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Studies on Metatherian Sex Chromosomes IV.* X Linkage of PGK-*A* with Paternal X Inactivation Confirmed in Erythrocytes of Grey Kangaroos by Pedigree Analysis

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Abstract

Pedigree and population data are presented for erythrocyte phosphoglycerate kinase A (PGK-*A*) allozymes in the two species of grey kangaroo. The pedigree data confirm the hypothesis that PGK-*A* is X linked, the paternally derived X chromosome being inactive in nucleated erythroblast precursors of the enucleate erythrocytes in females. The existence of different allozyme variants in eastern and western grey kangaroos further supports their division into separate species.

Introduction

Most macropodid species are monotypic for an allozyme of phosphoglycerate kinase A (PGK-*A*) designated N for 'normal', but populations of the two grey kangaroo species possess, in addition, variant allozymes which are distinct from each other (Cooper *et al.* 1971). The variant allozyme of the eastern grey kangaroo, *Macropus giganteus*, is designated VE for 'variant eastern' and has a more negative charge than N. The variant allozyme of the western grey kangaroo, *Macropus fuliginosus*, is designated VW for 'variant western' and has a more positive charge than N. The results of a preliminary investigation suggested that PGK-*A* is X linked in grey kangaroos, the paternally derived X chromosome being inactive in the nucleated erythroblast precursors of the erythrocytes of females (Cooper *et al.* 1971). We have now greatly extended the investigation and shown that the hypothesis as originally proposed is the only explanation compatible with the data.

Methods

Most of the captive animals used in this study were from our breeding colonies. The code numbers of those held by CSIRO are prefixed by G in the text, and of those held by Macquarie University are prefixed by SG. Blood was collected from the caudal vein of captive animals and from the heart or the throat of animals shot in the field. Up to 10 ml of blood was added to 10 ml of sterile citrated saline (5 g NaCl and 17·4 g dihydrated trisodium citrate to 1 litre with distilled water) and thoroughly shaken. The cells were washed once in 0·85% saline and preserved at −20°C in approximately 1·5 volumes of ethylene glycol–citrate solution (60 g dihydrated trisodium citrate and 400 ml of ethylene glycol made up to 1 litre with distilled water) per volume of packed cells. Starch gels were made from 36 g Electro-Starch (Otto Hiller, Madison, Wisconsin) and 300 ml of 0·01 M tris–citrate buffer, pH 7·3. After being degassed by a suction pump, the molten starch was poured into moulds 128 by 204 by 6 mm, covered with a glass plate, and allowed to cool for several hours before use. After the cover was removed, intact erythrocytes were applied to the gel by the technique of VandeBerg and Johnston (1976). The gels were electrophoresed overnight at

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6 V/cm at 4°C, the electrolyte buffer concentration being 10 times that of the gel buffer. Gels were stained according to the technique of Cooper *et al.* (1971), the NADH concentration being increased 1·5–2·5 times for photographic purposes.

