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SHORT PAPER

Glucose-6-phosphate dehydrogenase expression in heterozygous kangaroo embryos and extra-embryonic membranes

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SUMMARY

The electrophoretic expression of the *X*-linked enzyme glucose-6-phosphate dehydrogenase was examined in heterozygous *Macropus robustus* embryos and their extra-embryonic membranes. The amnion and allantois, like the somatic tissues of the embryo proper, showed paternal *X* inactivation while the avascular and vascular yolk sac cells showed evidence of activity of both maternal and paternal alleles.

1. INTRODUCTION

One of the two *X* chromosomes in each somatic cell of female eutherian mammals is inactivated early in embryonic development (Lyon, 1972). In female mouse and rat embryos, cytological studies established that *X* inactivation is random in tissues of the embryo proper but in the extra-embryonic yolk sac there is paternal *X* inactivation (Takagi & Sasaki, 1975; Wake, Takagi & Sasaki, 1976). Analysis of *X*-linked phosphoglycerate kinase (PGK) activity in mouse yolk sac further indicated that the endoderm is subject to paternal *X* inactivation whereas in the mesodermal component it is random (West *et al.* 1977; McMahon, Fosten & Monk, 1983).

The pattern of *X* chromosome activity has not been reported in kangaroo intra-uterine (embryonic) or early extra-uterine (pouch young) stages but later in pouch life and thereafter, almost all tissues exhibit paternal *X* inactivation (Cooper *et al.* 1975; Johnston *et al.* 1978). In this paper we report on the expression of *X*-linked glucose-6-phosphate dehydrogenase (G6PD) in embryonic and extra-embryonic tissues of intra-uterine heterozygous females and hemizygous males of a kangaroo, *Macropus robustus*.

2. MATERIALS AND METHODS

Crosses were made between female wallaroos (*M.r. robustus:* G6PD-F) and male euros (*M.r. erubescens:* G6PD-S) to provide embryos heterozygous for G6PD. Mating takes place in this species within hours of a female giving birth to a pouch young. If conception occurs, the embryo proceeds to the blastocyst stage when it enters embryonic diapause. Development resumes after the removal of pouch young (RPY) and birth takes place 28·5–34·5 days later (Calaby & Poole, 1971). All ages mentioned in this paper refer to days after removal of pouch young.

The ages of the embryos used in this study ranged from 21 to 25 days RPY. For each conceptus, various organs from the embryo proper and the three extra-embryonic membranes (amnion, allantois and yolk sac) were isolated for electrophoretic analysis.

The large yolk sac in kangaroos consists of two regions (avascular and vascular) which are delineated by a prominent blood vessel (the sinus terminalis) and these two regions were separated by cutting outside the sinus. The morphology of a 23-day embryo and the associated extra-embryonic membranes is shown in Plate 1. It is difficult to establish a precise age equivalence of a kangaroo embryo in relation to a mouse embryo. Organs commonly used as age indicators for the mouse show allometric growth patterns in embryo kangaroos. For example, the anterior limb bud of the kangaroo embryo develops earlier than the hind limb and various head elements, compared with the mouse. However, on general morphology we suggest that a 23-day kangaroo embryo is roughly equivalent to an 11-day mouse embryo.

Small portions of kangaroo embryonic and extra-embryonic tissues were transferred to 1 % sodium citrate for 6–8 min, fixed in methanol : glacial acetic acid (3 : 1), disaggregated in a drop of 60 % acetic acid on a slide at 40 °C and allowed to dry. The air-dried preparations were stained with 2 % aceto-orcein and mitotic metaphase spreads were examined to determine sex chromosome constitution.

