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# Single Nucleotide Primer Extension (SNuPE) analysis of the *G6PD* gene in somatic cells and oocytes of a kangaroo (*Macropus robustus*)

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**Summary**

cDNA sequence analysis of the X-linked glucose-6-phosphate dehydrogenase (*G6PD*) gene has shown a base difference between two subspecies of the kangaroo, *Macropus robustus robustus* (wallaroo) and *M. r. erubescens* (euro). A thymine residue in the wallaroo at position 358 in exon 5 has been replaced by a cytosine residue in the euro, which accounts for the previously reported electrophoretic difference between the two subspecies. This base difference allowed use of the Single Nucleotide Primer Extension (SNuPE) technique to study allele-specific expression of *G6PD* at the transcriptional level. We began by examining *G6PD* expression in somatic cells and observed complete paternal X inactivation in all somatic tissues of adult female heterozygotes, whereas we found partial paternal allele activity in cultured fibroblasts, thus confirming previous allozyme electrophoresis studies. In late dictyate oocytes from an adult heterozygote, the assay also detected expression of both the maternal and paternal alleles at the *G6PD* locus, with the maternal allele showing preferential expression. Thus reactivation of the inactive paternally derived X chromosome occurs during oogenesis in *M. robustus*, although the exact timing of reactivation remains to be determined.

## 1. Introduction

In mammals inactivation of one of the two X chromosomes in each somatic cell ensures that both females (XX) and males (XY) have the same dosage of X-linked gene products (Lyon, 1961). X chromosome inactivation is random and clonal in eutherians (specifically mice and humans) and genes subject to inactivation are, in most cases, completely repressed with no detectable expression of the allele carried on the inactive X chromosome. X inactivation in marsupials (kangaroos) is preferentially paternal and clonal and it is not uncommon to find locus-specific, tissue-specific and species-specific differences in the expression of the paternally derived X chromosome (Cooper *et al.*, 1993). At the glucose-6-phosphate dehydrogenase (*G6PD*) locus, complete paternal X inactivation has been observed in somatic cells of kangaroos (*Macropus robustus*, *M. rufogriseus*), and partial paternal allele expression has been detected in cultured fibroblasts (Johnston *et al.*, 1978). In the American marsupial, the Virginia opossum (*Didelphis virginiana*), paternal allele expression of *G6PD* in both somatic tissues and cultured fibroblasts was detected (Samollow *et al.*, 1987, 1989), although developmental variations in the levels of expression of the paternal allele have been reported in some tissues (Samollow *et al.*, 1995).

In the female germ line of humans and mice, reactivation of the randomly inactivated X chromosome has been observed at the onset of meiosis (leptotene) (Gartler *et al.*, 1975; Johnston, 1981; Kratzer & Chapman, 1981) during embryogenesis, but this is not the case in kangaroos. Earlier studies using an allozyme mobility difference of *G6PD* between two subspecies of the kangaroo *M. robustus*

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– *M. r. robustus*, the wallaroo (*w*) and *M. r. erubescens*, the euro (*e*) – showed that in predictyate (leptotene, zygotene, pachytene) and early dictyate oocytes of pouch young, no paternal allele expression was detected in heterozygotes (Johnston *et al.*, 1985). Insufficient *G6PD* activity was present in dictyate oocytes of adults to obtain a result from allozyme micro-electrophoresis (Briscoe *et al.*, 1983). The fact that reactivation of the paternally derived inactive X occurs in kangaroos is evident from pedigree data, where either the maternally or paternally derived X chromosome can be transmitted from mother to offspring in its active state (Johnston *et al.*, 1978). Given these results, it follows that reactivation must occur in female kangaroos later in oogenesis than the early dictyate stage, or possibly in the zygote or at an early post-zygotic stage.

