Aust. J. Biol. Sci., 1977, **30**, 431–43

Studies on Metatherian Sex Chromosomes VI.* A Third State of an X-linked Gene: Partial Activity for the Paternally Derived *Pgk-A* Allele in Cultured Fibroblasts of *Macropus giganteus* and *M. parryi*

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Abstract

The locus for the glycolytic enzyme phosphoglycerate kinase *A* (PGK-*A*) is sex-linked in the eastern grey kangaroo (*M. giganteus*) and the pretty-face or whiptail wallaby (*M. parryi*). Females older than the pouch life stage of development do not express their paternally derived allele in some tissues such as blood and liver. This phenomenon is called paternal X inactivation and is characteristic of kangaroos. This distinguishes them from eutherian females, which are mosaics of two kinds of cell brought about by random X inactivation of either the paternally derived or the maternally derived X chromosome. But in skeletal and cardiac muscle the paternally derived allele is weakly expressed. This paper reports data which show that in fibroblast cells cultured from tissue explants of these two species the paternally derived allele is also weakly expressed. This suggests the possibility of a form of mosaicism as in eutherians but with unequal proportions of the two types of cell. Our results show this is not so. Cloning experiments have been performed with cultured cells from three heterozygous *M. giganteus* females. A total of 49 clones was obtained and each clone was like its parent culture, i.e. there was a strongly active maternally derived allozyme and a weak paternally derived one. Two interpretations of these data are possible. Each cell may have a fully active maternally derived allele and a partly active paternally derived one. Or the clones themselves may be heterogeneous, such that one or the other of the X-linked alleles may switch on or off every few cell generations or less. Since X inactivation is known to be rather stable this latter hypothesis is considered the less likely. Autoradiography shows that the longer arm of the X chromosome in the cultured cells is late-replicating. Thus later replication of the X chromosome in kangaroos does not necessarily mean complete inactivity. Differences in morphology between the two X chromosomes in females are described.

Introduction

Electrophoretically detectable polymorphisms for the glycolytic enzyme phosphoglycerate kinase *A* (EC 2.7.2.3; ATP: 3-phospho-D-glycerate 1-phosphotransferase; PGK-*A*) have been described in the eastern grey kangaroo *Macropus giganteus*, the western grey kangaroo *M. fuliginosus*, and the pretty-face or whiptail wallaby, *M. parryi*. The characteristics of these polymorphisms are described in two preceding papers in this series (VandeBerg *et al.* 1977*a*, 1977*b*). In this paper we describe further data which show that the paternally derived allele is also expressed in mass cultures of fibroblast cells grown from tissue explants of *M. parryi* and *M. giganteus*. The phenotypes of PGK-*A* heterozygotes in cultured cells resemble those found in

muscle. We have performed cloning experiments which suggest that each cultured cell expresses the maternally derived allele completely and partially expresses the paternally derived one. In addition, our data establish that these cells have a late-replicating longer arm of their X chromosome.

Materials and Methods

Terminology

PGK-*A* designates the enzyme which has allozymes N (common to *M. giganteus* and *M. parryi*), VE (*M. giganteus* only), and VP (*M. parryi* only). *Pgk-A* is the locus symbol with alleles *Pgk-A*^{N}, *Pgk-A*^{VE} and *Pgk-A*^{VP}. The phenotypes of heterozygotes are described by writing the allozyme produced by the maternally contributed allele first, and that of the paternally contributed one second e.g. N/(VE) and VE/(N).

Animals

These were described by VandeBerg *et al.* (1977*b*), with the exceptions noted in the footnotes to tables of this paper.

Cell Culture

(i) *Collection of tissue*

From the live animal in the field, tissue was most conveniently taken from the ear while the animal was anaesthetized (Brietal Sodium = sodium methohexital, Eli Lilley, administered at 7·5–15 mg/kg). The area was scraped with a sterile scalpel blade to remove all traces of hair and swabbed with ether followed by 70% ethanol to sterilize it. A circular piece of tissue about 0·5 cm in diameter was taken using a specially constructed sterile stainless steel punch. The biopsy was placed in collection medium consisting in every 100 ml of 2 mg amphotericin B (Fungizone), 100 mg chlortetracycline hydrochloride, 4 mg chloramphenicol, 100 mg kanamycin sulphate, 6 mg sodium penicillin G and 10 mg streptomycin sulphate, made up in Ham's F10 medium. The bottle was kept warm and was not put in a refrigerator. Post-mortem collection of body wall, heart, lung, and kidney was done as soon as possible after death of the animal, although successful cultures were initiated from necropsies taken 4 h after death. Tissue of about the same size as the biopsy was treated as above.

