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A variation in mouse kidney pyruvate kinase activity determined by a mutant gene on chromosome 9

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SUMMARY

Pyruvate kinase activity was studied in kidney extracts of mice from various sources. One C57BL/6J female among several hundred examined expressed activity approximately one half of the normal level. This animal transmitted the trait, and a single gene responsible for the activity difference was identified and mapped to chromosome 9. Linkage with *Mod-1* and *dilute* was established. A spontaneous mutation in the C57BL/6J strain apparently was responsible for the variant pyruvate kinase.

1. INTRODUCTION

Pyruvate kinase (PK, E.C. 2.7.1.40) catalyses the transfer of a phosphate group from phosphoenolpyruvate to adenosine diphosphate with co-production of pyruvate and adenosine triphosphate. In mice and other mammals the enzyme exists in multiple forms (Cardenas & Dyson, 1978). Cell hybridization experiments have identified chromosome 9 as the apparent location of one *Pk* locus, *Pk-3* (Lalley, Francke & Minna, 1978). In this report the discovery of genetic variation which results in reduced PK-3 activity is described.

2. MATERIALS

Mice were obtained from the Jackson Laboratory. Nucleotides were obtained from P-L Biochemicals, Milwaukee, Wisconsin. Enzymes, substrates, and Triton detergent were obtained from Sigma Chemical, St Louis, Missouri. Other chemicals were of reagent quality and were obtained from local suppliers.

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3. INSTRUMENTATION

Assays were performed with a GeMSAEC portable centrifugal analyser. This instrument has seventeen cuvettes arranged on the perimeter of a small rotor. Sixteen of the cuvettes were used for sample assays while the seventeenth was the reference cuvette. In operation, after a rapid acceleration phase which mixed and transferred the reagents and tissue extracts into the cuvettes, each cuvette was monitored sequentially as it passed a stationary photo-multiplier. The data were accumulated in an LSI-11 computer and analysed by a least-squares best-fit procedure to determine the initial rate of the reaction. Temperature was controlled at 30 °C. The instrument has been fully described by Burtis *et al.* (1972, 1973) and Tiffany *et al.* (1972, 1973). Rotors were loaded with an automatic sample loader (Burtis *et al.* 1974) that gave rapid and precise loading of the small volumes required. The advantage of this instrument is that it provides greater efficiency to the analysis of enzyme activities than is possible by spectrophotometers of conventional design.

4. METHODS

Tissue. Kidneys were removed after methoxyflurane anaesthesia. Each kidney was homogenized with 0·6 ml buffer. Insoluble material was removed by centrifugation at 18000 ***g*** for 20 min. The supernatant was frozen at −70 °C and stored in small aliquots until needed.

Pyruvate kinase activity assays. All pyruvate kinase activity assays were performed with the fast analyser. The final reagent concentrations were (*a*) 1·0 mM phosphoenol pyruvate, (*b*) 1·2 mM adenosine diphosphate, (*c*) 0·5 mM reduced nicotinamide adenine dinucleotide, (*d*) 0·4 mM magnesium chloride, and (*e*) 0·04 M potassium phosphate buffer, pH = 8·0. The principle of the assay is the coupling of pyruvate production from phosphoenol pyruvate (Reaction 1) to pyruvate reduction by lactate dehydrogenase (Reaction 2).

$$\text{PEP} + \text{ADP} \rightleftharpoons \text{Pyruvate} + \text{ATP} \quad (1)$$

$$\text{Pyruvate} + \text{NADH} \rightleftharpoons \text{Lactate} + \text{NAD} \quad (2)$$

The progress of the latter reaction is monitored as NADH oxidation at 340 nm. The extract already contained sufficient lactate dehydrogenase to catalyse the second reaction faster than the first; therefore, the addition of lactate dehydrogenase to the reaction mixture was not necessary.

Pyruvate kinase electrophoresis. The enzyme was separated by horizontal starch gel electrophoresis using a Tris-citrate, pH 8·0, system of buffers (Johnson *et al.* 1980*b*).