Most of the earlier studies on X inactivation in marsupials utilized allozyme electrophoresis. It is evident that more sensitive techniques for the detection of X-linked gene expression at the transcriptional level are required. Reverse Transcription–Polymerase Chain Reaction (RT–PCR) is a useful technique for studying X inactivation at the transcriptional level. The Single Nucleotide Primer Extension (SNuPE) technique allows the allele-specific expression of genes to be analysed (Singer-Sam & Riggs, 1993). Only a single base difference between the two alleles being analysed is required in heterozygotes. Several studies have used SNuPE to observe differential expression of autosomal and X-linked genes in eutherians during early development and gametogenesis (Singer-Sam *et al.*, 1990; Buzin *et al.*, 1994; Lebon *et al.*, 1995) and to study genomic imprinting (Singer-Sam *et al.*, 1992; Szabó & Mann, 1995). In this study a sequence difference in the X-linked *G6PD* gene was detected between two subspecies of *M. robustus*. Based on this nucleotide difference, the SNuPE assay was used to study allele-specific expression of *G6PD* in both adult somatic cells and late (adult) dictyate oocytes.

## 2. Materials and methods

Females representing two subspecies of *M. robustus*, maintained in captivity at the Macquarie University Fauna Park, were used in this study. Samples were obtained from two *M. r. robustus* (wallaroo), four *M. r. erubescens* (euro), six *we* (wallaroo female × euro male) hybrids and two *ew* (euro female × wallaroo male) hybrids.

Blood samples were obtained from the caudal vein. Other tissues, including kidney, heart, spleen, brain, lung, skeletal muscle and small intestine samples, were taken from a *we* hybrid and from an *ew* hybrid that had been killed by injection of pentobarbital sodium. Ovaries and uterus were also obtained from a *we* hybrid. Tissues were dissected into small pieces in PBS− (phosphate-buffered saline without magnesium or calcium), immediately transferred to liquid nitrogen and later stored at −70 °C. For oocyte collection, ovaries were dissected from the reproductive tract of the *ew* hybrid and placed in PBS−. After rupturing large ovarian follicles, the released oocytes were taken up and expelled from a micropipette with an internal tip diameter slightly less than or the same as the oocyte diameter, resulting in the removal of adhering follicle cells. A total of 15 oocytes were isolated and maintained in PBS− prior to treatment.

Fibroblasts from the 8 female hybrids were derived from ear pinna biopsies. Collection procedures and culture conditions were as previously described by Cooper *et al.* (1977). Prior to electrophoresis, cultured fibroblasts were detached with 0·1% trypsin and resuspended in culture medium. Preparation of cell lysates, Cellogel electrophoresis and staining for *G6PD* were as previously described by Johnston *et al.* (1978).

RNA was isolated from tissues, cultured fibroblasts, blood and oocytes using total RNA isolation reagent (Advanced Biotechnologies Ltd). The standard protocol supplied by the manufacturer was used for tissues and cultured fibroblasts; however, modifications were made to the initial steps of RNA extraction from blood and oocytes. An aliquot of 500 $\mu$l of blood was centrifuged at 2000 rpm for 10 min. The supernatant was removed and 1 ml of RNA isolation reagent added. Fifty microlitres of RNA isolation reagent was added to the oocytes in a Petri dish before being transferred to an Eppendorf tube at 4 °C. Subsequent steps in the RNA isolation technique were modified accordingly to account for the small amount of sample present in oocytes. RNA extraction was then carried out according to the manufacturer's instructions. After their concentration and purity were determined, the samples were separated into 5 $\mu$l aliquots and stored at −70 °C. Reverse transcription was performed using a reverse transcription kit (Promega). Samples were separated into 5 $\mu$l aliquots and stored at −70 °C.

Primers for PCR amplification and sequencing of the two subspecies were designed from the *G6PD* cDNA sequence of the wallaroo, *M. robustus* (Loebel *et al.*, 1995). PCR products were sequenced at the Westmead Hospital DNA Sequence and Synthesis Facility, Westmead, NSW.

The region of *G6PD* cDNA containing the base difference in exon 5 (see Fig. 1) was amplified by PCR using the following primers: (EX4F 5′ TGGCTGTTCCGTGATGGGCTTCTC 3′, EX8R 5′ CAAGAGGTGGTTCTGCATCACGTC 3′).