(ii) *Initiation of cultures*

All procedures were carried out in a laminar flow hood with sterile apparatus. A single piece of tissue was cut up very finely in embryo extract (Commonwealth Serum Laboratories, C.S.L.), the pieces were transferred by Pasteur pipette to a culture flask, distributed evenly over one surface, and then chicken plasma (C.S.L.) was dropped over them. Excess plasma was removed, and the flask placed in an incubator at 35°C for 15–20 min. Excess liquid was then removed and 15–20 ml of pre-warmed medium (Ham's F10 with 20% foetal calf serum) was added with the bottle in an upright position. The bottle was gassed with sterile filtered 5% CO_2, placed in the incubator at 35°C and *slowly* placed in a horizontal position to allow the medium to cover the tissue. Growth of cells out from the explants appears after 2–6 days and when at least $2{\cdot}5 \times 10^5$ cells were present the first transfer was made. Cells were removed from the cell surface by first washing with phosphate-buffered saline without calcium or magnesium (PBS^-, i.e. water to 1 litre, NaCl 8 g, KCl 0·2 g, KH_2PO_4 anhydrous 1·15 g, phenol red 0·02 g), then incubating in 0·05% pronase (pronase 50 mg; EDTA tetrasodium 100 mg, PBS^- 100 ml). Thereafter the cells were passaged at 1-week intervals with an initial inoculum of 1×10^5–5×10^5 cells per 75-cm^2 bottle. Cells were stored indefinitely frozen under liquid nitrogen in 10% dimethylsulphoxide in the foetal-calf-supplemented Ham's F10. Then 1×10^6–4×10^6 cells were placed in each vial and the initial freezing in liquid nitrogen vapour was carried out using a Linde BF-5 biological freezer with a temperature drop of 1°C per minute. The temperature of 35°C was chosen because it is near the body temperature of most kangaroos, but no systematic study of the effect of temperature on growth has been performed.

(iii) *Cloning procedures*

With kangaroo cells cloning has been a difficult problem. The following procedures have been used.

Procedure 1. The cells to be plated at low density for clone initiation were taken from an early subculture (passages 1–6). A suspension of *single* cells was made by incubating the cells in pronase for 5–10 min and then gently pipetting the suspension 10–15 times. Lux or Falcon 75-cm^2 plastic tissue culture flasks were then seeded with 10–100 cells, depending upon plating efficiency. After incubation for 6–7 days the vessels were thoroughly scanned under an inverted microscope for single colonies whose positions were marked with a Texta colour. Clones which occupy an entire viewing field under low power (0·4 cm diameter) are ready for transfer which is usually 10–20 days after initial plating. To remove the clones, Lux or Falcon flasks were broken on their upper surface by a sharp blow. Cloning rings were made from Bellco aluminium culture tube closures (Cat. No. 2005-00016) which had their tops removed to create an open cylinder. The end applied to the plastic was smeared with silicone high vacuum grease of approximately D1400. The cloning ring was placed over the clone, which had previously been washed with PBS$^-$, and the pronase was applied. The transfer was best monitored under the inverted microscope, so that one could be sure that all cells were freed and taken up with a Pasteur pipette for transfer to a 25-cm^2 Falcon tissue culture flask. When sufficient cells had been grown in the flasks the cells were removed for electrophoresis. This procedure worked with only one batch of foetal calf serum for SG9 (Australian Laboratory Services batch 012).

Procedure 2. This is a feeder layer technique which worked with all three lines. It was devised by one of us (J.A.M.G.). The cells to be cloned were first grown for two passages in HAT medium (10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and $1{\cdot}6\times10^{-5}$ M thymidine dissolved in Ham's F10 supplemented with 20% foetal calf serum). All further growth was also carried out in HAT medium. The cells were then plated at a density of 100–200 cells per 75-cm^2 flask together with a feeder layer of $7{\cdot}5\times10^5$ HAT-sensitive cells. These were from a *Cricetulus griseus* peritoneal cell line which lacked thymidine kinase (American Type Culture Collection number CCL 14·1 B14-150). The HAT-sensitive feeder layer took 1–2 weeks to die and come off the surface of the flask. When it had largely disappeared 5–20 colonies of kangaroo cells 1–10 mm in diameter usually remained. The hamster and kangaroo cells were easily distinguishable morphologically and their PGK-*A* mobilities were also different. Control flasks without the feeder layer showed no colonies at all for the three cell lines mentioned in this paper (except for SG9 in the presence of batch 012 serum). The larger colonies were then transferred to 25-cm^2 flasks together with $2{\cdot}5\times10^5$ feeder layer cells, and treated as in procedure 1. This technique is de scribed in more detail elsewhere (Graves and Hope 1977).

(iv) *Preparation of cells for electrophoresis*

Cells were removed in the usual manner and pelleted in a Durham tube by spinning at 750 rev/min for 5 min. As much supernatant as possible was removed. Tubes were then centrifuged for a further 2 min to sediment any moisture adhering to the sides. This moisture was also removed. This step is important if the ratio of cells to lysing fluid is to be accurately controlled. Cells were lysed by freezing and thawing in liquid nitrogen after the addition of gel buffer (10 μl added to 1×10^5–$2{\cdot}5\times10^5$ cells) or without addition of any fluid. The lysates were then applied to the filter paper insert for electrophoresis.

(v) *Electrophoresis*

This was performed as in Beutler (1969) with the modifications described in Cooper *et al.* (1971).

(vi) *Autoradiography*

Terminal DNA synthesis in the X chromosomes of SG9 was studied by autoradiography. Two techniques were used.

