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Dictyate Oocytes of a Kangaroo (*Macropus robustus*) Show Paternal Inactivation at the X-linked *Gpd* Locus

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Abstract

Purified samples of large numbers of dictyate oocytes from 13 *M. robustus* pouch young heterozygous for glucose-6-phosphate dehydrogenase type and six homozygous controls were examined electrophoretically to determine activity states at the *Gpd* locus. Like somatic cortical and medullary cells, oocytes expressed only the maternal phenotype irrespective of the direction of the cross. No evidence was found of reactivation of the inactive (paternal) allele or inactivation of both maternal and paternal alleles. It was therefore concluded that unlike eutherian dictyate oocytes, only a single (maternal) allele is active in each dictyate oocyte in *M. robustus*. The stage of reactivation of the paternal allele remains to be determined.

Introduction

Inactivation of one of the two X chromosomes in cells of female eutherian mammals provides dosage compensation relative to the single (active) X of hemizygous males (Lyon 1972). Inactivation is established early in the development of female eutherians and is random from cell to cell except in the derivatives of the trophectoderm and primitive endoderm where the paternal X is preferentially inactivated (Takagi and Sasaki 1975; Frels *et al.* 1979). On the other hand, oocytes have both X chromosomes genetically active. This totipotency of oocyte X chromosomes involves reactivation of the inactive X in the oogonial or preleptotene precursor (Gartler *et al.* 1975; Johnston 1981; Kratzer and Chapman 1981) presumably associated with some event(s) that lead to meiosis.

In female kangaroos, the pattern of X inactivation differs from that of eutherians in several ways. At the few X-linked loci examined electrophoretically in pouch young, juveniles and adults, a variety of somatic cell and tissue types exhibit paternal X inactivation while a few tissues *in vivo* or *in vitro* show partial or complete activation of the paternal allele (Cooper *et al.* 1975; Johnston *et al.* 1978). Kangaroo germ-line cells have been examined for activity of the X-linked *Gpd* gene in *Macropus robustus* (Robinson *et al.* 1977). There was preferential inactivation of the paternal allele in oogonia-rich ovarian extracts and in extracts of oocytes rich in leptotene to diplotene stages of prophase I. There was no electrophoretic evidence of reactivation of the inactive (paternal) allele. This result provided strong support for the view that both alleles were not active in *M. robustus* oocytes. However, it did not rule out the unique possibility amongst mammalian cells that both X-linked alleles were inactive, with the detectable glucose-6-phosphate dehydrogenase (G6PD) the product of long-lived enzyme-messenger RNA synthesized in premeiotic germ cells or the product of ovarian somatic

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cells. The proposition that both alleles were inactive became more plausible with our recent finding of extremely low G6PD levels in single dictyate oocytes from Graafian follicles of *Macropus eugenii* (tammar wallaby) adults (Briscoe *et al.* 1983). For example, mouse oocytes at about the same stage have up to 285 times more G6PD activity per picolitre of cytoplasm than tammar wallaby oocytes. This result led us to question whether perhaps the proportion of oocytes to somatic cells in our earlier experiment with *M. robustus* may not have been high enough to allow detection of different forms of the enzyme. It was clear that additional information from large, more homogeneous samples of oocytes free of somatic cells was needed to resolve the question of G6PD expression in these cells. To this end we have recovered larger numbers of primordial follicles from mid-term female pouch young of *M. robustus* and removed the adhering follicle cells to provide a rich, uniform extract of early dictyate oocytes. The present paper deals with the electrophoretic phenotypes of such extracts obtained from G6PD heterozygotes and provides evidence that there is paternal X inactivation in oocytes at the early dictyate stage.

Materials and Methods

Animals

Two subspecies of *M. robustus* have different electrophoretic forms of the X-linked G6PD (Richardson *et al.* 1971). Crosses between female wallaroos (*M. r. robustus*: G6PD-F) and male euros (*M. r. erubescens*: G6PD-S) produce fertile female hybrids heterozygous for G6PD type. These are designated Gpd^F/Gpd^S with only the maternally derived G6PD–F form being expressed in somatic tissues (Johnston and Sharman 1975). The reciprocal cross between female euros and male wallaroos results in Gpd^S/Gpd^F heterozygotes. The ages of the female pouch young examined ranged from 110 to 180 days.