To stain the gels for pyruvate kinase, a three-step procedure was developed. First, the slice was incubated for 10 min in 50 ml of a solution containing (*a*) 0·1 M-KCl, (*b*) 5 mM $MgCl_2$, (*c*) 4 mM phosphoenol pyruvate, (*d*) 2 mM ADP, (*e*) 1 mM NADH, (*f*) 50 i.u. LDH, and (*g*) 0·1 M Tris-base adjusted to pH 8·0 with HCl. Second, the first solution was removed and the gel incubated at 37 °C for 50 min. Finally, the bands were developed by adding 50 ml of a solution con-

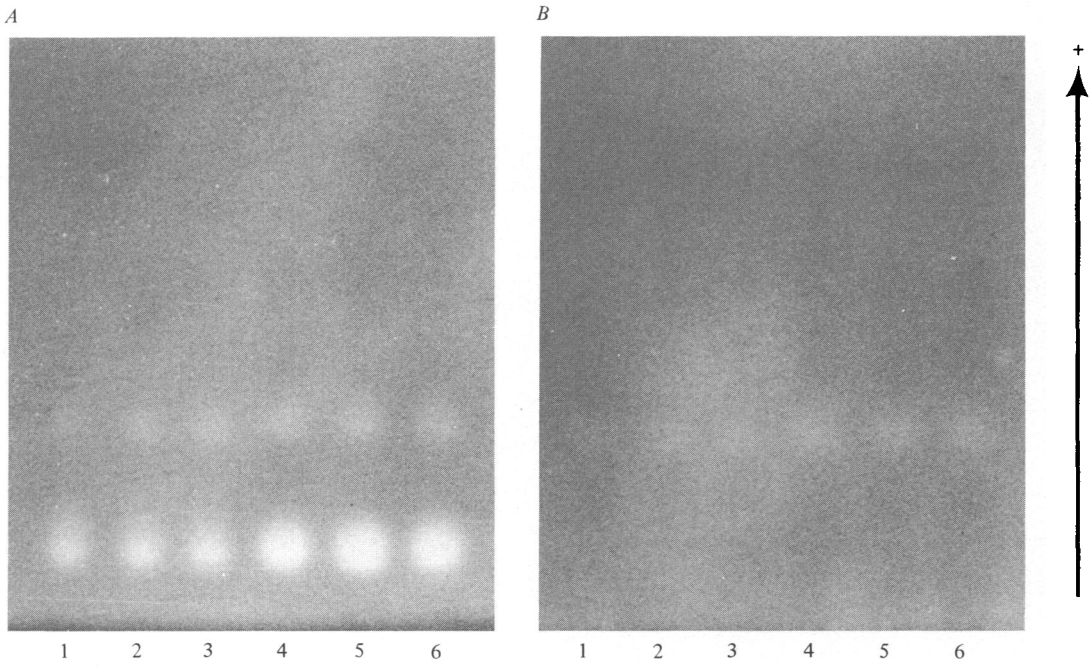

Fig. 1. Photograph of starch gels stained for pyruvate kinase, A, with phosphoenol pyruvate present in the stain solution, and B, in the absence of phosphoenol pyruvate. Samples 1–3 are DBA/2J males; samples 4–6 are DBA/2J females. The arrow indicated migration towards the anode.

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taining 0·75 mM NBT and 0·075 mM PMS. Pyruvate kinase activity was expressed as a white spot on a blue background (Fig. 1 A). The specificity was confirmed by eliminating the phosphoenol pyruvate from the first solution and continuing the same procedure (Fig. 1 B). When this was done, only one band disappeared (pyruvate kinase) but a minor area of activity remained (probably due to unspecific tetrazolium oxidase activity).