(1) *Mass cultures.* Cells were treated with colcemid (0·3 μg/ml) and labelled with 0·5 μCi/ml of 5 methylthymidine deoxyriboside (Radiochemical Centre, Amersham, England) 4 h before fixing. The thymidine was labelled with tritium of specific activity 5·9 Ci/mmol. Cells were harvested with 0·1% trypsin, given hypotonic treatment for 10 min with a solution of 1 part culture medium : 5 parts distilled water, fixed in 3 parts methanol : 1 part glacial acetic acid and slides prepared by air-drying. Autoradiographs were made using a 1 : 1 dilution of Kodak NTB-2 emulsion and were exposed for 6 weeks. These were developed in D19B for 10 min and fixed for 15 s in Kodak Rapid Fix. Slides were stained with Giemsa (Gurr's R 66). Cells with grain totals of less than 500 were scored for the number of grains over the shorter satellited arm and the longer non-satellited arm of the two X chromosomes and over the two number-7 chromosomes. χ^2 values were calculated on the

hypothesis that the numbers of grains over the two X chromosomes in each cell were equal and these χ^2 values were then summed. The same analysis was performed for the number-7 chromosomes.

(2) *5C5 clone.* [*Methyl*-^{3}H]Thymidine of specific activity 22 Ci/mmol (Radiochemical Centre, Amersham, England) was added, 6 h before beginning hypotonic treatment, at a concentration of 0·3 μCi/ml and colcemid was added 4·5 h later at a concentration of 0·1 μCi/ml of culture medium. Cells were harvested with 0·05% pronase suspended in 5 ml Ham's F10 with 10% foetal calf serum, centrifuged at 800 rev/min for 5 min and resuspended in 3 ml 0·53% KCl for 12–15 min at 37°C before fixation in methanol–acetic acid (3 : 1 v/v). Air-dried slides were stained with orcein and all metaphase cells photographed. The slides were dipped in a 1 : 1 dilution of Kodak NTB-2 nuclear track emulsion, dried, exposed for 3 days and autoradiographs were developed for 1·5 min in Dektol developer.

Table 1. PGK-*A* phenotypes in blood, muscle, and cultured cells of some eastern grey kangaroos (*M. giganteus*)

All cells were cultured from ear explants except for SG60, for which muscle fascia was used

Animal No.	Sex	PGK-*A* phenotypes			Parents and their PGK-*A* blood phenotypes	
		Blood	Skeletal muscle	Cultured cells	♀ parent	♂ parent
SG9	♀	VE[A,B]	VE/(N)[A]	VE/(N)	SG4, VE[C]	SG56 or SG62,N
SG60	♀	N[A]	N/(VE)[A]	N/(VE)	SG8,[A] N	SG33,[A] VE
G318	♀	VE	—	VE/(N)	G68, VE[D]	G40,[E] N
SG56	♂	N	N	N	SG14, N	SG33, VE
SG62	♂	N	N	N	SG25, N	SG33, VE
SG3	♀	N		N	Not known	Not known
SG14	♀	N		N		
SG54	♀	N		N		
SG63A[F]	♂	N		N		

[A] As reported in VandeBerg *et al.* (1977*b*).
[B] Was VE/(N) at 9 months and VE at 15 months.
[C] SG4 was VE/(N) in her skeletal muscle.
[D] Hybrid offspring of G13 (*M. giganteus*) × G15 (*M. fuliginosus*).
[E] *M. fuliginosus* (see VandeBerg *et al.* 1977*a*).
[F] Chromosomally XXY.

Results

Mass Cultures

Cultures were initiated from nine *M. giganteus* individuals (Table 1), fourteen *M. parryi* individuals (Table 2), and six individuals from other species and interspecies crosses (Table 3) and examined electrophoretically for PGK-*A*. The PGK-*A* phenotypes of *M. parryi* found are illustrated in Fig. 1. For *M. giganteus*, three phenotypes were found: N, N/(VE), and VE/(N) and these are illustrated in the previous paper (VandeBerg *et al.* 1977*b*). The phenotypes of the tissues of two of these animals, SG9 and SG60, have been reported in that paper. One other animal, G318, had the VE/(N) phenotype in its cultured cells. Both SG9 and G318 were the produce of matings of VE × N blood phenotypes and SG60 was the product of the reciprocal mating: the allele with most activity in the cultured cells is from the mother. By contrast two males, SG56 and SG62, the product of matings of N × VE blood phenotypes, had only the N allozyme in their blood, in all other tissues and in their cultured cells. Attempts have been made to set up VE males in culture but these attempts were unsuccessful due to fungal contamination. However, the VE

phenotype has been found in male muscle (VandeBerg *et al.* 1977*b*) and in muscle from both males and females during the course of a population survey for PGK-*A* variation (F. R. Sherman *et al.*, unpublished data). In the latter investigation the four phenotypes [N, VE, N/(VE), and VE/(N)] were found in females in the expected Hardy–Weinberg ratios.

Table 2. PGK-*A* phenotypes in blood and cultured cells of some pouch young *M. parryi* and in the blood and muscle of their female parents

Animal No.	Sex	PGK-*A* phenotypes			Female parent's PGK-*A* phenotype		
		Blood	Cultured cells	Tissue cultured	Animal No.	Blood	Muscle
C2	♀	VP	VP/(N)	Ear	—	—	—
6	♀	VP	VP/(N)	Body wall	5	N	N/(VP)
15	♀	N	N/(VP)	Body wall	14	N	N
24	♀	N	N/(VP)	Kidney	23	VP	VP/(N)
33	♀	N[A]	N	Body wall	32	N	N/(VP)
47	♀	VP[A]	VP	Body wall	46	VP	VP
49	♀	VP	VP	Body wall	48	VP	VP
8	♂	N	N	Kidney	7	VP	VP/(N)
10	♂	VP	VP	Testis	9	VP	VP
17	♂	N	N	Testis	16	VP	VP/(N)
20	♂	N	N	Body wall	19	N	N/(VP)
31	♂	N[1]	N	Body wall	30	VP	VP/(N)
43	♂	N	N	Body wall	42	N	N/(VP)
55	♂	N[1]	N	Body wall	54	N	N

[A] Typed from body wall or leg muscle.

