Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6611–6614, November 1983
Genetics

Correction of a genetically caused enzyme defect by somatic cell hybridization

(glucose-6-phosphatase induction/enzyme-deficient mutant liver cells)

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Contributed by Salome G. Waelsch, July 25, 1983

ABSTRACT **Liver cells obtained from newborn mice homozygous for any one of several overlapping deletions in chromosome 7 fail to express a number of liver-specific differentiated traits. Among these is the activity of the membrane-bound liver-specific enzyme glucose-6-phosphatase (Glc-6-*P*ase; D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9). Previous studies have led to the suggestion that the region of the genome covered by these deletions includes genes that normally regulate the expression of structural genes encoding liver-specific enzymes and proteins mapping elsewhere in the genome. To find out whether the deficiency of Glc-6-*P*ase may be caused by the deletion of the relevant structural gene, mouse liver cells homozygous for the deletion c^{14CoS} were hybridized with 2S Faza rat hepatoma cells, and the hybrid cell cultures were analyzed for mouse and rat Glc-6-*P*ase activity. Hybrids showed expression of mouse Glc-6-*P*ase activity, proving that the structural gene for this enzyme is not included in the deletion c^{14CoS} in chromosome 7. In the hybrid cells the rat hepatoma genome apparently contributes a factor that activates the structural gene of the mouse and corrects its failure of expression, which most likely resulted from the deletion of an essential regulatory or processing gene. By using as a marker glucose-6-phosphate isomerase (Glc-6-*P*Iase; glucosephosphate isomerase, D-glucose-6-phosphate ketolisomerase, EC 5.3.1.9), known to map on chromosome 7, this entire chromosome could be excluded as a possible carrier of the Glc-6-*P*ase structural gene. In addition, the structural genes for Glc-6-*P*ase and for tyrosine aminotransferase (TyrATase; L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5), another enzyme deficient in lethal deletion homozygotes, were shown to map on two different chromosomes. Together with our previous studies of TyrATase gene regulation, the present experiments suggest that the region of the mouse genome defined by the deletions includes one or more genes regulating the expression of several structural genes that map on different chromosomes and that encode liver-cell-type specific traits.**

Studies of the effects of several overlapping lethal deletions on chromosome 7 of the mouse have provided strong indications for the presence of tissue-specific regulatory genes in the corresponding region of the intact chromosome. These appear to be instrumental in the differentiation of various traits of liver parenchymal cells, including liver-specific enzymes and proteins. The structural genes coding for some of these proteins are known to map elsewhere in the genome, but their expression appears to be subject to regulation by a gene or genes mapping in the deleted chromosomal region (1). In the case of tyrosine aminotransferase (TyrATase; L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5), somatic cell hybridization experiments provided evidence that even though the TyrATase structural gene is not expressed in newborn mice homozygous for the deletions, it is intact and maps in a chromosome other than that carrying the deletions. This conclusion is based on the observation that mutant mouse liver–rat hepatoma cell hybrids, when induced for TyrATase, produced not only rat but also mouse liver TyrATase (2). We report here experiments with glucose-6-phosphatase (Glc-6-*P*ase; D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9), another hepatic enzyme that shows virtually no activity in the deletion mutants, resulting in abnormally low blood sugar levels and early postnatal death (3). Somatic cell hybridization experiments were designed to find out whether the structural gene for Glc-6-*P*ase maps inside the deletion on chromosome 7.

MATERIALS AND METHODS

The experimental design was similar to that reported in another series of experiments (2).

Mutant Mice. Mice carrying the lethal deletion c^{14CoS} were used in these experiments. The relevant strains are maintained in our mouse colony. Matings of mice heterozygous for chinchilla (c^{ch}) as the normal allele and the c^{14CoS} deletion were used to produce deletion-homozygous newborn, identified as albinos with unpigmented eyes, which die within a few hours after birth.