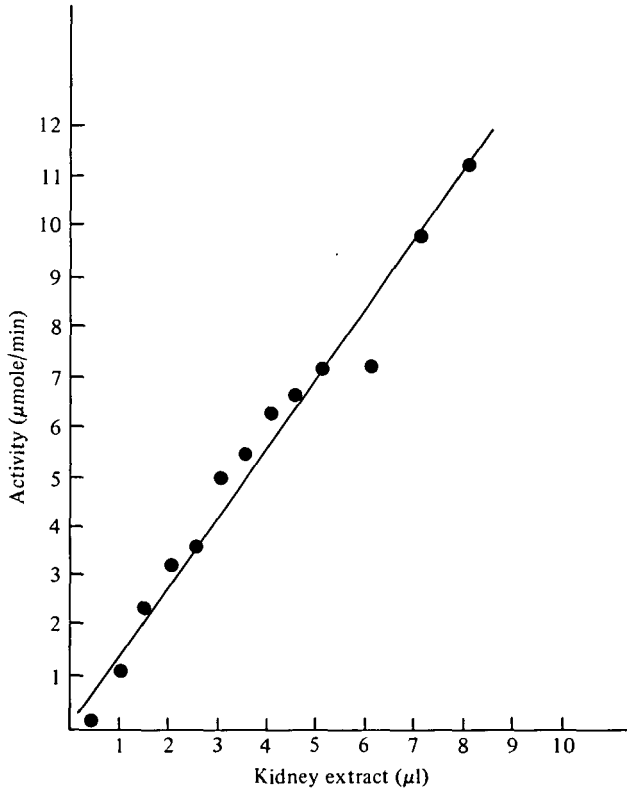

Fig. 2. Graph of PK activity with increasing amounts of kidney extract. Values are based on a pooled extract of several animals.

Cytoplasmic malic enzyme. Genotypes controlling electrophoretic variation of malic enzyme (MOD-1) were determined by procedures described elsewhere (Johnson *et al.* 1980*a*).

5. RESULTS AND DISCUSSION

Pyruvate kinase is one of several enzymes which has been screened for variation among C57BL/6J, DBA/2J and their F_1 progeny in an attempt to explore methods of detecting germinal mutations in mice (Johnson, 1979; Johnson *et al.* 1980*b*). Techniques so far employed have utilized electrophoresis and activity analysis. Variation detected by electrophoresis has been reported previously (Johnson *et al.* 1980*b*). Described here is the first case of variation revealed by activity screening.

The sample with which investigation for variation was concerned included 200 C57BL/6J parental females, 35 parental DBA/2J males and 1500 F_1 offspring. Additionally, a number of stock animals were analysed in order to establish baseline values.

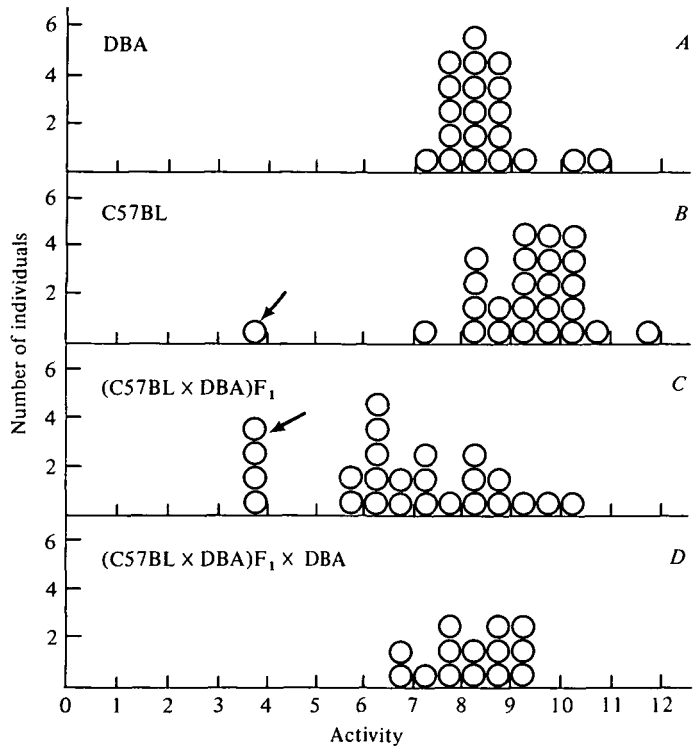

Fig. 3. Distribution of PK activities in individual kidney extracts from female animals from different sources. The arrow in segment B identifies the variant parental C57BL animal, and the arrow in segment C, her four variant F_1 progeny.