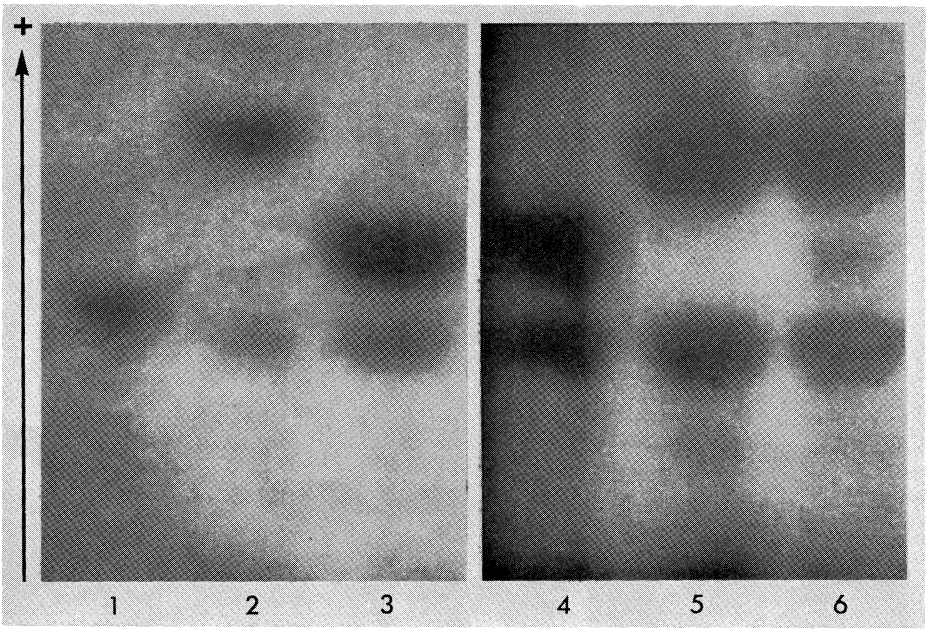


Fig. 1. PGK-*A* phenotypes of grey kangaroos. First gel (channels 1–3) showing the three allozymes: *1*, western grey kangaroo, type VW; *2*, eastern grey kangaroo, type VE; *3*, eastern grey kangaroo, type N. Second gel (channels 4–6) showing the unusual type encountered in eastern grey kangaroos; *4*, N; *5*, VE; *6*, VE/(N). The slow form common to all channels with mobility slightly slower than VW is PGK-*B*, which is inherited in an autosomal codominant manner (Cooper *et al.* 1971; VandeBerg *et al.* 1973*a*).

Results

All but 2 of the 347 animals typed for PGK-*A* expressed a single allozyme in their erythrocytes, either N, VE, or VW (Fig. 1). The two exceptions were both females bred from phenotypically VE mothers and N fathers. One was SG9 from which blood was taken when the animal was approximately 11 months old and again $3\frac{1}{2}$ months later. The first sample on repeated runs expressed predominantly the VE band, but a distinct trace of N was also present. Only the VE band could be detected in the second sample. The other animal, G475, was bled at the ages of approximately 9, 13·5, and 20 months. It consistently expressed predominantly VE with a trace of N (Fig. 1). This phenotype is designated VE/(N). The reciprocal phenotype, which was never observed in erythrocytes of any animal, would be designated N/(VE). Cultured fibroblast cells of SG9 also expressed VE/(N), and fibroblasts of SG60, a progeny of the reciprocal mating N♀ × VE♂, expressed N/(VE) (Cooper *et al.* 1975*a*, 1975*b*). To aid understanding, we offer our interpretation at this point. We believe these phenotypes result from slight expression of the paternally derived PGK-*A* type along with much higher activity of the maternally derived type.

The level of paternal X activity which could be detected in erythrocytes if it were present was determined from the experiments in which lysates (3 volumes of gel buffer per volume of packed cells) of N and VE were mixed in various ratios prior to electrophoresis. After lengthy staining, the weakly active band could be just detected in the reciprocal mixtures of 1 : 127, i.e. less than 1% paternal X activity is probably detectable in lysates. Since higher activity is gained by our routine technique of applying intact cells to a chromatography paper insert than by applying lysates (VandeBerg and Johnston 1976), the level of detection of paternal X activity in routine typing of erythrocytes is probably even greater.

Table 1. Breeding data for the inheritance of erythrocyte PGK-*A* in grey kangaroos