With the exception of C2 all the *M. parryi* were pouch young of the females whose PGK-*A* phenotypes for both blood and tissues have been described by VandeBerg *et al.* (1973, 1977*b*). These adult females fell into four phenotypic classes—N, VP, N/(VP), and VP/(N). As with the analogous variation in *M. giganteus*, the

Table 3. PGK-*A* phenotypes in cultured cells of several species and some species hybrids

Except for *fuliginosus* and *giganteus* all these species are monotypic for PGK-*A*. For species hybrids the female parent is given first. All cells were cultured from ear explants

Animal No.	Species	Sex	PGK-*A*	Animal No.	Species	Sex	PGK-*A*
R8	*Macropus rufogriseus*	♀	N	B2	*Wallabia bicolor*	♀	N
O31	*Macropus robustus erubescens* × *M. r. robustus*	♀	N	RG1	*Macropus rufogriseus* × *M. giganteus*	♀	N
OK1	*Macropus robustus robustus* × *Megaleia rufa*	♀	N	G180	*Macropus fuliginosus*	♀	N

phenotypes of skeletal muscle and cultured cells resemble each other closely. Only VP and N occur in the cultured cells of males whilst all four phenotypes are found in the cultured cells from the female pouch young (Fig. 1 and Table 2). This sex difference is also found amongst the adults in which the female heterozygotes occur in Hardy–Weinberg proportions (VandeBerg *et al.* 1973).

It should be emphasized that in *M. parryi* the cultured cells and skeletal muscle were not typed from the same animals. The tissues of the pouch young themselves had an activity of PGK-*A* too low to type reliably for possible partial expression of the putative paternal allozyme. The fact that the fibroblasts derived from the presumed heterozygous female pouch young had either the VP/(N) or N/(VP) pattern suggests that the same patterns would be found in tissues of these animals if a more sensitive staining technique were available.

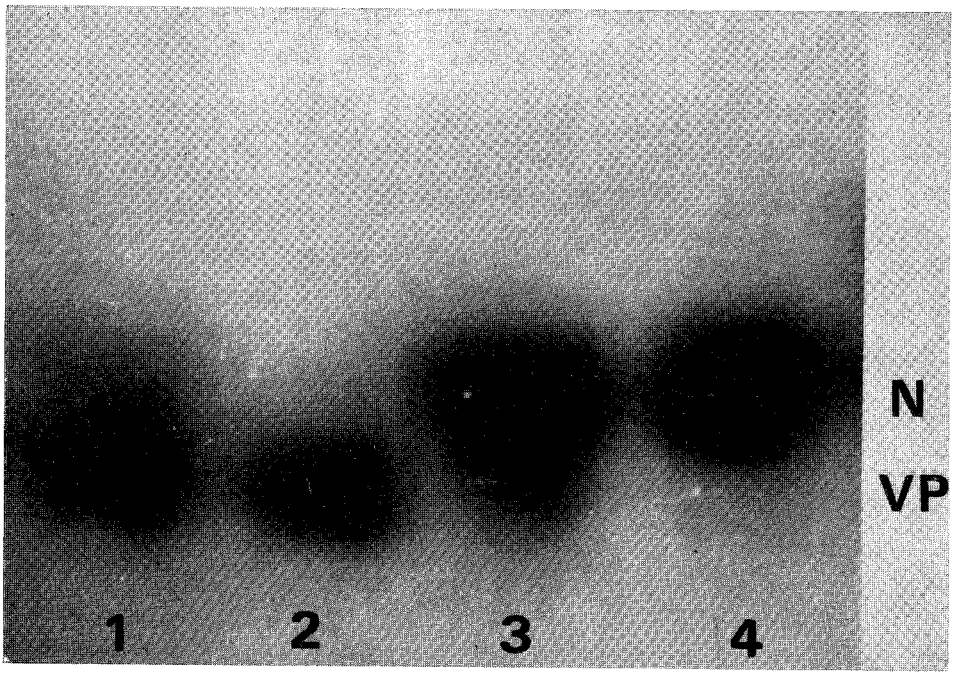

Fig. 1. Starch-gel electrophoresis showing four phenotypes which occur for PGK-*A* in cultured cells of *M. parryi*. *1*, VP/(N) ≡ *Pgk-A*^{VP}/*Pgk-A*^{N}. *2*, VP ≡ *Pgk-A*^{VP}/*Pgk-A*^{VP}. *3*, N/(VP) ≡ *Pgk-A*^{N}/*Pgk-A*^{VP}. *4*, N ≡ *Pgk-A*^{N}/*Pgk-A*^{N}. In each case the maternally contributed allele, or the allozyme produced by it, is written first.

The pouch young were selected for culturing because their tissue explants grow in culture more readily than do explants from adults. Later work, however, indicates that adult tissue can be taken in the field and cultures initiated some days later in the laboratory, so that in the future it may be possible to obtain data on the cultured cells and tissue phenotypes from the same *M. parryi* animals.

