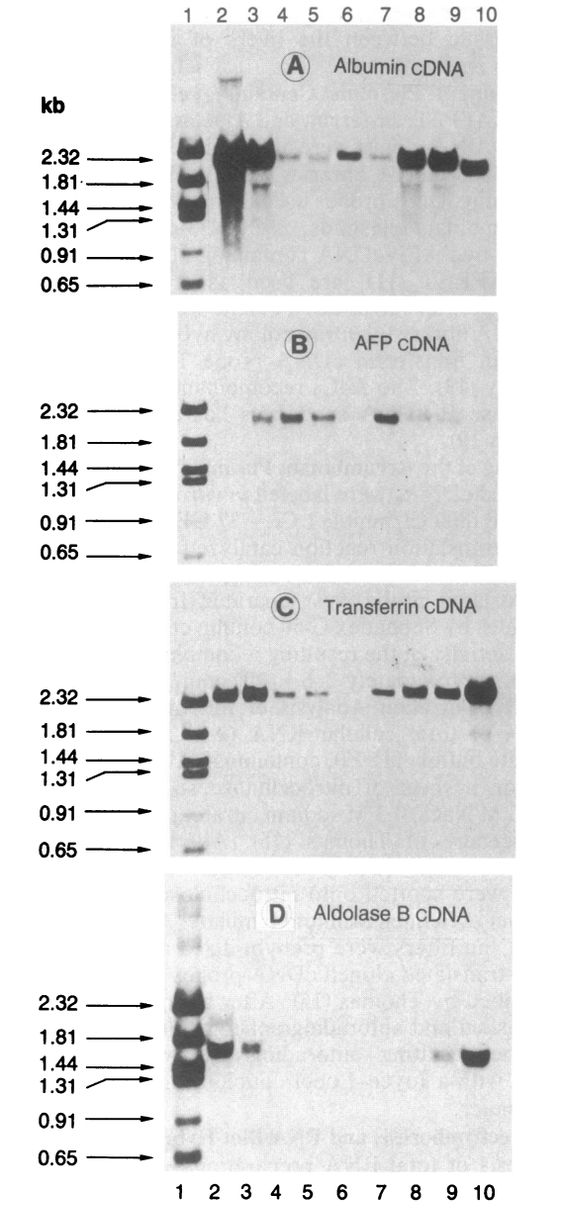

FIG. 3. Size analysis of albumin, AFP, transferrin, and aldolase B mRNA sequences in total RNA preparations from normal and mutant mouse tissues by gel blot hybridizations to ^{32}P-labeled cloned cDNA probes. Whole cell RNA samples (2 μg) from normal mouse adult liver (lane 2), livers of newborn deletion mutants (lane 8) and normal littermates (lane 9), 19-day (lane 3) and 15-day (lane 6) normal fetuses, yolk sac of 19-day (lanes 4 and 5, two different preparations) and 15-day (lane 7) normal embryos, and normal rat adult liver (lane 10). The RNA samples were separated on a vertical formaldehyde/1.2% agarose slab gel, transferred to nitrocellulose paper, hybridized with the ^{32}P-labeled mouse albumin cDNA (*A*), AFP cDNA (*B*), rat transferrin cDNA (*C*), or aldolase B cDNA (*D*) probes, and autoradiographed. For *D*, the nitrocellulose paper was hybridized and subsequently exposed for 24 hr at −70°C, using two intensifier screens. The arrows point to the size markers shown on lane 1, which are *Bgl* I, *Taq* I, and *Alu* I restriction fragments of the plasmid pBR322. kb, Kilobases.

RNA preparations show a discrete main band characteristic of the hybridization to each respective cDNA probe. This excludes the possibility of a differential degradation of particular RNA preparations. Furthermore, in the blots hybridized to the albumin (Fig. 3*A*) or AFP (Fig. 3*B*) cDNA probes the bands observed migrate in the position expected for a functional mature albumin or AFP mRNA molecule (2100–2200 nucleotides) (18). This is also true for transferrin mRNA, for which the sizes of the main bands in the autoradiograms of cDNA hybridization (Fig. 3*C*) correspond to those of functional mature transferrin mRNA molecules in the rat—i.e. 2.4 kb (13). The minor band of higher molecular weight observed in certain RNA preparations in the blot hybridization with the albumin cDNA probe (Fig. 3*A*, lane 2, adult, and lane 3, 19-day fetus) might correspond to a processing intermediate of the primary transcript. Similar weak bands migrating in the same position were also seen on the original autoradiograph of the mutant and normal littermate RNA preparations, in contrast to the positive print, where they are not visible (Fig. 3*A*, lanes 8 and 9). In comparison to the major mature albumin mRNA band they show similar relative intensities in the two RNA preparations.