Cells, Media, and Culture Conditions. Mouse liver cells were obtained from newborn deletion homozygotes and their normal littermates. The rat hepatoma cells (designated 2S Faza), a gift from Mary Weiss, are hypertetraploid with a mean number of 89 and a range of 77–111 chromosomes. They are resistant to 8-azaguanine and 6-thioguanine and sensitive to aminopterin. Culture media and cell fusion methods were identical to those described (2).

Induction of Glc-6-*P*ase. Cell hybrid cultures and rat hepatoma cells were induced by exposing them to a medium as described in the legend to Table 1. The induced cell cultures were shipped as frozen pellets from New York to Boston for analysis and could be kept frozen for several weeks without significant change in determined values.

Glc-6-*P*ase Assays. The cell cultures were sonicated in a buffer of 0.005 M sodium citrate/0.14 M KCl, pH 7/1 mM EDTA and centrifuged for 1 hr at 31,000 × *g* at 3°C. The pellet containing the microsomal membranes was dispersed in the same buffer. Aliquots were taken for protein determinations (4) and for incubation in 0.02 M glucose 6-phosphate (pH 6.8), specially purified to remove inorganic phosphate, for 30 min at 30°C,

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Abbreviations: Glc-6-*P*ase, glucose-6-phosphatase; Glc-6-*P*Iase, glucose-6-phosphate isomerase; TyrATase, tyrosine aminotransferase.

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and the reaction was stopped by addition of 10% trichloroacetic acid. After centrifugation, inorganic phosphate was measured by the Ames method (5) in a final volume of 1 ml. To determine a true blank (consisting of preformed inorganic phosphate and of phosphate liberated by other phosphatases), the Glc-6-*P*ase was first completely inactivated for 30 min at 37°C at pH 5 before incubation with glucose 6-phosphate as in the experimental sample.

Mouse and rat Glc-6-*P*ase can be distinguished by measuring the rate of enzyme inactivation at pH 5.4 at 30°C in 0.05 M acetate/bovine serum albumin at 5 mg per ml. Under these conditions the rate is about 2.9 times faster for the rat enzyme than for the mouse enzyme (Table 2).

By using a differential thermometer, the water bath (Tecam, TU-14 Tempunit) was found to be constant within ±0.02°C. After a suitable warming period, a zero time sample of 0.2 ml was removed and mixed with 0.2 ml of 0.3 M imidazole buffer (pH 6.8) and put on ice. Similar samples were removed after 5, 10, 15, and 20 min of incubation. Enzyme activity (c) was determined as described above. After subtracting the blank, the data were plotted as log c versus time and the best straight line was used for calculation of the first-order velocity constant (k).

The method has been tested by analyzing known mixtures of mouse and rat enzymes with good agreement down to 10% mouse enzyme. The percentage of mouse enzyme in the mixture can be read from a graph by plotting % mouse enzyme versus k, in which $k = 0.011$ min^{-1} corresponds to 100% mouse enzyme, and $k = 0.0316$ min^{-1} corresponds to 0% mouse enzyme. The k value from this graph for 10% mouse enzyme is 0.0295 min^{-1}. To minimize the effect of variations in the k values of rat and mouse enzyme in different cell cultures as well as other sources of error such as age of sample, each of the experimental samples was analyzed simultaneously with rat hepatoma cells grown and processed at the same time and made as comparable to the experimental samples as possible. Theoretically, induced mouse–rat cell hybrids containing no mouse enzyme should show the same k value as the rat hepatoma cells. Actually, the 17 induced hybrid cell cultures in Table 2, deemed negative for mouse enzyme, yielded an average k value of 0.0313 min^{-1}, as compared to 0.0316 min^{-1} for the rat hepatoma cells.

Glucose-6-Phosphate Isomerase (Glc-6-*P*Ise) as Marker for Chromosome 7. Because the structural locus for Glc-6-*P*Ise (glucosephosphate isomerase, D-glucose-6-phosphate ketolisomerase, EC 5.3.1.9) maps on chromosome 7, this enzyme served as a marker for presence or absence of this chromosome in the cell cultures. Its electrophoretic identification was carried out as described (2).