PCR reactions were carried out in a total of 30 $\mu$l; containing 1 × *Taq* buffer (500 mM-KCl, 100 mM-Tris-Cl (pH 9·0 at 25 °C), 1% Triton X-100), 1·5 mM-$MgCl_2$, 0·2 mM-dNTPs (dATP, dGTP, dCTP, dTTP), 0·12 mM of each primer and 1·5U of *Taq* DNA

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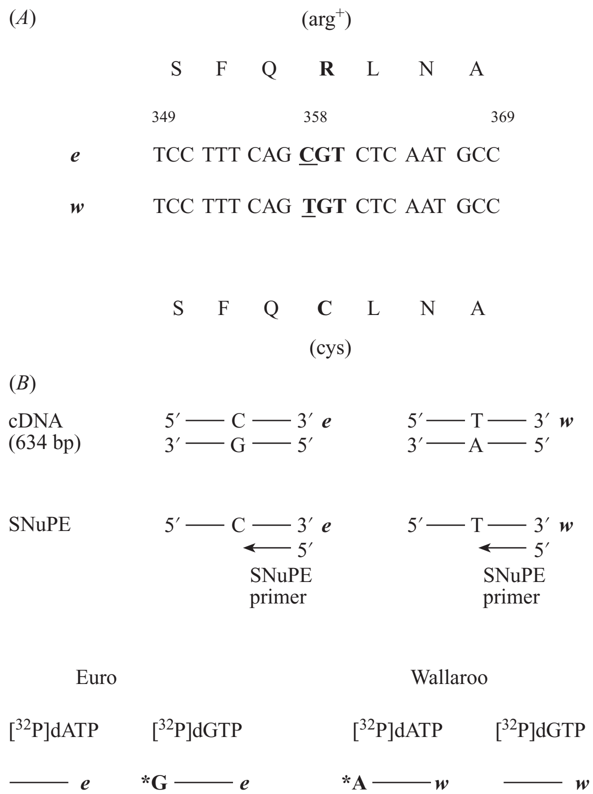


Fig. 1. Single Nucleotide Primer Extension (SNuPE) assay for allele-specific *G6PD* expression in *M. robustus* cDNA. (*A*) Comparison of *G6PD* cDNA sequence (exon 5, position 349–369) showing the base difference between *M. r. robustus* (***w*** = wallaroo) and *M. r. erubescens* (***e*** = euro). (*B*) In the SNuPE assay, [$^{32}$P]dATP is specific for the wallaroo allele as it is complementary to T (the base next to the SNuPE primer), and [$^{32}$P]dGTP is specific for the euro allele, being complementary to the next base C. The product, after one PCR cycle, consisted of the SNuPE primer and the allelic specific radioactive nucleotide added. (***w***, wallaroo; and ***e***, euro; *, band present).

polymerase and 2·5 $\mu$l of cDNA. A 'hot' start was used with samples heated to 95 °C for 5 min before the *Taq* polymerase was added. The thermocycles were one cycle at 95 °C for 30 s followed by 38 cycles of denaturation at 95 °C, annealing at 58 °C for 45 s, an extension at 72 °C for 90 s and a final extension at 72 °C for 10 min. PCR products were run on a 2% agarose gel in 1 × TAE buffer at 100 V, the observed 634 bp band was cut out and the cDNA extracted using Bresaclean (Bresatec). Purified PCR products were resuspended in 10 $\mu$l of s$H_2O$.

The SNuPE assay was based on Singer-Sam & Riggs (1993). The SNuPE primer (3′ CAGAGTTA-CGGGTGTACTTG 5′) was designed so that the 3′ end of the primer was just 5′ to the base difference between the two subspecies.