Oocyte Isolation

Ovaries were removed from pouch young and placed in phosphate-buffered saline without calcium and magnesium (PBS^-). The medulla region was dissected from the ovary and kept at 4°C until required for electrophoresis. The remainder of the ovary was transferred to 1 ml of PBS^- containing 0·1% (w/v) collagenase and 0·02% (w/v) deoxyribonuclease I (Eppig 1976) for 20 min at 37°C. According to Eppig (1976) the presence of deoxyribonuclease I prevents the clumping of cells which is probably caused by association with DNA released from damaged cells. During this time dissociation was physically assisted by the use of scissors and a finely drawn Pasteur pipette. The enzyme action was arrested by the addition of 0·3% (w/v) bovine serum albumin (BSA) in PBS^- solution. The cells were then washed twice in PBS^-.

Oocyte Separation

Isolated cells were separated by rate zonal sedimentation for 1 h at unit gravity in polycarbonate tubes containing 10 ml of Hepes-buffered Hams F10 culture medium stabilized with a 2–4% gradient of BSA at 4°C (Trotman and Tate 1983). Fractions (0·5 ml) were removed carefully from the top of the tube and transferred to a multi-well plate where they were microscopically examined for cell type. Oocyte rich and somatic cell rich fractions were collected separately, washed twice in PBS^- and transferred to siliconized microcentrifuge tubes.

Electrophoresis

After centrifugation, the oocyte and somatic cell fractions were lysed by freezing and thawing in 1–2 μl of lysing solution (Johnston *et al.* 1978). Medulla and G6PD-F/G6PD-S kidney mixtures were homogenized 1 : 3 (w/v) in lysing solution. The samples were applied to Cellogel and electrophoresis was carried out in 0·1 M lithium borate–0·0024 M EDTA buffer, pH 9·0, for 2 h with a voltage gradient of 14 V cm^{-1}. The gel stain was the same as in Johnston *et al.* (1978).

Results

Examination of conventionally prepared paraffin sections of *M. robustus* pouch young ovaries showed that from 110 to 180 days post partum the cortex contained