The phenotypes for all individuals are stable for at least the first 6–10 passages, during which the typings were made. SG9 and SG60 have been run at least 15 times each, and except when activity is low, as judged by the speed at which the bands appear, have always exhibited the same phenotype. With very low activity only the maternally derived allozyme appears. All of the *M. parryi* cultures were run from three to six times. Typing these cultures presents some difficulty because of the closeness of the N and VP bands on the gel. In particular N/(VP) may be confused with N which has trailed due to faulty loading. But when it is clear that all samples have entered the gel properly, the distinction can be made without ambiguity.

Cloning

Cultures used for cloning came from ear explants of SG9, SG60, and G318. Using procedure 1, 13 clones were grown for SG9 to sufficient size to give a PGK-*A* typing. The concentration of $NADH_2$ was reduced to one-half the normal concentration, i.e. from 0·42 to 0·21 mM $NADH_2$, to facilitate detection of weaker bands. The resultant lower level of fluorescence makes it difficult to reproduce the photographs of the gels for publication and so a diagram is shown in Fig. 2. In both the mass cultures and in the clones VE appeared first on staining and the N band appeared later. The clones and the mass culture had phenotypes which were indistinguishable. With prolonged staining the paternal allozyme may eventually show a band of the same proportions as the maternal allozyme. This does not imply equal activity, which is judged instead by the rate at which the $NADH_2$ is oxidized.

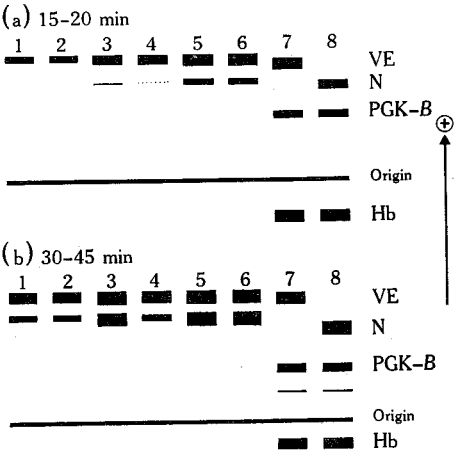

Fig. 2. (*a*) Diagrammatic representation of a PGK gel 15–20 min after application of the stain to mass cultures and clones of SG9 which was phenotypically VE/(N). Note how VE has stained more strongly than N. *1*, Clone, 50 000 cells. *2*, SG9 second passage, 50 000 cells. *3*, Clone, 140 000 cells. *4*, SG9, second passage, 140 000 cells. *5*, SG9, second passage, 500 000 cells. *6*, SG9, second passage, 3×10^6 cells. *7*, VE blood. *8*, N blood. VE and N from blood have fractionally lower mobility than VE and N in cultured cells, for unknown reasons. (*b*) Same gel after 30–45 min. Note how the N band has appeared in some where it was absent at 15–20 min (1 and 2) and has grown stronger in the others. (This experiment was done with cloning procedure 1.) Hb, haemoglobin.

By procedure 2 (the feeder layer technique) 23 clones were derived from the SG9 mass culture, 3 from G318, and 10 from SG60. Each of these was indistinguishable from its parent mass culture. Some clones exhibited the hamster PGK-*A* in addition, which was easily distinguishable from the VE and N bands by its slower mobility.

Autoradiography

Both the mass cultures and a single clone from SG9 (5C5 clone) had a late-replicating X chromosome in each cell (Figs 3 and 4). Late replication appeared to be a property of only the non-satellited (i.e. long) arm of the X chromosome (Fig. 3); the χ^2 analysis for this arm in the mass cultures gave a highly significant result ($\chi^2_{125} = 1118 \cdot 10$, $P \ll 0 \cdot 001$). For the satellited arm $\chi^2_{87} = 72 \cdot 3$, $P = 0 \cdot 26$, which is a non-significant result.

Since the difference between counts over the X homologues was accentuated by the labelling procedure (which ensured that cells reaching mitosis were labelled only during a brief interval of the S phase), it was possible in almost all cells to equate the X chromosome having the higher grain count with the late-replicating X chromosome. It is on this basis that the average grain counts for the late ('hot') and early ('cold') replicating X chromosomes are given (Table 4).

The 5C5 clone had few dividing cells in it and so was labelled for 5·5 h. Fourteen cells were scored and $\chi^2_{14} = 24{\cdot}5$, $0{\cdot}025 < P < 0{\cdot}05$, was obtained for the difference between the non-satellited arms. It is probable that this clone also had a late-replicating non-satellited arm.