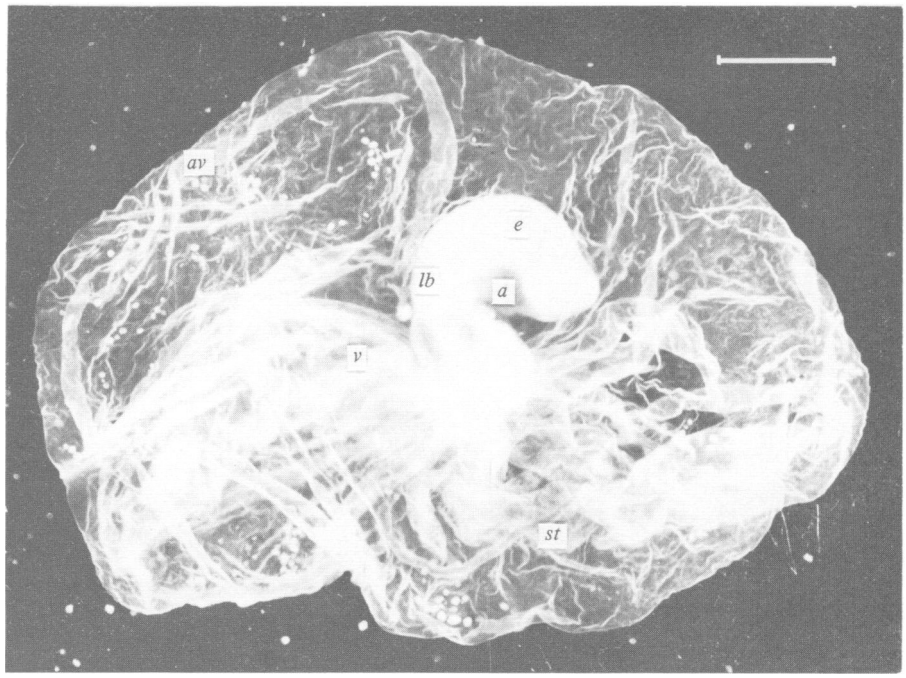
Tissues for electrophoresis were homogenized in an equal volume of lysing solution (Johnston *et al.* 1978) and applied to Cellogel (Chemetron). Electrophoresis was carried out in 0·1 M lithium borate – 0·024 M EDTA buffer, pH = 9·0, for 4 h with a voltage gradient of 14 V cm^{-1}. The gel stain was the same as in Johnston *et al.* (1978).

3. RESULTS AND DISCUSSION

Limb bud, brain and tail of the embryo, and amnion, allantois and yolk sac (avascular and vascular regions) from each of four specimens (2 ♀♀ and 2 ♂♂) were examined for G6PD expression. Electrophoretic results are shown in Plate 2. The control G6PD-F and G6PD-S kidney mixtures (tracks 1, 4, 6 and 9) gave two separate bands, one in the fast position and the other in the slow position. Limb bud (track 8), brain, tail, amnion (track 7) and allantois (track 10) of females showed a single band in the fast position. The hemizygous males also exhibited a fast band in these tissues as did the vascular and avascular yolk sac (track 5). The slightly faster mobility of the yolk sac sample compared with the controls illustrates the minor mobility differences observed between some of the tissue types.

The results obtained from the female yolk sac are clearly different from those of all other tissues examined from either sex. In reaching the limits of this electrophoretic system (with twice the normal running time, to maximize band separation and resolution) there was still some band coalescence and slight enzyme dissociation. Given these constraints we interpret the avascular yolk sac (track 2), as showing a weak band in the fast position of equivalent mobility to the male yolk sac (see track 5). Between this and the slow band was located a heavily staining intermediate band. G6PD is a dimer in kangaroos (Johnston *et al.* 1978), so the presence of an intermediate band represents heterodimer formation and only occurs if both alleles are active in the same cell. (In the most unlikely event that the intermediate band does not represent heterodimer formation, it would follow that some yolk sac cells express a two-banded phenotype and therefore show random *X* inactivation). The vascular yolk sac (track 3) also shows evidence of heterodimer formation but there is a preponderance of the faster migrating allozyme and some band coalescence.

It has been previously established that the paternally derived *Gpd* allele is inactivated in somatic tissues of heterozygous kangaroos ranging in age from adults to 26-day-old pouch young (Johnston *et al.* 1978). In the present study, the finding that the Gpd^F allele derived from the female wallaroo parent was the only allele expressed in embryonic tissues indicates that paternal *X* inactivation had occurred in these *M. robustus* embryos. This is the earliest stage of development that paternal *X* inactivation has been demonstrated in a marsupial. The amnion and allantois also showed evidence of paternal



M. robustus 23 day embryo removed from the uterus and still surrounded by the amnion and extensive yolk sac. Projected scale, 5 mm. *a*, Amnion; *av*, avascular yolk sac; *e*, embryo; *lb*, limb bud; *st*, sinus terminalis; *v*, vascular yolk sac.