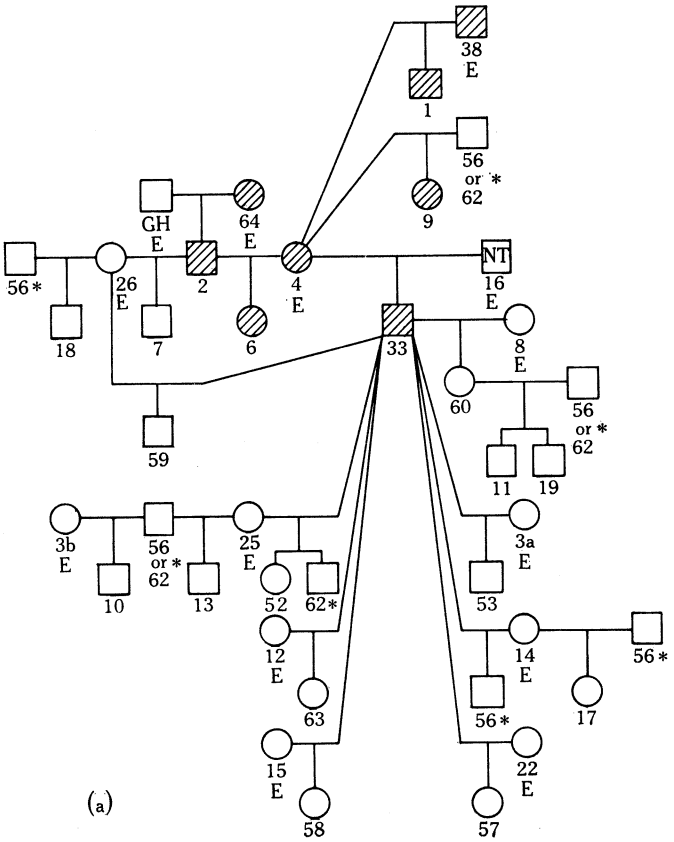
Phenotypes of parents		Offspring			
		Normal		Variant	
♀♀	♂♂	♀♀	♂♂	♀♀	♂♂
N	N	60	65	0	1[A]
N	?	13	4	0	0
?	N	7	4	0	0
N	VE	4	6	0	0
VE	N	3	3	4	6
VE	?	0	1	1	1
VE	VE	0	0	1	1
VW	N	1	1	1	0

[A] This individual was G476, the mother of which was typed as N, but this may have been an error. See the pedigree in Fig. 2 and the discussion of this point in the text.

The breeding data are presented in Table 1 with SG9 and G475 included with the VE phenotypes. Neither the VE/(N) nor the N/(VE) phenotype was observed in erythrocytes of the five other female or nine male offspring from VE♀ × N♂ matings nor in erythrocytes of the four female and six male offspring from N♀ × VE♂ matings shown in Table 1.

Three pedigrees showing the inheritance of VE and N phenotypes are presented in Fig. 2. These pedigrees show all known direct ancestors and descendants of phenotypically VE individuals tested. SG9 and G475 are indicated as VE types in the pedigrees. G15 and G40, which are important animals in one of these pedigrees, have also sired many progeny not shown in the pedigrees of VE inheritance. These matings involved only N phenotypes and are presented in the pedigrees in Figs 3 and 4*a*. A pedigree showing the inheritance of VW is shown in Fig. 4*b*. (For completeness a single pedigree involving the VP variant in the prettyface wallaby, *Macropus parryi*, is shown in Fig. 4*c*.)