Table 1. Summary of induction of Glc-6-*P*ase in 2S Faza cells

Additions	Specific activity 0 hr	24 hr	48 hr	72 hr	96 hr
Dex/Bt_2cAMP	10		26	32	29
(1×) aa and Dex/Bt_2cAMP	9		59	101	138
(2×) aa and Dex/Bt_2cAMP	12		62	114	148
(1×) aa	19		44	96	133
(1×) aa and Dex/Bt_2cAMP	14		97	142	201
(1×) 10 essential aa and Dex/Bt_2cAMP	36	58	148	152	178
(1×) 10 nonessential aa and Dex/Bt_2cAMP	13	28	154	152	244
(1×) 10 nonessential aa and Dex/Bt_2cAMP	10	22	75	171	197
(1×) 6 nonessential aa and Dex/Bt_2cAMP	11	15	35	62	86
(2×) 6 nonessential aa and Dex/Bt_2cAMP	28	63	105	107	100
(2×) Ala, Gly, Cys, Ser and Dex/Bt_2cAMP	26	57	100	117	103

The growth medium (McCoy's 5a) contained glucose, amino acids, and 15% fetal calf serum. Additions were 1 μM dexamethasone (Dex)/0.5 μM dibutyryl cAMP (Bt_2cAMP) and amino acids (aa) in the same (1×) or double (2×) the concentration found in rat serum (6). For each induction time, the specific activity is given in nmol/min/mg protein in the microsomal fraction.

RESULTS

It is not known what physiological inducer is responsible for the increase in Glc-6-*P*ase activity shortly after birth, but the process can be accelerated by the injection of glucagon or dibutyryl cAMP into newborn mice or mice *in utero*. In adult animals, glucocorticoids readily induce Glc-6-*P*ase activity.

In cell cultures in which the 2S Faza cells alone were used as test object, neither dexamethasone nor dibutyryl cAMP at various doses has much inducing effect *per se*. Rather, their action appears to be that of increasing the effectiveness of added amino acids (Table 1). The mechanism of induction has not been fully explored. The initial experiments in Table 1 show that with the concentration of the 20 amino acids at twice the level normally found in rat serum (2×), the effect is only slightly higher than that obtained with the normal (1×) concentration, indicating that the system is nearly saturated with amino acids. When the 10 essential amino acids (or the 10 nonessential amino acids) alone are added, the induction is about as strong as with the complete amino acid mixture. However, when only 6 nones-

Table 2. Induction of Glc-6-*P*ase in mouse livers, rat hepatoma cells, and hybrid cell cultures

Sample	Glc-6-*P*ase specific activity, nmol/min per mg membrane protein: Uninduced	Induced	No. of samples	k, min^{-1}	% Glc-6-*P*ase: Mouse	Rat
Normal adult mouse livers induced *in vivo* with dexamethasone	49	386	6	0.011 ± 0.0005	100	
Rat hepatoma cells	16	215	14	0.0316 ± 0.0004		100
Mutant mouse liver–rat hepatoma cell hybrids	31	347	9	0.0269* ± 0.0007	22.8 (14.1–37.1)	77.2
		262	8	0.0314	0	100
Normal mouse liver–rat hepatoma cell hybrids	24	297	9	0.0278* ± 0.0004	18.7 (13.4–26.3)	81.3
		270	9	0.0312	0	100

Samples were incubated simultaneously in the same water bath. k values were determined at pH 5.4, 30°C, and are shown as mean ± SD.
*Significantly different from rat hepatoma cells (Fisher's t test; $P < 0.01$), but not significantly different from each other. Hybrid cell cultures having 0% to <10% mouse enzyme have been grouped together in the zero column.