SNuPE reactions were carried out in a total of 10 $\mu$l; containing 1 × *Taq* buffer, 4·0 mM-$MgCl_2$, 1 $\mu$M-SNuPE primer and 0·5U *Taq* DNA polymerase, 10 ng of purified cDNA, and 1 $\mu$Ci of [$^{32}$P]dATP or [$^{32}$P]dGTP. The radioactive nucleotides [$^{32}$P]dATP and [$^{32}$P]dGTP (3000 Ci/mmol, 10 $\mu$Ci/ ml) were diluted 10-fold in s$H_2O$ and 1 $\mu$l of each diluted radioactive nucleotide was added to each sample separately, just prior to incubation. SNuPE consisted of one cycle of denaturation at 95 °C for 1 min, annealing at 42 °C for 2 min and extension at 72 °C for 1 min. Two microlitres of loading dye was added to 2 $\mu$l of each sample, which was then run on a 15% denaturing polyacrylamide gel at 100 V (Hoefer minigel system; Pharmacia Biotech Inc.). Gels were exposed to X-ray film overnight at −80 °C.

## 3. Results

Sequencing analysis of *G6PD* cDNA from the two subspecies of the common wallaroo, *M. robustus*, revealed that the sequence described by Loebel *et al.* (1995) was from the DNA of the euro, *M. r. erubescens*, not the wallaroo, *M. r. robustus*. A cytosine residue in the euro at position 358 in exon 5 has been replaced by a thymine residue in the wallaroo (Fig. 1*A*). This substitution accounts for the electrophoretic mobility differences of *G6PD* found in this study (data not shown) and in previous studies (see Johnston *et al.*, 1978, 1985). A positively charged arginine in the euro results in a slower migrating form of the *G6PD* enzyme (*G6PD*-S). By contrast, the wallaroo, which has a neutrally charged cysteine, has a faster migrating form of the enzyme (*G6PD*-F). This base difference was then used to investigate differential expression of the euro and wallaroo *G6PD* alleles at the transcriptional level.

Allozyme electrophoresis of cultured fibroblasts from the 8 female hybrids confirmed that they were heterozygous for *G6PD* (data not shown). Heterodimer formation was evident, indicative of both alleles being active in the same cell. In all cases there was preferential expression of the maternally derived allele. Fig. 1*B* illustrates the allele-specific SNuPE assay designed on the basis of the base difference detected between the two subspecies. Fig. 2 shows the controls used for the SNuPE assay and the results obtained for different cell types of *M. robustus*. Expression of the wallaroo allele was observed when [$^{32}$P]dATP was added to the reaction mix, whereas the euro allele was expressed when [$^{32}$P]dGTP was added. A negative control exhibited no allelic expression when the opposite radioactive nucleotide, [$^{32}$P]dGTP for the wallaroo and [$^{32}$P]dATP for the euro, was added to the reaction mix. The difference in level of expression in all assays can be explained by the higher specific activity of [$^{32}$P]dATP when the assay was performed. Fig. 2*A* shows the SNuPE results from cultured fibroblasts. In cultured fibroblasts of the female *we* heterozygote, preferential expression of the maternal