The population data for the frequencies of VE and VW in individuals shot or caught in the wild are shown in Tables 2 and 3 respectively. The occurrence of two different allozymes in *M. giganteus* and *M. fuliginosus* is another piece of evidence which supports their division into separate species as was shown on other grounds by Kirsch and Poole (1972). The VE population data are given by States, because only a few animals in the study originated from any particular locality. The VW population data are given by the three commonly recognized subspecies: *Macropus fuliginosus ocydromus* of south-western Western Australia; *M.f. melanops* of southern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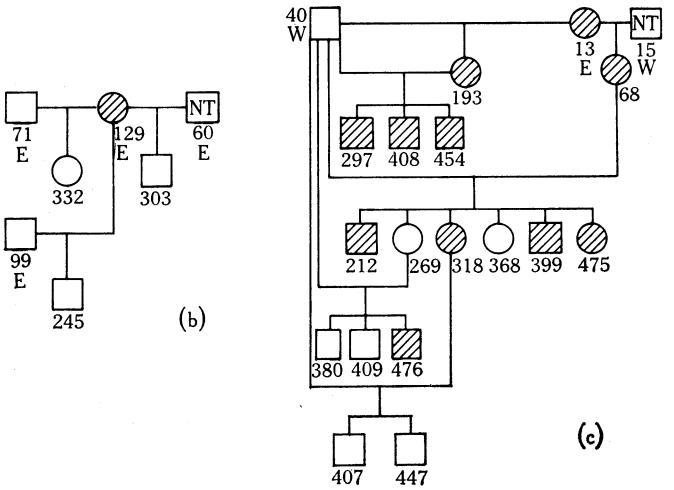
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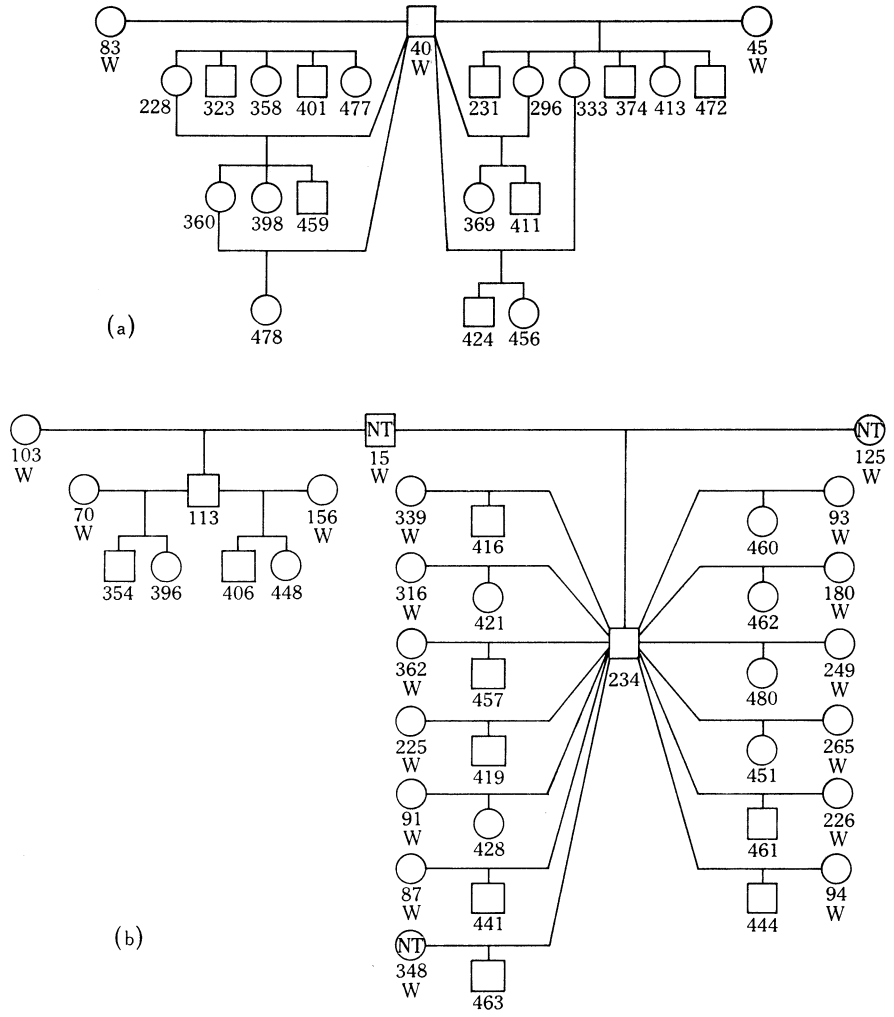

Fig. 3. Grey kangaroo pedigrees showing the absence of the VE allozyme in descendants of G40 and G15 from matings with other *M. fuliginosus*. The animals are from the CSIRO colony. See caption of Fig. 2 for other details.

Fig. 2. Grey kangaroo pedigrees of erythrocyte VE inheritance. Squares are males; circles females. The open symbols indicate animals phenotypically N, and the shaded symbols refer to animals phenotypically VE. NT signifies that the animal was not tested. The species of all animals originating from outside the pedigree is designated as E for eastern grey (*M. giganteus*) or W for western grey (*M. fuliginosus*). Pedigree (*a*) is of animals from the Macquarie University colony (prefixed by SG in the text), except for GH (Ghinty) and 64 which are from the colony maintained by Dr T. H. Kirkpatrick at the Department of Primary Industries Research Station, Warwick, Qld. Note that SG56 and SG62 appear in several places in the pedigree, as indicated by the asterisk. It is uncertain which of these males sired several of the progeny, as indicated, since both had access simultaneously to the does which gave birth at a particular time. Pedigrees (*b*) and (*c*) are of animals from the CSIRO colony (prefixed by G in the text). The phenotype of G269 given as N may represent an error in typing as discussed in the text.