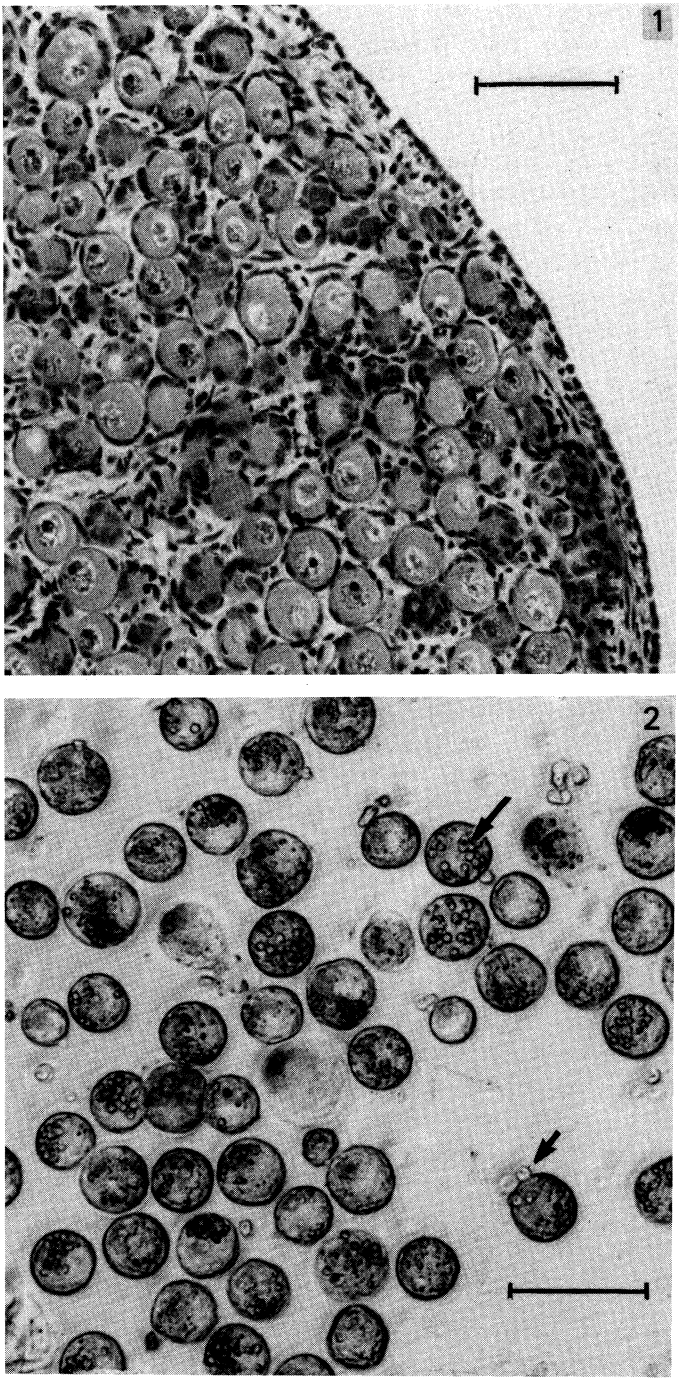

Fig. 1. Histological section of part of cortical region of 130-day ovary of *M. robustus*. Note numerous dictyate oocytes surrounded by flattened follicle cells. Bar = 50 μm.

Fig. 2. Oocyte-rich suspension obtained after treatment of ovary with collagenase-deoxyribonuclease I and concentration in a BSA gradient. Note yolk droplets inside oocytes (long arrow) and a few follicle cells in suspension or attached to oocyte surface (short arrow). Bar = 50 μm.

large numbers of primordial follicles, each composed of a dictyate oocyte and an investing layer of squamous follicle (somatic) cells (Fig. 1). Oogonia and non-follicular predictyate leptotene to diplotene oocytes were rare and most of them atretic. The dictyate stage which follows diplotene is characterized by elongation of the bivalents into a diffuse, coiled network of chromatin strands and a large, intensely stained nucleolus (Fig. 1). A few oocytes remain in this arrested state until shortly before ovulation but most become atretic and are resorbed later in pouch life or after pouch exit. The age range of pouch young used for oocyte recovery thus provided the greatest number of non-atretic oocytes at the same stage of development.

The collagenase–deoxyribonuclease I treatment of Eppig (1976) was successful in dissociating the various ovarian cell types. After being subjected to the BSA gradient the cells separated out as follows: somatic cells occurred in the first four fractions collected; the next two fractions were discarded because they contained a mixture of oocytes and somatic cells: oocytes at varying densities were found in the remainder of the fractions, the last four of which were discarded because of the presence of aggregated cells. The oocyte fractions used for electrophoresis contained very few somatic cells (Fig. 2).

Oocytes, cortical somatic cells and medulla from nine Gpd^F/Gpd^S heterozygotes, four Gpd^S/Gpd^F and six homozygous controls were examined electrophoretically for G6PD expression. The control G6PD-F and G6PD-S kidney mixture gave two bands, one in the fast position and the other in the slow position (Fig. 3, tracks 5 and 7). Oocyte fractions from Gpd^F/Gpd^S heterozygotes produced a band in the maternal G6PD-F position (track 3). Only the maternal G6PD-S phenotype was expressed in oocyte fractions of Gpd^S/Gpd^F heterozygotes (tracks 4 and 8).