Fig. 2 shows a graph of the response in PK activity to increasing amounts of enzyme (kidney extract), under the assay conditions used for screening. The relationship is linear and demonstrates the ability of the assay to detect differences should they occur.

Table 1 presents typical activity values seen for DBA/2J, C57BL/6J, plus F_1 and backcross offspring. The values are presented graphically in Fig. 3 (females) and Fig. 4 (males). It is apparent upon examining these data that there are both sex and strain differences. In the DBA strain, females have appreciably higher activity than males, and in the C57BL strain, females typically have slightly higher values. Likewise, among F_1 and backcross offspring, females usually show somewhat higher activity than males. Between strains, the C57BL strain seems to be generally higher in activity than the DBA strain, but the differences are much greater for males than for females.

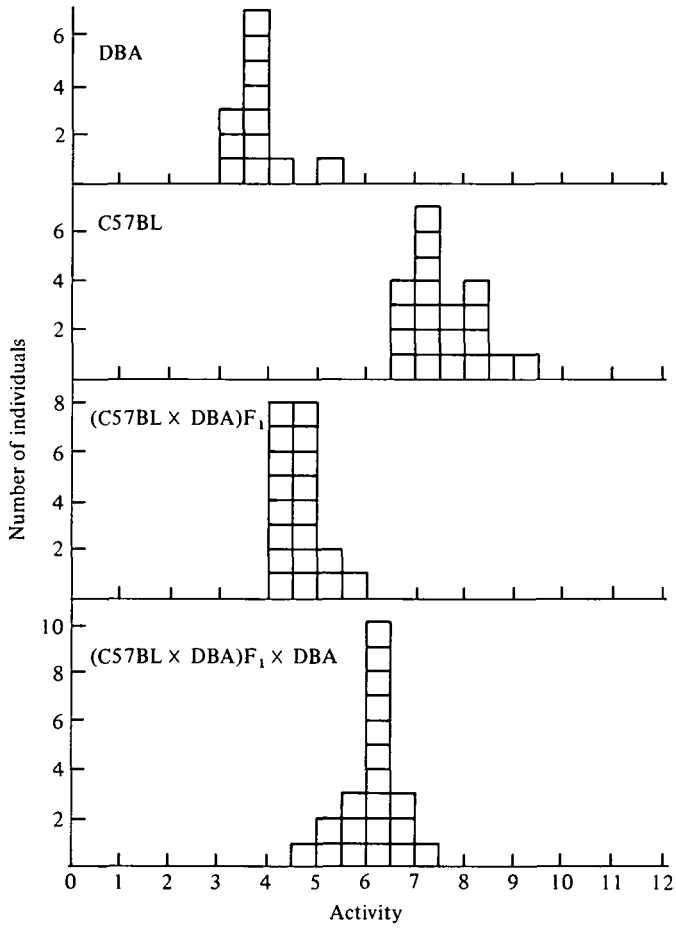

Fig. 4. Distribution of PK activities in kidney extracts in males. Arrangement follows that of Fig. 3.