South Australia, western Victoria, and south-western New South Wales; and *M.f. fuliginosus* of Kangaroo Island off the South Australian coast. Kirsch and Poole (1972) were inclined not to recognize *M. f. ocydromus* as a subspecies distinct from

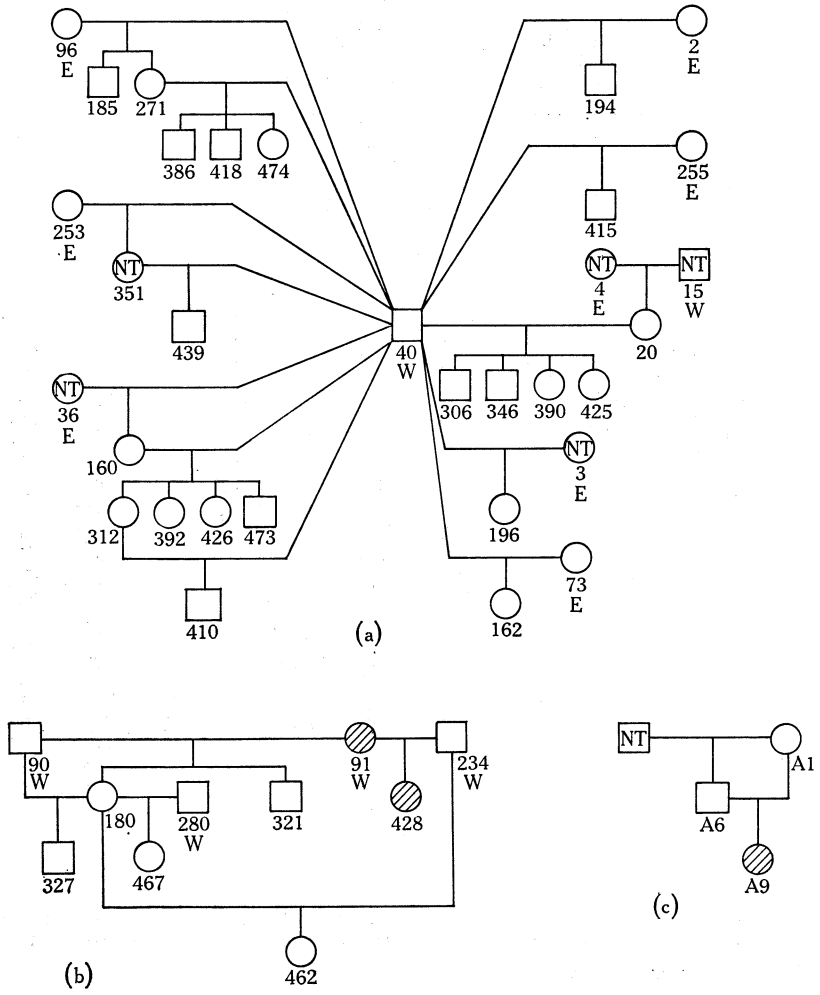

Fig. 4. (*a*) Grey kangaroo pedigree showing the absence of the VE allozyme in descendants of G40 and G15 from matings with *M. giganteus* of phenotype N. (*b*) Grey kangaroo pedigree of erythrocyte VW inheritance; open symbols indicate animals phenotypically N, and shaded symbols indicate animals phenotypically VW. (*c*) Prettyface wallaby (*Macropus parryi*) pedigree of erythrocyte VP inheritance (VP is the variant which occurs in this species); open symbols indicate N and shaded symbols indicate VP. The animals shown in (*a*), (*b*) and (*c*) were from the CSIRO colony. See Fig. 2 legend for other details.

M. f. melanops, but the distinction is maintained in this paper because the two variant individuals not known to be related were both from Western Australia. It is emphasized, however, that the data do not indicate a significant difference between the gene frequencies of the two populations ($P = 0{\cdot}65$ in Fisher's exact 2×2 contingency table test).