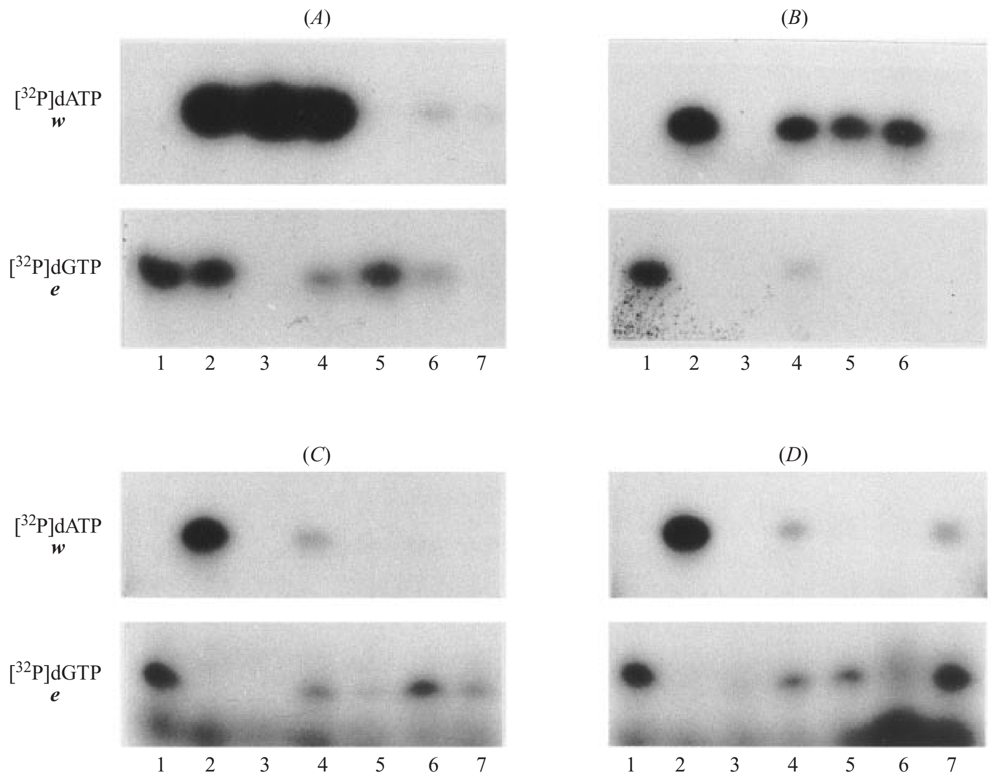


Fig. 2. Allele-specific expression of *G6PD* in different cell types of *M. robustus*. The top row of bands represents expression detected by the radioactive nucleotide [$^{32}$P]dATP, which is specific for the wallaroo (***w***) allele, and the lower row represents expression detected by [$^{32}$P]dGTP, which is specific for the euro (***e***). (*A*) Controls, top row: lanes (1) no cDNA; (2) 5 ng ***e*** plus 5 ng ***w*** cDNA; (3) 10 ng ***w*** cDNA. Controls, lower row: lanes (1) 10 ng ***e*** cDNA; (2) 5 ng ***e*** plus 5 ng ***w*** cDNA; (3) no cDNA. Samples: lanes (4) ***we*** hybrid cultured fibroblasts; (5) ***e*** cultured fibroblasts; (6) ***ew*** hybrid cultured fibroblasts; (7) ***w*** cultured fibroblasts. (*B*) Controls, top row: lanes (1) no cDNA; (2) 10 ng ***w*** cDNA; (3) 10 ng ***e*** cDNA. Controls, lower row: lanes (1) 10 ng ***e*** cDNA, (2) no cDNA, (3) 10 ng ***w*** cDNA. Samples: lanes (4) ***we*** hybrid cultured fibroblasts; (5) ***we*** ovary (6) ***we*** uterus. (*C*) Controls: lanes (1)–(3) as in (*B*). Samples: lanes (4) ***ew*** hybrid cultured fibroblasts; (5) ***ew*** kidney; (6) ***ew*** brain; (7) ***ew*** heart. (*D*) Controls: lanes (1)–(3) as in (*B*). Samples: lanes (4) ***ew*** hybrid cultured fibroblasts; (5) ***ew*** liver; (6) blastocyst (unsexed); (7) ***ew*** oocytes.

wallaroo allele and partial activity of the paternal euro allele were evident. The female *ew* heterozygote showed preferential expression of the maternal euro allele and partial activity of the paternal wallaroo allele. In blood samples, expression of the maternal allele was observed in both the *we* and *ew* female heterozygotes, but no paternal allele activity was detected (data not shown). On the SNuPE gel in Fig. 2*B*, only maternal wallaroo allele expression was detected in somatic tissues from female *we* heterozygotes. There was activity of only the maternal euro allele in the somatic tissues of the female *ew* hybrid (Fig. 2*C*). In the oocytes of the female *ew* heterozygote, activity of both the maternal euro and paternal wallaroo allele was evident, with the maternal allele showing preferential expression (Fig. 2*D*).