In the case of aldolase B (Fig. 3*D*), the molecular sizes of the main bands in the autoradiograms of cDNA hybridizations are also consistent with previous observations concerning the size of functional, mature aldolase B mRNA molecules in the rat—i.e. 1.70 kb (9). The less intense band, corresponding to an RNA species of approximately 2 kb, observed with the aldolase B cDNA probe (Fig. 3*D*, lanes 2, 3, 8, and 9) probably represents a precursor of the mature mRNA molecule.

DISCUSSION

The phenotype of newborn mice homozygous for certain lethal albino deletions on chromosome 7 includes deficiencies of liver-specific enzymes as well as of serum proteins. These are thought to be the result of the deletion of genes instrumental in the regulation of parenchymal liver cell differentiation (1). However, until now the particular levels of regulation remained unspecified and could concern either synthesis or processing of mRNA, or message translation—i.e., protein synthesis and processing. The experimental results presented here make it possible to identify different levels of regulation of gene expression at which the deletions intervene: one for the serum proteins, the other for a liver-specific enzyme. Amounts of mRNA molecules encoding the three serum proteins albumin, AFP, and transferrin were shown to be undiminished in the livers of newborn homozygous mutants. In fact, in the case of one of these (AFP) its mRNA level appeared to be 2-fold higher in the livers of deletion mutants than in those of normal littermates. Thus the genetic defect responsible for the deficiency of plasma protein synthesis does not appear to involve alterations in the transcription of these genes but rather abnormalities of processes beyond those of transcription—i.e., translational or posttranslational.

Among various mechanisms that might account for the serum protein defects is the regulation of transport of mRNA molecules from the nucleus to the cytoplasm. It is more likely, however, that the ultrastructural alterations of rough endoplasmic reticulum and Golgi apparatus, characterizing parenchymal liver cells of these mutants (1, 2), might play a causal role in the decreased synthesis as well as the secretion of serum proteins. Indeed, synthesis of these secretory proteins is known to take place on membrane-bound polysomes (21), and the ultrastructural abnormalities in liver cells of homozygous deletion mutants include loss of membrane-bound ribosomes and disaggregation of polysomes (2). Such polysomes isolated from the livers of newborn albino deletion homozygotes have been shown (22) to be less efficient than those obtained from normal littermates in incorporating radioactive leucine into protein in a cell-free system, even after the addition of excess liver mRNA. Conceivably, mRNA molecules encoding the plasma proteins albumin, AFP, and transferrin accumulate in the cytoplasm of the mutants without becoming engaged in translation (22).

It is interesting to note here that various studies of albumin and AFP gene expression during liver development of rodents have shown these to be regulated primarily at the transcriptional level (17, 18, 23–25). The serum protein deficiencies of the deletion homozygotes, however, appear to be the result of a defect in steps operating on a different level—i.e., one beyond that of transcription.

The reason for the consistent 2-fold increase in AFP mRNA level found in livers of newborn mutants is not obvious. It is tempting to attribute the higher steady-state level of AFP mRNA to a retardation of liver development in the deletion mutants. Such a developmental retardation should be reflected in a concomitant deficiency of albumin mRNA levels, which normally increase postnatally (17). The failure to find lower amounts of albumin mRNA in mutant livers argues against the assumption of developmental retardation as an explanation for increased AFP levels.

In contrast to the serum proteins, an effect of the lethal albino deletion (c^{14CoS}) on the control of transcription or message stability of a gene expressing a liver-specific enzyme was established for aldolase B. The livers of newborn deletion homozygotes showed one-fourth normal aldolase B mRNA levels. Even though several other developmentally regulated liver-specific enzymes are known to have severely reduced activities in deletion homozygotes, measurements of aldolase B enzyme activities have not been reported (3). In this context it should be noted that dramatically reduced levels of tyrosine aminotransferase mRNA levels in albino deletion homozygotes were demonstrated recently (26).

The data presented here provide strong evidence for normal transcription of the genes encoding the three serum proteins albumin, AFP, and transferrin, all of them deficient in newborn mice homozygous for certain radiation-induced deletions at and around the albino (*c*) locus on chromosome 7. The deficiencies in synthesis of these serum proteins must therefore be ascribed to effects of the deletions on other levels of control—e.g., posttranscriptional, translational, or posttranslational. Even though lack of message stability has at this time not been ruled out as the possible cause of reduced aldolase B mRNA levels in the mutants, the observed decrease is consistent with the interpretations of the other liver enzyme deficiencies in albino deletion homozygotes. In the case of those it was assumed that the deletions included a gene or genes encoding a factor concerned specifically with the transcriptional regulation of other structural genes expressing liver cell type-specific traits (27). The nature of the regulating factor remains to be identified, but it might be analogous to the "transcriptional regulatory protein" postulated by Yamamoto (28) to interact with specific enhancer elements in the modulation of transcription.

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