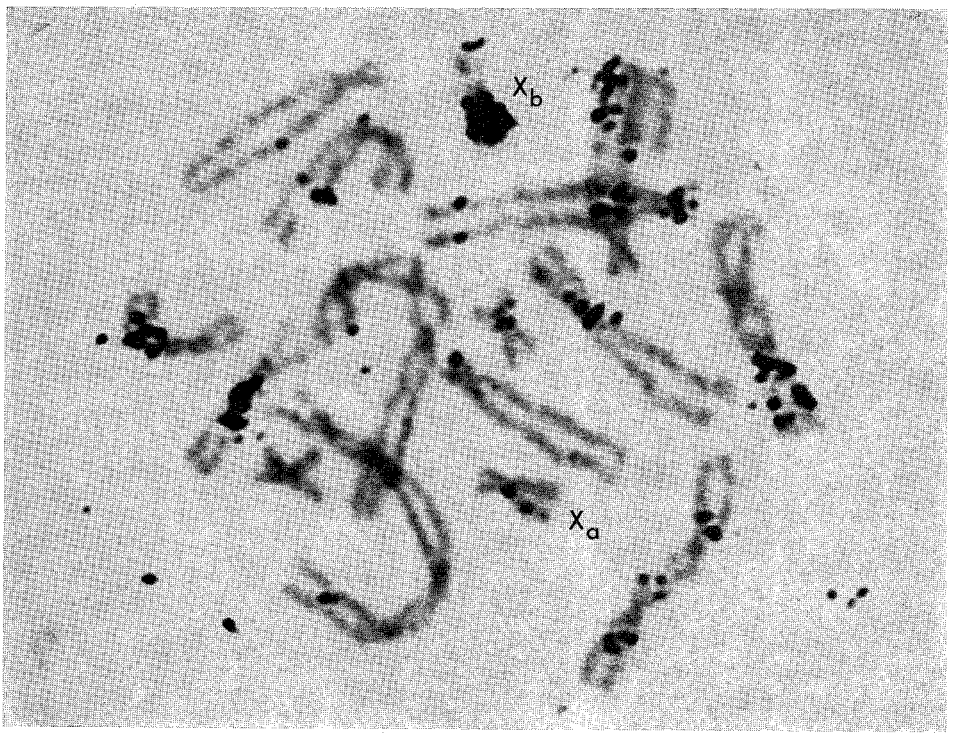

Fig. 3. Late-labelling X (X_b) in mass cultures of SG9. The alternate X chromosome (X_a) replicates synchronously with most of the autosomes.

Graves (1967) noted that one of the two X chromosomes (X_1) in cultured lymphocytes of female grey kangaroos had a distinct secondary constriction in the short arm whereas the secondary constriction of the alternate X chromosome (X_2) was much less prominent. Hayman and Martin (1965) noted a similar difference in the size of the secondary constrictions of the X chromosomes in lymphocyte cultures of *Potorous tridactylus*. In this study we have confirmed that one of the two X chromosomes of cultured grey kangaroo lymphocytes has a distinct secondary constriction, assumed to be the nucleolar organizer region, in its short arm but, contrary to earlier expressed opinions, this is the late-replicating X chromosome (Fig. 5). Johnston (unpublished data) has also found that in *P. tridactylus* lymphocytes the X chromosome with the more prominent secondary constriction is late-replicating. We have called the late-replicating X chromosome with the prominent secondary constriction X_b and its non-late-replicating, presumably active, homologue X_a: thus our $X_a = X_2$ and our $X_b = X_1$ of Graves (1967). In cultured fibroblasts from clone 5C5 the X chromosome with the secondary constriction in its short arm (X_b) is also the late-replicating one but the alternate X chromosome (X_a) has a morphology different

from that of X_a in lymphocytes (Fig. 4). In fibroblasts X_a, which replicates synchronously with most of the autosomes, as does X_a in lymphocytes, usually has a pronounced constriction on the long arm side of the centromere as well as a secondary constriction in the short arm (cf. Figs 4 and 5). X_b (late-replicating) and X_a chromosomes in fibroblasts may be distinguished on satellite size, those of X_b always being smaller than those of X_a in the same cell (Fig. 4).

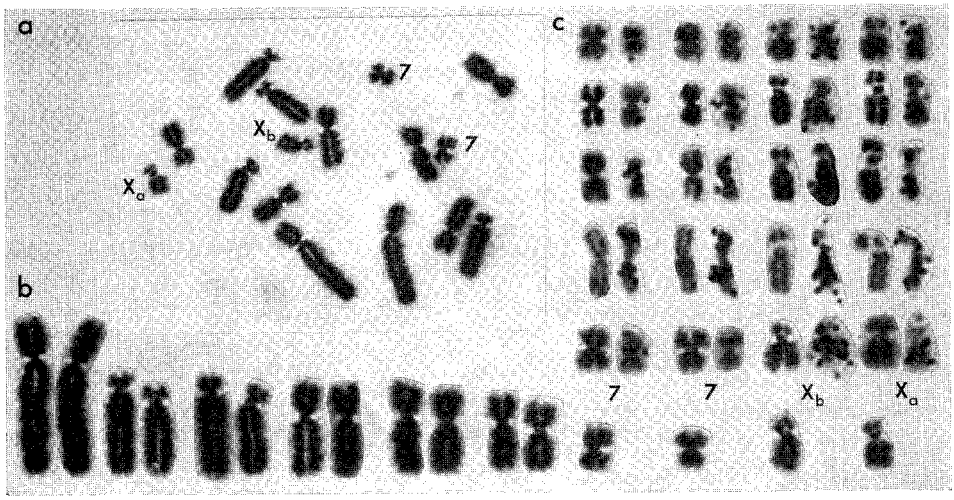

Fig. 4. (*a*) Pre-autoradiography photograph of the chromosomes from one cell of [*methyl*-^{3}H]-thymidine-labelled clone 5C5 from SG9. (*b*) Karyotype from cell shown in (*a*); this cell completed DNA synthesis before label was added to the culture. (*c*) Partial karyotypes from five other cells showing only number-7 autosomes and X chromosomes; post-autoradiography photographs of each chromosome are shown on the right-hand side. X_b was the later replicating of the two X chromosomes but there were no significant replication differences between the No. 7 pair or any other pair of autosomes. Note the morphological differences between X_a and X_b chromosomes (see text and cf. Fig. 5).