In contrast to the usually encountered form of X linkage, with X linkage *and* paternal X inactivation no difference is expected for the frequency of the PGK phenotypes between males and females (Cooper 1976). For the data on *M. giganteus* in Table 2, a 2×2 contingency test for association between sex and PGK type for the totals yields $\chi_1^2 = 0{\cdot}99$, $0{\cdot}50 > P > 0{\cdot}30$. The frequency of VW in Table 3 is too low to allow a significance test.

Table 2. Population data by States for the PGK-*A* polymorphism in erythrocytes of *M. giganteus* individuals not known to be related

Separate gene frequencies for the samples from the different areas are not given because no significant difference exists among the five groups of known origin. Using the Brandt and Snedecor formula $\chi_4^2 = 2{\cdot}37$, $0{\cdot}6 < P < 0{\cdot}7$. The overall gene frequency of the variant is 0·13

Territory or State	*n*	Normal ♀♀	Normal ♂♂	Variant ♀♀	Variant ♂♂
Qld	21	16	4	1	0
N.S.W.	46	22	17	6	1
Vic.	10	4	5	0	1
A.C.T.	18	6	10	1	1
S.A.	6	2	4	0	0
Unknown	30	16	8	4	2
Totals	131	66	48	12	5

Table 3. Population data by subspecies for the PGK-*A* polymorphism in erythrocytes of *M. fuliginosus* individuals not known to be related

Subspecies	*n*	Normal ♀♀	Normal ♂♂	Variant ♀♀	Variant ♂♂	Gene frequency of VW
M.f. ocydromus	35	19	14	2	0	0·06
M.f. melanops	27	18	9	0	0	0·00
M.f. fuliginosus	4	3	1	0	0	0·00

The N, VE, and VW allozymes of PGK-*A* appear to be equally active on gels. Both VE and N are equally heat stable, VW being untested for this parameter. Only slight inactivation of VE and N was observed after heating lysates for 32 min at 50°C, but lability was much more pronounced at 55°C (Fig. 5). PGK-*B*, which is autosomally inherited and expressed to a lesser extent than PGK-*A* in kangaroo soma (Cooper *et al.* 1971; VandeBerg *et al.* 1973*a*), was not affected by heating at 55°C.

Discussion

We shall assume that VE is determined by an allele $Pgk\text{-}A^{VE}$, N by the allele $Pgk\text{-}A^{N}$, and VW by the allele $Pgk\text{-}A^{VW}$. For convenience these symbols will be abbreviated to *VE*, *N*, and *VW*. The most common mode of inheritance of enzyme and other protein variation is autosomal codominance, a mode which clearly is not applicable to erythrocyte PGK-*A* of grey kangaroos, because (1) a double-banded codominant phenotype typical of animals heterozygous for a monomeric enzyme

was never observed, and (2) the pedigree data are inconsistent with codominant inheritance (e.g. VE♀♀ × N♂♂ produced only N and VE offspring). X-linked codominance is similarly ruled out. X-linked dominance of *VE* is ruled out by the production of N offspring by VE fathers, and X-linked dominance of *N* is ruled out by the production of VE offspring by N fathers. Since crosses within the species *Macropus giganteus* yielded those results, the hypothesis of X linkage with failure to activate the paternal X in species hybrids is not compatible with the observations.