Table 3. Mouse Glc-6-*P*ase activity in liver cell hybrid colonies

Fusion		Colonies		Colonies with mouse Glc-6-*P*ase activity		Colonies without mouse Glc-6-*P*ase activity	
Type	No.	No. derived	No. analyzed	No.	%	No.	%
Mutant mouse liver–rat hepatoma cells	9	19	17	9	53	8	47
Normal mouse liver–rat hepatoma cells	10	21	18	9	50	9	50

sential amino acids (asparagine, aspartic acid, glutamine, glutamic acid, proline, and tyrosine) are added, or when the 4 amino acids that were omitted and that are convertible to pyruvate are added, a definite decrease in induction takes place. Induction after >96 hr has not been investigated, but there is still an increase in Glc-6-*P*ase activity between 72 and 96 hr, except when the induction is not maximal, as in the last two experiments in Table 1, in which the Glc-6-*P*ase activity levels off after 48 hr of induction.

Glc-6-*P*ase activity in microsomes prepared from induced rat hepatoma cell cultures showed very little latency when tested with detergents or mannose 6-phosphate as substrate. This was true also when unfrozen 2S Faza cell cultures were used and when they were gently homogenized in an all-glass homogenizer in isotonic sucrose solution. Control preparations of microsomes from mouse or rat liver showed strong latency. The "leakiness" of the hepatoma microsome preparations makes it difficult to decide whether the rat hepatoma enzyme contains the associated glucose 6-phosphate transporter protein that was described by Arion *et al.* (7) and shown to be controlled by a separate gene.

Nine fusions were made between mutant mouse liver and 2S Faza rat hepatoma cells. These yielded 19 colonies for enzyme analysis. Control material was provided from 10 fusions of normal littermate liver and 2S Faza rat hepatoma cells, which yielded 21 colonies to be assayed for Glc-6-*P*ase. The results are summarized in Tables 2 and 3. They clearly demonstrate the expression of mouse Glc-6-*P*ase activity in at least 50% of the hybrid cells with the homozygous chromosomal deletion responsible for failure of enzyme expression in the parental liver cells. The normal mouse liver–rat hepatoma cell hybrids gave the same results—i.e., mouse Glc-6-*P*ase activity was expressed in 50% of the colonies and was of a magnitude similar to that of the mutant mouse liver cell hybrids. This indicates

Table 4. Association of expression of mouse Glc-6-*P*ase and Glc-6-*P*Iase

Hybrid	No. of colonies				
	Glc-6-*P*ase$^+$ GPI$^+$	Glc-6-*P*ase$^-$ GPI$^-$	Glc-6-*P*ase$^-$ GPI$^+$	Glc-6-*P*ase$^+$ GPI$^-$	Total
Mutant mouse liver–rat hepatoma cells	8	3	5	1	17
Normal mouse liver–rat hepatoma cells	6	4	1	7	18

Table 5. Association of expression of mouse TyrATase and Glc-6-*P*ase in cell hybrid colonies

Hybrid	No. of colonies				
	Tyr-ATase$^+$ Glc-6-*P*ase$^+$	Tyr-ATase$^-$ Glc-6-*P*ase$^-$	Tyr-ATase$^+$ Glc-6-*P*ase$^-$	Tyr-ATase$^-$ Glc-6-*P*ase$^+$	Total
Mutant mouse liver–rat hepatoma cells	4	5	3	5	17
Normal mouse liver–rat hepatoma cells	2	6	3	7	18

that induction of the mouse enzyme in the normal liver cell hybrids also is dependent on activating agents formed in the rat genome.

The mouse liver enzyme Glc-6-*P*Iase can be distinguished electrophoretically from the corresponding rat enzyme and, because its structural gene maps on chromosome 7, it can serve as a marker for this chromosome. The results are summarized in Table 4. Eleven of the 17 mutant mouse liver cell hybrids showed concordance of activities of both enzymes; 6 of the hybrids showed discordance. In the cell hybrids, the presence of chromosome 7 appears not to be essential for the expression of Glc-6-*P*ase activity, and this chromosome is therefore excluded as that carrying the structural gene for this enzyme. The results obtained with normal mouse liver cell hybrids support this conclusion.