Discussion

The data presented by VandeBerg *et al.* (1973) for *M. parryi* and VandeBerg *et al.* (1977*b*) for *M. parryi* and *M. giganteus* show clearly that the heterozygotes express both *Pgk-A* alleles in skeletal muscle and some other tissues. The *M. giganteus* data also show that the level of expression is dependent upon parental source, the allele which comes from the father having a lower level than the maternally derived allele. The data presented here establish that the *Pgk-A* alleles in cultured cells behave in the same way.

In the absence of the cloning results these asymmetrical patterns seen in the *Pgk-A* heterozygotes could have been explained in four ways. In the first the culture is assumed to be a mosaic containing two types of cell, a majority expressing VE only and a minority expressing N only. This hypothesis is very similar to the random X chromosome inactivation characteristic of eutherian mammals, except that in the eutherians the proportions of the two kinds of cells are usually equal. In the second a mosaic is also postulated. The majority type has only the VE allele active whilst in the minority type both alleles are fully active. The gonads of eutherian females presumably represent such a mosaic, having germ cells with two active X chromosomes

Table 4. χ^2 **Tests for a 1 : 1 ratio of grains in each cell for the longer non-satellited arms of the X chromosomes, their shorter satellited arms, and over both arms of the number-7 autosomes**

Total grains per cell	Non-satellited arms of the X chromosomes				Satellited arms of the X chromosomes				Number-7 autosomes		
	χ^2 for 1 : 1 ratio of grains in each cell	No. of cells	Average No. of grains per arm: 'Hot'	'Cold'	χ^2 for 1 : 1 ratio of grains in each cell	No. of cells	Average No. of grains per arm attached to: 'Hot' n-s[A]	'Cold' n-s	χ^2 for 1 : 1 ratio of grains in each cell	No. of cells	Average No. of grains per both chromosomes in each cell
1–25	34·6	10	3·9	0·2	4·0	3	0·4	0·0	1·0	1	0·1
26–50	318·7	26	14·6	1·5	4·0	5	0·3	0·1	13·0	7	0·5
51–75	261·1	23	22·9	6·2	13·4	17	1·3	1·0	7·5	9	0·4
76–100	135·1	18	22·9	8·7	7·5	14	3·3	2·9	8·1	13	1·8
101–200	249·2	23	28·7	9·2	27·3	23	6·5	5·9	26·6	23	5·0
201–300	87·4	11	35·0	17·2	7·3	11	12·5	11·2	9·2	11	11·1
301–500	95·0	14	37·9	21·4	8·8	14	13·5	11·3	9·1	14	15·5
Totals	1181·10	125	—	—	72·30	87	—	—	74·5	78	—

[A] n-s, non-satellited.

(Gartler *et al.* 1975) and somatic cells with only one active X chromosome. In the third the culture is assumed to consist of only one type of cell in which the maternal allele is fully expressed and the paternal allele partly expressed. In the fourth, unstable gene action is postulated such that in one cell generation (say) an allele may manifest itself fully and in the next be switched off. Such a hypothesis may take various forms, e.g. (1) in one generation the maternal allele could be on and the paternal one off and in the next *vice versa*, or (2) the maternal allele could be on all the time and the paternal allele could switch on and off from one generation of cells to the next. In either case intraclonal heterogeneity would result. The cloning results reported in this paper are compatible with the third and fourth hypotheses.

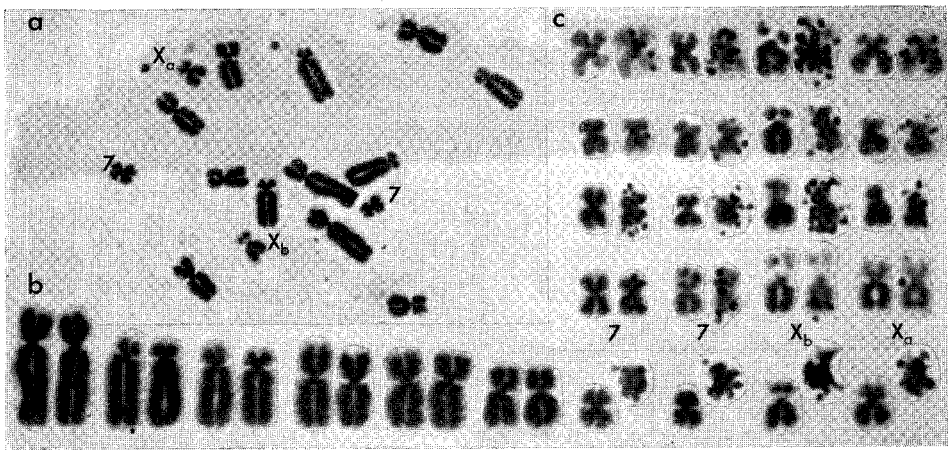

Fig. 5. (*a*) Pre-autoradiography photograph of the chromosomes from one cell of [*methyl*-^{3}H]-thymidine-labelled lymphocyte culture of female SG26. Thymidine (specific activity 22 Ci/mmol) was added to the culture at a concentration of 0·25 μCi/ml 5·5 h before beginning harvest of cells. (*b*) Karyotype from cell shown in (*a*) with post-autoradiography photographs of number-7 autosomes and X_a and X_b sex chromosomes shown above and right of corresponding chromosome from pre-autoradiograph. (*c*) Partial karyotypes from five other cells showing only number-7 autosomes and X chromosomes with post-autoradiography photograph of each chromosome on its right-hand side. X_b was the later replicating of the two X chromosomes but there were no obvious replication differences between the No. 7 pair or between any other pair of autosomes. Note the morphological differences between X_a and X_b chromosomes (see text and cf. Fig. 4).