Table 1. *Pyruvate kinase specific activities* ($\pm$ *standard deviation*) *in kidney samples from normal animals of different types*

Animal type	Specific activity
DBA/2J, ♀♀	8·3 ± 0·8
DBA/2J, ♂♂	3·8 ± 0·6
C57BL/6J, ♀♀	9·0 ± 0·9
C57BL/6J, ♂♂	7·5 ± 0·7
(C57BL/6J × DBA/2J) F_1, ♀♀	7·4 ± 1·3
(C57BL/6J × DBA/2J) F_1, ♂♂	4·5 × 0·4
(C57BL/6J × DBA/2J) F_1 × DBA/2J, ♀♀	8·1 × 0·9
(C57BL/6J × DBA/2J) F_1 × DBA/2J, ♂♂	6·2 × 0·7
(C57BL/6J × DBA/2J) F_1 × C57BL/6J, ♀♀	6·7 × 0·9
(C57BL/6J × DBA/2J) F_1 × C57BL/6J, ♂♂	6·2 × 0·5

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The genetic basis for the normal strain difference is unknown, but it appears polygenic. Thus, on examining males (where the strain difference is most extreme), F_1 animals show values intermediate between the parental types. Also, backcross offspring tend to average in the intermediate range between F_1 and parental types. Activities in all groups seem to be normally distributed, although the data are rather limited for precise description.

Electrophoretically, all groups look the same except that males stain more lightly than females (Fig. 1).

In the course of screening for variation, one C57BL female was found which had PK activity only about half that of normally expected values. PK activity of that unusual female and the remainder of her family is shown in Fig. 5. There were 7 offspring produced from the female, 6 females and 1 male. Four of the six female offspring had reduced activity, as the mother did. The one male offspring, and the male parent were normal. All the exceptional individuals were clearly outside normally expected values (Fig. 3). The variant animals were examined by electrophoresis. They displayed a band of activity that migrated the same as that of normal individuals, and the variant bands could usually be observed to be somewhat lighter in staining intensity than normal.

In order to pursue the inheritance of the variant PK activity several matings were performed. Offspring 'G' (Fig. 5) was mated with several DBA/2J females, but none of the offspring expressed activity levels significantly lower than that observed in comparable crosses from families in which all individuals had normal activities. It was not possible to remate the variant parental animal because it died between the time of surgery and discovery of the variation.

Three of the 4 variant F_1 females were mated with DBA/2J males and produced litters. The results are shown in Fig. 6. Generally, activity values formed a bimodal distribution with approximately equal numbers under each peak. The average low value was approximately the same as observed originally; and thus segregation as due to a single major Mendelian factor would seem to account adequately for the pattern.

The observation of the single variant C57BL/6J parental animal and the inheritance pattern in the progeny suggest that the parent was heterozygous for a mutant gene that occurred in a previous generation. From the one case, it would appear that mutation occurred in an animal involved in one of the final stages of production at Jackson Laboratory, the supplier of the parental C57BL and DBA animals.

The backcross mice were examined for MOD-1 and dilute phenotypes as they were scored for PK activity, this to determine if linkage could be detected. Although activity did not behave perfectly as a discrete character, it was sufficiently well defined, especially in females, to observe that almost all of the individuals in the low PK activity group carried the *Mod-1*^{b} and *D* genes (characteristic of C57BL). Likewise, high PK activity co-segregated with the *Mod-1*^{a} and *d* genes of the DBA strain. This is illustrated in Fig. 6 for dilute and nondilute. *Mod-1*, not illustrated, appeared similarly (Johnson *et al.* 1980*c*). Together, the results locate