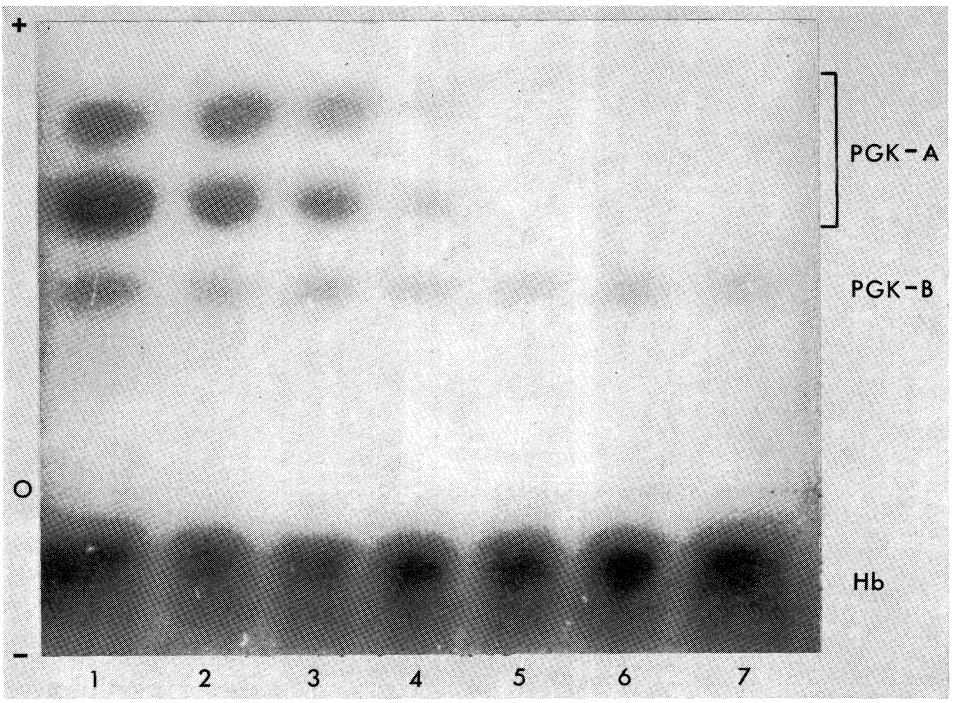

Fig. 5. Gel showing equal heat lability of N and VE allozymes. A mixture of equal volumes of N and VE lysates was heated at 55°C, and an aliquot was removed from the mixture and placed on ice after each of the following periods of heat treatment: *1*, no heating; *2*, 1 min; *3*, 2 min; *4*, 4 min; *5*, 8 min; *6*, 16 min; *7*, 32 min. Note the stability of PGK-*B* relative to PGK-*A*. The PGK-*B* bands are faint and not readily reproduced; they are, however, readily seen on the original gel even in channel 7.

The hypothesis of autosomal inheritance with *VE* dominant is inconsistent with the data. Because *VE* is low in frequency, most phenotypically VE individuals are putative heterozygotes on this hypothesis. Therefore, both VE♀ × N♂ and N♀ × VE♂ matings should give close to a 1 : 1 ratio of N : VE progeny. However, the difference between the 6 : 10 ratio of N : VE observed for VE♀♀ × N♂♂ and the 10 : 0 ratio for N♀♀ × VE♂♂ is highly significant in Fisher's exact 2 × 2 contingency table test ($P < 0{\cdot}01$). One VE male, SG33, was responsible for the production of 9 of the 10 N offspring from N♀♀ × VE♂♂; he never produced a VE offspring (see pedigree in Fig. 2).

The hypothesis of autosomal inheritance with *N* dominant is also inconsistent with the data. The crosses G13 × G40 and G13 × G15 are interspecific eastern × western grey kangaroo, and both gave a VE progeny (see pedigree in Fig. 2). Therefore,

if this hypothesis were valid, both G40 (phenotypically N) and G15 (not tested) would have to be at least heterozygous for *VE*. But no VE was observed among 139 western greys of which 66 were not known to be related. Also, G40 gave only N to 40 of his 41 offspring in matings with N females. The result for the exceptional offspring, G476, is thought to be due to an error in the pedigree as is discussed shortly. Furthermore, as shown in the pedigrees in Figs 3 and 4*a*, G40 has sired 15 N progeny when mated to his own N daughters, and two N progeny when mated to two of the above 15 N progeny. The exact probability of this result occurring under this hypothesis, assuming all of the females from outside the pedigree were homozygous for *N*, is $P = 0{\cdot}162$. If any of the eastern grey females in the pedigrees were heterozygous for *VE*, then the probability would be even lower.

The hypothesis which is consistent with all the data, except the phenotypes of the mother–offspring combination of G269 and G276, is that PGK-*A* is X linked and that the paternally derived X chromosome is inactive in erythroblasts of females. The two exceptional grey kangaroos, SG9 and G275, which expressed the VE/(N) phenotype in their erythrocytes, seem to confirm the rule since both were females from the cross VE♀ × N♂ and both were therefore heterozygotes. At a later sampling, activity of the N allozyme was not detectable in erythrocytes of SG9. Apparently the paternal X was not completely inactive in at least some erythroblasts of G475 nor of SG9 at the first sampling.