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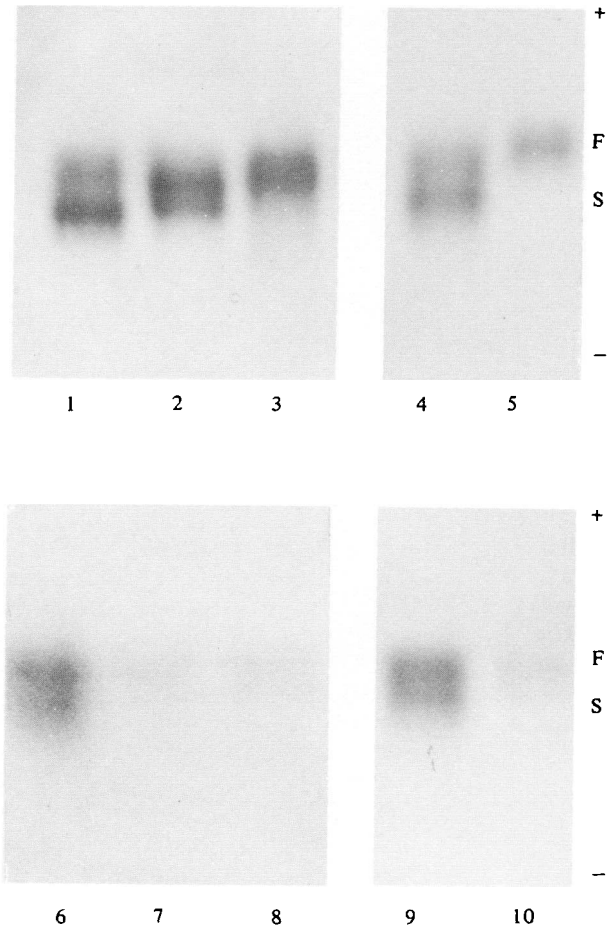

G6PD electrophoretic phenotypes (F, fast; S, slow) from 21 to 25 day *M. robustus* embryonic and extra-embryonic tissues and control tissue mixtures. 1, 4, 6 and 9, 1:1 mixtures of F and S kidneys; 2, avascular yolk sac from heterozygote; 3, vascular yolk sac from same heterozygote; 5, avascular yolk sac from hemizygous male; 7, amnion from heterozygote; 8, limb bud from heterozygote; 10, allantois from heterozygote.

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X inactivation but because of the low level of G6PD activity obtained from these membranes, paternal allele expression in a small proportion of cells cannot entirely be ruled out.

In the yolk sac of kangaroos the avascular region is bilaminar (trophectoderm and endoderm) and the vascular region is trilaminar, with the inclusion of mesoderm between the two layers (Sharman, 1961). The avascular yolk sac phenotype (Plate 2) showed ample evidence of expression of both *Gpd* alleles, however we have yet to determine if all cells of the trophectoderm and endoderm layers escape inactivation. Satisfactory isolation of the two layers is required prior to electrophoresis but our attempts to enzymatically separate them have so far been unsuccessful. Isolation of the three layers of the vascular region is also required although the greater activity of the faster migrating allozyme (track 3) may reflect paternal *X* inactivation of mesodermal cells.

Examination of other *X*-linked loci is needed to determine if the behaviour of the *Gpd* locus represents a general pattern for *X*-linked genes in kangaroo yolk sac cells. Almost all kangaroo female somatic cells exhibit paternal *X* inactivation, but partial activation of the paternal allele has been observed in cultured fibroblasts for PGK and G6PD (Cooper *et al.* 1975; Johnston *et al.* 1978) and in some tissues *in vivo* for PGK (VandeBerg, Cooper & Sharman, 1977). These observations together with the present yolk sac result encourage the view that in kangaroos, imprinting or regulation of paternal *X* inactivation is more labile than in eutherians.