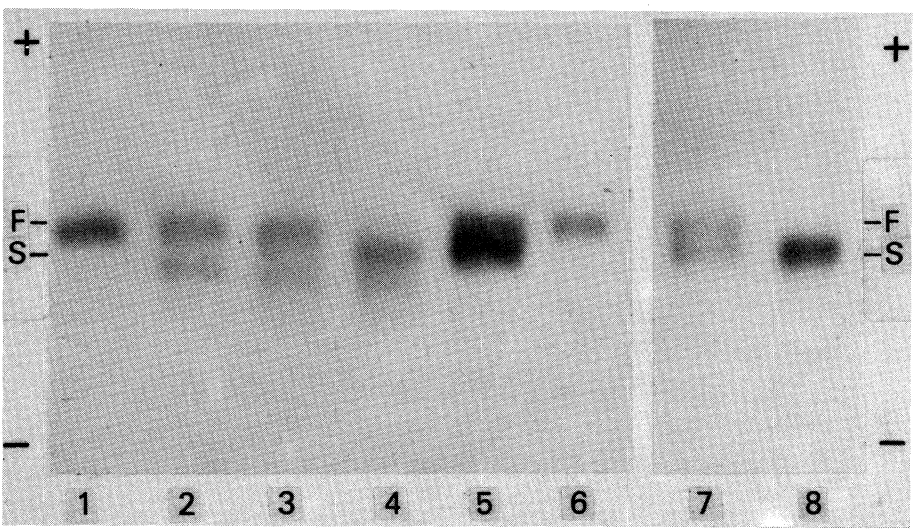

Fig. 3. G6PD electrophoresis phenotypes (*F*, fast; *S*, slow) from oocyte samples from *M. robustus* pouch young and control mixtures. Tracks 5 and 7, 1 : 1 mixtures of *F* and *S* kidneys; 1, medulla *F/S*; 2, oocytes *F/F*; 3, oocytes *F/S*; 4, oocytes *S/F*; 6, cortical somatic cells, *F/S*; 8, oocytes *S/F*.

Minor sub-banding was observed in some oocyte samples (tracks 2, 3 and 4), however, the sub-band from track 3 does not represent paternal allele expression because the position of the band does not correspond with the normal G6PD-S position. In addition, oocytes from Gpd^F/Gpd^F homozygotes also displayed similar sub-banding (track 2).

The somatic cortical cell (track 6) and medullary cell (track 1) samples expressed only the maternal allele.

Discussion

Dictyate oocytes removed from Graafian follicles of mice and humans have provided evidence that both X chromosomes are active at this stage. In human G6PD heterozygotes, maternal, paternal and intermediate bands were present following electrophoresis of several oocytes (Gartler *et al.* 1972). Dictyate oocytes removed from the ovaries of adult XX mice possess twice the activity of those of XO mice for the sex-linked enzymes G6PD (Epstein 1969), hypoxanthine phosphoribosyltransferase (HPRT) (Epstein 1972) and phosphoglycerate kinase (PGK) (Kozak *et al.* 1974).

The dictyate oocytes recovered from kangaroo pouch young were much less mature than those used for eutherian investigations, being at the primordial follicle rather than the Graafian follicle stage but it is clear that at this early dictyate oocyte stage, unlike eutherians, reactivation of the inactive (paternal) allele has not occurred. Whether or not reactivation of the paternal allele occurs later in development of preovulatory kangaroo oocytes remains to be established. We were unable to determine electrophoretic phenotypes of dictyate oocytes from Graafian follicles because the numbers obtainable were too low to provide sufficient enzyme for resolution on Cellogel. Polyacrylamide microgel methods using one or a few oocytes presented separation problems. That reactivation of the paternal allele for G6PD does occur at some stage of oocyte development (or in the ovum or early embryo) is clear from *M. robustus* pedigree data (Johnston and Sharman 1975). They show that progeny derived from females heterozygous for *Gpd* may inherit either allele in its active form, indicating that both alleles are potentially active.

On the basis of the results described here we conclude that it is extremely unlikely that both alleles are inactive in *M. robustus* early dictyate oocytes. Somatic cell inclusion in samples is so low following BSA gradient separation that the single band in the maternal position in heterozygotes could not reasonably be attributed solely to somatic cells. Also it seems highly improbable that the band is due to the presence of long-lived messenger RNA from earlier (premeiotic) stages (Lyon 1977) given that the dictyate oocytes in samples used for electrophoresis were probably from 50 to 120 days old.

It has often been assumed that activity states at the *Gpd* locus represent activity states at most, if not all, X-linked loci in eutherian mammals and that inactivation of the paternal allele for *Gpd* indicates inactivation of the entire paternally-derived X chromosome. Such an assumption may not be valid for marsupials (Cooper *et al.* 1977) and transcriptional patterns at other X-linked loci in kangaroo oocytes need to be established before statements are made regarding the behaviour of the entire paternally derived X chromosome.

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