No examples of clones showing the kinds of cells postulated in the first and second hypotheses were found, i.e. no clones with only one allele active or with both equally active were found. But in view of the limited number of clones obtained it is necessary to ask at what level these cells could have existed within the mass culture and gone undetected, at the usual 95% level of significance. This is given by the equation

$$(1-x)^n = 0{\cdot}05,$$

where n is the number of cells of a single kind, and x is the maximum proportion of cells of other types which could have existed and not been detected at the 95% level. For SG9, $n = 36$ and $x = 0{\cdot}08$, so that these results indicate that at least 92% of the cells in the SG9 culture must have the partly active paternal X chromosome and a

maximum of 8% could have the other phenotypes postulated in the first two hypotheses. For SG60, $n = 10$ and $x = 26\%$, and for G318 $n = 3$ and $x = 67\%$.

The only direct way of deciding between the third and fourth hypotheses is to examine the products of a single cell, which is not at present technically possible. Intraclonal heterogeneity implies that alleles switch between activity and inactivity every few cell generations or less. Because X chromosome inactivation is known to be highly stable this fourth hypothesis must be regarded as the less likely.

Our conclusion is that it is likely that an X-linked gene may exist in at least three states—active, inactive, and partially active. The partially active state has not been previously described. Like the state of complete inactivity characteristic of X-linked genes in blood cells, the state of partial activity in kangaroos is a parental source effect, the gene on the paternally derived chromosome being less active than its maternally derived allele. This conclusion has some implications for an understanding of the mechanism of X chromosome inactivation. It could be that the primary mechanism which causes the whole chromosome to be inactive can be set at at least three levels—off, on, and partly on. Or it could be that the primary mechanism is set in the same way in both blood cells and in cultured cells and muscle, and that a secondary mechanism is capable of partly overriding the primary mechanism at some loci.

The cultured cells of this investigation, which morphologically resemble fibroblasts, had differential chromosome replication for their longer arms but not for their shorter arms. Cultured lymphocytes from *M. giganteus* have a late-replicating X chromosome (Graves 1967; *M. major* of this reference = *M. giganteus*). No data are available for the two chromosome arms of *M. giganteus* separately, but in a closely related species *M. robustus* the shorter arm does not show differential replication between the two X chromosomes in lymphocytes (Sharman, unpublished data). In lymphocytes of *M. giganteus* there is no detectable expression of the paternally derived allele (VandeBerg 1975). If it is assumed that the shorter arm in the *M. giganteus* lymphocytes behaves like that in *M. robustus* lymphocytes, then the expression of the *Pgk-A* locus in cultured cells and its lack of expression in lymphocytes does not appear to be correlated with any change in replication patterns. Simply on a size basis it seems more likely that the *Pgk-A* locus is carried on the larger arm. If it is, our results imply that partial activity of the *Pgk-A* locus is possible on a late-replicating segment of the X chromosome.

The confusion over which X chromosome is late-replicating in lymphocytes (Hayman and Martin 1965; Graves 1967) presumably arose because the X chromosome of males, which must be fully active, has a pronounced secondary constriction. In this respect it resembles the late-replicating X chromosome in the females rather than the active X chromosome.

Our results do not prove that there is a partial breakdown of dosage compensation, as opposed to X chromosome inactivation, although they strongly suggest that this is so. We have not ruled out the possibility that both alleles in females have, in sum, the same activity as the one allele in males.

If female kangaroos do have more activity for X-linked genes than do males, it could be that sex determination in kangaroos is more dependent upon the X chromosome–autosome ratio than is the case in eutherians. From the limited data available, this appears to be so. Sharman *et al.* (1970) have shown that the Y chromosome is male-determining in kangaroos. However, if one compares their XO tammar (*M. eugenii*) with a eutherian XO (human or mouse), the former is somewhat more

masculine than either of the latter. This tammar possessed an empty scrotum and gonads which contained both testicular and ovarian elements but in all other respects it was essentially female. Similarly their XXY tammar was somewhat more feminine than a human XXY. Its body weight was in the feminine range and it possessed a pouch although its other characteristics were masculine. While the presence or absence of a Y chromosome is unquestionably the principal factor in sex determination in both groups, it seems that the X chromosome–autosome ratio has some residual effect upon the degree of maleness or femaleness in kangaroos. Perhaps the system of sex determination in present-day therian mammals is ultimately derived from one which depended upon an X chromosome–autosome balance.

Acknowledgments

We thank Mr W. E. Poole for material from kangaroo No. G318, Carolyn Murtagh for her assistance in the early stages of this work, and Mr D. Pye and Mr A. MacGregor for help with cell culture techniques. This work was carried out under grants from the Australian Research Grants Committee to D.W.C., G.B.S. and J.A.M.G. and Macquarie University Research Grants to D.W.C. and G.B.S.

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Manuscript received 29 March 1977