Functionally, the yolk sac in eutherians and marsupials is not a phylogenetic remnant (Lillegraven, 1976). The eutherian yolk sac remains relatively small and weakly vascular and eventually regresses to become incorporated in the gut after the development of the chorio-allantoic placenta. The marsupial yolk sac becomes extremely large and persists to the end of gestation as the yolk sac placenta (Lillegraven, 1976), acting as an exchange organ and as an endocrine organ (Heap, Renfree & Burton, 1980). Paternal *X* inactivation in the yolk sac of eutherians was regarded as somehow advantageous for the interaction between mother and embryo after implantation (Wake, *et al.* 1976). We see no selective advantage in female kangaroo yolk sac having both alleles active at the *Gpd* locus especially with the presumed doubling of gene product relative to genetic males.

A comparison of the pattern of *X* inactivation in mouse extra-embryonic membranes with those of a kangaroo shows that they are quite different. Mouse amnion, allantois and the mesoderm layer of the yolk sac (like tissues of the embryo proper) are randomly *X* inactivated while the yolk sac endoderm exhibits paternal *X* inactivation (reviewed in West, 1982). Kangaroo amnion, allantois and the mesoderm layer of the yolk sac (like tissues of the embryo proper) appear to show paternal *X* inactivation, while some cells of the yolk sac trophectoderm and/or endoderm lack *X* inactivation. However, these marsupial/eutherian differences must be treated with caution in relation to phylogenetic implications because some of the germ layer lineages of extra-embryonic membranes of kangaroos are less well known than in the mouse.

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REFERENCES

Calaby, J. H. & Poole, W. E. (1971). Keeping kangaroos in captivity. *International Zoo Yearbook* **11**, 5–12.

Cooper, D. W., Johnston, P. G., Murtagh, C. E., Sharman, G. B., Vandeberg, J. L. & Poole, W. E. (1975). Sex-linked isozymes and sex chromosome evolution and inactivation in kangaroos. In *Isozymes*. Vol. III. *Developmental Biology* (ed. C. L. Markert), pp. 559–573. New York: Academic Press.

HEAP, R. B., RENFREE, M. B. & BURTON, R. D. (1980). Steroid metabolism in the yolk sac placenta and endometrium of the tammar wallaby, *Macropus eugenii*. *Journal of Endocrinology* **87**, 339–349.

JOHNSTON, P. G., SHARMAN, G. B., JAMES, E. A. & COOPER, D. W. (1978). Studies on metatherian sex chromosomes. VII. Glucose-6-phosphate dehydrogenase expression in tissues and cultured fibroblasts of kangaroos. *Australian Journal of Biological Sciences* **31**, 415–424.

LILLEGRAVEN, J. A. (1976). Biological considerations of the marsupial-placental dichotomy. *Evolution* **29**, 707–722.

LYON, M. F. (1972). *X*-chromosome inactivation and developmental patterns in mammals. *Biological Reviews* **47**, 1–35.

MCMAHON, A., FOSTEN, M. & MONK, M. (1983). *X*-chromosome inactivation mosaicism in the three germ layers and the germ line of the mouse embryo. *Journal of Embryology and experimental Morphology* **74**, 207–220.

SHARMAN, G. B. (1961). The embryonic membranes and placentation in five genera of diprotodont marsupials. *Proceedings of the Zoological Society of London* **137**, 197–220.

TAKAGI, N. & SASAKI, M. (1975). Preferential inactivation of the paternally derived *X* chromosome in the extraembryonic membranes of the mouse. *Nature* **256**, 640–642.

WAKE, N., TAKAGI, N. & SASAKI, M. (1976). Non-random inactivation of *X*-chromosome in the rat yolk sac. *Nature* **262**, 580–581.

WEST, J. D. (1982). *X* chromosome expression during mouse embryogenesis. In *Genetic Control of Gamete Production and Function* pp. 49–92. Proceedings of the Serono Clinical Colloquia on Reproduction, no. 3. London: Academic Press.

WEST, J. D., FRELS, W. I., CHAPMAN, V. M. & PAPAIOANNOU, V. E. (1977). Preferential expression of the maternally derived *X* chromosome in the mouse yolk sac. *Cell* **12**, 873–882.

VANDEBERG, J. L., COOPER, D. W. & SHARMAN, G. B. (1977). Studies on metatherian sex chromosomes. V. Activity of the paternally derived allele at the *X* linked *Pgk-A* locus in some tissues of kangaroos *Macropus giganteus* and *M. parryi*. *Australian Journal of Biological Sciences* **30**, 421–430.