## 4. Discussion

It has long been assumed from allozyme electrophoresis studies that inactivation of the paternally derived allele was complete at the *G6PD* locus in kangaroo somatic tissues of female heterozygotes (Johnston *et al*., 1978). By identifying the base difference responsible for the electrophoretic difference between the two subspecies of *M. robustus*, it has been possible to utilize a more sensitive allele-specific assay and to confirm that the paternally derived allele is not being transcribed in these tissues. This finding rules out the possibility that allozyme electrophoresis was failing to detect very low levels of paternal allele expression. It should be pointed out that the Virginia opossum shows variable expression of the paternally derived allele in all tissues that have been examined (Samollow *et al*., 1987). Thus we confirm our previous conclusion of species-specific differences in the behaviour of sex-linked loci between Australian and American marsupials.

Cultured fibroblasts are the only cells to have shown electrophoretic evidence of paternal allele expression at the *G6PD* locus in kangaroos, with activity of the paternal allele always less than that of the maternal allele. Again, the SNuPE technique reflects the electrophoresis results, with partial activity

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of the paternal allele being observed in reciprocal heterozygotes. The functional basis for this difference is not known.

The most significant finding of this study was that the SNuPE technique was sensitive enough to detect allele-specific expression of both the maternal and paternal alleles at the *G6PD* locus in dictyate oocytes from an adult *ew* female heterozygote. Previous studies involving allozyme electrophoresis have indicated complete paternal X inactivation in oocytes from different-aged pouch young of *M. robustus*. Ovaries from pouch young containing large numbers of predictyate oocytes showed only maternal allele expression at the *G6PD* locus (Robinson *et al.*, 1977). To overcome possible contamination effects of somatic follicle cells showing only maternal inheritance, large numbers of dictyate oocytes were isolated and electrophoresed from older pouch young, but again no paternal allele expression was observed (Johnston *et al.*, 1985). Unlike eutherian mammals, there was insufficient *G6PD* activity present in isolated adult oocytes to determine the status of X chromosome activity using allozyme electrophoresis (Briscoe *et al.*, 1983).

Evidence for the reactivation of the inactive X chromosome in oocytes has been predicted from pedigree data, where both the maternal and paternal allele can be transmitted to the next generation in its active form (Johnston *et al.*, 1978). Thus, using the more sensitive allele-specific SNuPE technique, it has been possible to show for the first time that the paternally derived allele at the *G6PD* locus is reactivated in adult dictyate oocytes in kangaroos. The exact timing of reactivation during oogenesis in kangaroos is still not known. We cannot rule out the possibility that the more sensitive SNuPE technique may have detected faint paternal activity in predictyate and early dictyate oocytes from pouch young. However, we feel that this is unlikely given the good correlation between the allozyme and SNuPE results obtained in this study. We have shown that reactivation does not occur at the post-dictyate, pronuclear or zygote stages.

Allozyme electrophoretic studies on *Mus caroli* have shown that both *G6PD* alleles are equally active in late oocytes of heterozygotes (Kratzer & Chapman, 1981). This finding contrasts with the results presented here where there is preferential expression of the maternal allele in *M. robustus* late oocytes. We exclude the possibility of follicle contamination because the oocytes were completely freed of adhering cells. The results obtained for *M. robustus* oocytes are very similar to those obtained for cultured fibroblasts, lending support to the hypothesis that the reactivated paternally derived allele never attains the same level of activity as the active maternally derived allele. It has been shown that *M. robustus* cultured fibroblasts exhibiting paternal *G6PD* activity have a late-replicating paternally derived X chromosome (Johnston & Robinson, 1986). We suggest that late DNA replication is the most likely explanation for the paternally derived X chromosome never producing as much gene product as the active maternally derived X chromosome in kangaroos.

Preferential maternal allele expression has now been demonstrated in late oocytes of *M. robustus*, in cultured fibroblasts of this species and in the somatic tissues of the North American opossum. This lends further support to the hypothesis that marsupial cells possessing a reactivated paternally derived allele never show as high a level of paternal allele activity compared with the maternally derived allele.

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