The mother–pouch young combination which is inconsistent with the hypothesis of paternal X inactivation deserves some mention. The most likely hypothesis is that an error occurred in the typing of G269, which might really have had the VE phenotype. Unfortunately, G269 was sampled only once prior to its death, which occurred before the discrepancy in the pedigree was known. Multiple typings of the sample stored in our freezers under the number G269 have always given the N phenotype, but it is possible that a mistake could have occurred at some stage of the collection, processing, and cataloguing of the sample. The blood sample putatively from G269 was collected and processed along with 75 other blood samples in the brief span of several days. Pedigree errors are bound to occur occasionally when dealing with large numbers of samples from any species. Another hypothesis is that G269 is a true exception to the rule that the paternally derived X is inactive in erythroblasts. Its own mother G68 was phenotypically VE; so G269 could have inherited *VE* from G68 and *N* from G40, but expressed only the paternally derived X. If this hypothesis is correct, then G269 is the only observed instance of *maternal* X inactivation in all the studies to date of PGK-*A*, G6PD, and the DNA replication patterns in marsupials. This hypothesis will deserve serious consideration only if the observation can be repeated on family data in which there is no possibility of error.

The pedigree data indicate that the paternally derived *Pgk-A* gene can be transmitted to the offspring in active form. Seven N offspring were born to VE mothers, and two N offspring were born to a VW mother. The frequencies with which the paternal and maternal alleles of heterozygous females are transmitted do not depart from Mendelian expectations. Only those females known to be heterozygous by virtue of their parentage can be used in our calculation, since it would be biased if we included mothers whose heterozygosity was known solely because they had produced offspring unlike themselves. The mothers under consideration are SG60, G68, G193, and G318, which together transmitted their maternal allele nine times and their paternal one three times ($\chi^2_1 = 3{\cdot}0$, $0{\cdot}10 > P > 0{\cdot}05$) (Table 4). Further

data would be highly desirable. In the closely related species *Macropus parryi* polymorphic for PGK-*A*, five heterozygous mothers transmitted their active allele and seven transmitted their inactive one (VandeBerg 1975). One caution should be mentioned in regard to the interpretation of the transmission data for grey kangaroos. Much of it is derived from hybrids, and even though it is clear that the rule of paternal X inactivation is not violated in kangaroo hybrids, a great deal more data would be required to demonstrate the presence or absence of a hybrid effect on the transmission of genes to the next generation.

Table 4. Data on the frequency of transmission of the active and inactive *Pgk-A* alleles from heterozygous grey kangaroo females to their progeny

Species code: E, *M. giganteus*; H, hybrid of *M. giganteus* ♀ × *M. fuliginosus* ♂; H/W, backcross of above hybrid ♀ × *M. fuliginosus* ♂

Species or hybrid	Code No.	Mother's		Transmitted	
		Active allele	Inactive allele	Active allele	Inactive allele
E	SG60	*N*	*VE*	2	0
H	G68	*VE*	*N*	4	1
H	G193	*VE*	*N*	3	0
H/W	G318	*VE*	*N*	0	2
Totals				9	3

The hypothesis of X linkage with complete paternal X inactivation has not been advanced for any organism other than kangaroos. Unless data are available from reciprocal crosses it is difficult to distinguish between this hypothesis and hypotheses involving autosomal dominance. Our confidence in the hypothesis as the valid explanation for the grey kangaroo data is increased by the results of our population study on the PGK-*A* phenotypes in the muscle of the prettyface wallaby (VandeBerg *et al.* 1973*b*). In this species, heterozygotes can be distinguished by the electrophoretic patterns obtained from muscle. As expected on the basis of X linkage, they occur only in females in the proportion predicted by the Hardy–Weinberg law. In another two papers in this series, data will be given which show that grey kangaroo heterozygotes can also be distinguished in the same way, and again they occur only in females.

Acknowledgments

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