Because neither the structural gene for TyrATase (2) nor that for Glc-6-*P*ase maps on chromosome 7, it appeared interesting to evaluate the possible association of these two enzyme activities in the mutant and control cell hybrid cultures. Table 5 shows the results of such a tabulation. The two enzymes are expressed independently of each other in both mutant and normal cell hybrids, indicating independent assortment of the respective structural genes. Therefore, they are assumed to map on two different chromosomes.

DISCUSSION

Glc-6-*P*ase, an enzyme primarily expressed in liver and kidney, shows little or no activity in these organs in newborn mice homozygous for a deletion on chromosome 7 that is responsible for their perinatal lethality (1). The experiments reported here have served, however, to demonstrate inducibility and expression of this membrane-bound enzyme in the same mouse liver cells homozygous for the deletion after they had been fused to 2S Faza rat hepatoma cells. TyrATase, a cytosolic enzyme not expressed in liver cells of deletion homozygotes, has been shown previously to be inducible in mouse–rat cell hybrids (2). In neither case can the lack of expression of the enzyme be caused by the deletion of its structural gene. As pointed out (1), other liver-specific differentiation products, in particular several serum proteins that are deficient in the presence of the homozygous deletion in chromosome 7, are known to have their respective structural genes on other chromosomes. Thus, the deletion affects the expression of at least five proteins without the elimination of their structural genes. It is important to emphasize that these protein and enzyme deficiencies are specific and that the mutant liver is capable of synthesis of normal amounts of other proteins, demonstrating the existence of an intact protein synthesizing machinery (8).

The specificity of effects implicates the deleted portion of chromosome 7 in the regulation of expression of a number of

structural genes that map elsewhere and encode liver-specific proteins, including enzymes. The degree of specificity of the deletion effects is further emphasized by the ultrastructural abnormalities of various intracellular membranes, that are restricted to liver parenchymal cells, while most other cell types remain perfectly normal.

Of the cell hybrid cultures, a higher percentage shows inducibility of mouse Glc-6-*P*ase than was the case with TyrATase. Whereas only ≈25% of all cell hybrid cultures expressed mouse TyrATase activity in mutant as well as normal hybrid cells, >50% did so in the case of Glc-6-*P*ase, which could be because of the different, more prolonged, and more effective inducing method.

Nothing is known at this time of the molecular nature of the factor(s) contributed by the rat genome and responsible for inducing the expression of mouse Glc-6-*P*ase activity in cell hybrids with an enzyme-deficient mouse liver cell parent. Whatever it may be, this factor must be instrumental in the normal differentiation of this liver-specific trait, and it appears to be encoded in the portion of the genome that is deleted in mutants lacking the enzyme. At this time it cannot be decided whether the factor is identical with or different from that responsible for induction of TyrATase expression. Correction of the two enzyme deficiencies in mutant cell hybrids suggests that the segment of chromosome 7 identified by the deletions includes a gene or genes regulating or processing the expression of structural genes mapping elsewhere and encoding liver cell-specific traits. Such regulation of cell-type specificity is reminiscent of Herskowitz's concept of master regulatory genes and proteins derived from the analysis of the mating-type locus in yeast (9), which may serve as a model for similar systems of regulation of gene expression in other cell types.

We gratefully acknowledge the continued generosity of Dr. Mary Weiss in providing us with 2S Faza cells. We thank Leslie Pick and Susan G. Schiffer for their help with the initial cell hybrid cultures and Lorna Chang for excellent technical assistance. This work was supported by grants from the National Science Foundation (PCM80-25507), the National Institutes of Health (GM-27250 and GM-19100), and the American Cancer Society (RD-80 and CD-38). Funds were also provided by friends of Washington University (St. Louis, MO) through Chancellor William H. Danforth, for which C.F.C. expresses his gratitude.

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