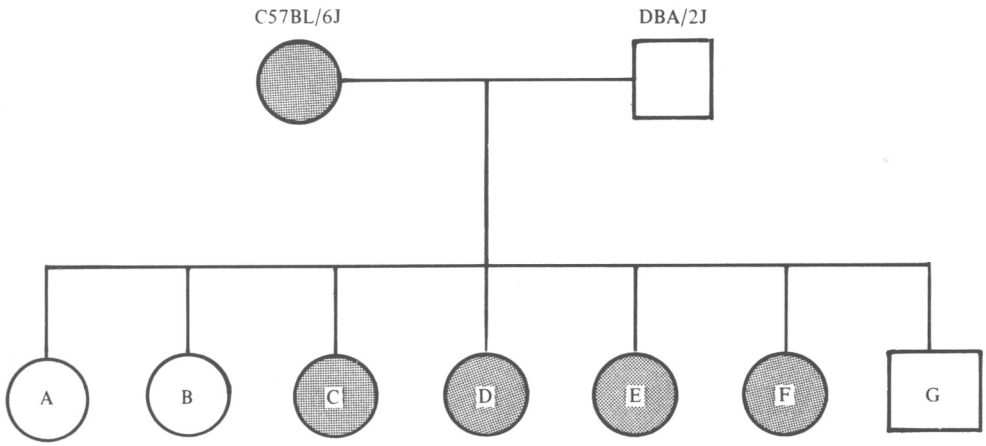

Fig. 5. The family of animals which first showed variation in PK activity.

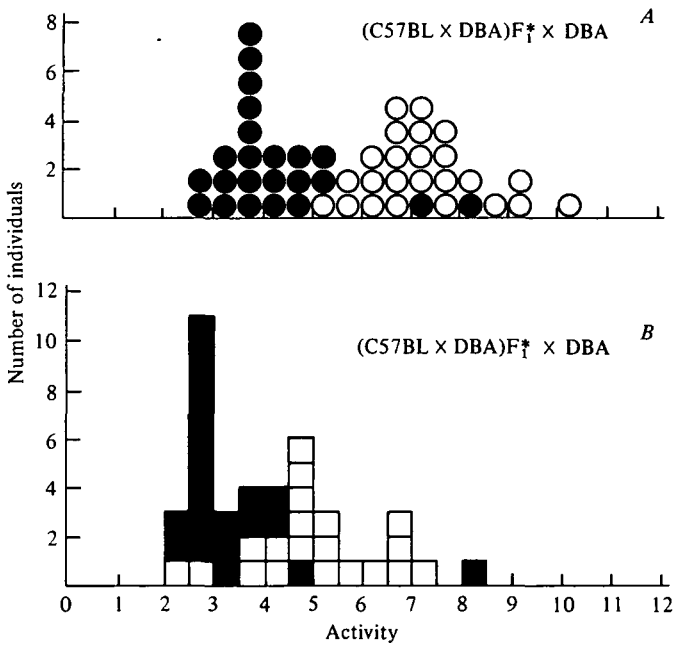

Fig. 6. PK activities in the progeny of the backcross between the variant F_1 animals (F_1*) and DBA animals. Open circles indicate the dilute phenotype (d/d), solid circles non-dilute (D/d). The upper distribution depicts females, the lower one males.

the controlling factor for reduced PK activity to chromosome 9. By other means, a gene designated *Pk-3* has been localized to chromosome 9 by Lalley *et al.* (1978), and that gene apparently controls the major PK found in kidney (Cardinas & Dyson, 1978). While there remains some question as to whether or not this is the same locus as that which the presently reported variation concerns, it is at least a reasonable working hypothesis to suppose so. Thus, the normal allele that deter-

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mines PK activity at levels regularly found in DBA/2J and C57BL/6J strains is provisionally designated *Pk-3^a*, while the variant allele responsible for reduced activity is termed *Pk-3^r*.

Homozygotes for the mutant *Pk-3^r* gene have not yet been produced. It is possible that such a genotype will result in the complete absence of PK-3 and, furthermore, may be lethal. Additional genetic analyses are in progress and characterization of the normal and mutant gene products is planned.

This work was the product of a project that is concerned with the investigation of various approaches to the detection of germinal mutations in mice. The centrifugal analyser, because of its efficiency, allows a variety of detection concepts which would otherwise not be practical to be pursued effectively. One is the concept of detection based on enzyme activity differences caused by mutation; and, in this regard, the present report is a successful demonstration of the instrument for this purpose. Activity as a general indicator is conceivably capable of providing recognition of mutations at structural enzyme loci as well as at other loci concerned with regulation or modification of the structural gene products. Thus, by change in a single phenotypic parameter, activity, mutational events at more than one locus might be conveniently detected. The value of the concept will ultimately be determined as more samples and more variant enzymes are subjected to similar analyses.

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