Expression of the maternally derived X chromosome in the mural trophoblast of the mouse

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SUMMARY

Only the maternally derived allelic form of the X-chromosome-linked enzyme phosphoglycerate kinase (PGK-1) is observed in the mural trophoblast of heterozygous female progeny in $F_3$ and backcross matings. We have demonstrated that this expression of the maternally derived PGK-1 is not a result of maternal tissue contamination nor of selection of cells expressing the maternal X chromosome ($X^m$). Our results suggest that the expression of $X^m$ in mural trophoblast is a consequence of nonrandom X-chromosome inactivation in trophectoderm cells.

INTRODUCTION

Preferential expression of the maternal X chromosome ($X^m$) has been reported in certain extra-embryonic membranes of rodents by Takagi & Sasaki (1975) and Wake, Takagi & Sasaki (1976). This nonrandom expression of $X^m$ was demonstrated in mouse yolk sac, mouse chorion, and rat yolk sac using fluorescent staining of the asynchronously replicating, and presumably inactive, X chromosome. A cytological marker was used to follow the maternal or paternal origin of the X chromosome. The asynchronously replicating X chromosome was found to be of paternal origin ($X^p$) in a large proportion of the mitotic figures.

The preferential expression of the maternally derived X chromosome in mouse yolk sac has been independently verified, using our electrophoretic variant of the X-linked enzyme phosphoglycerate kinase (PGK-1) as a marker of X-chromosome expression (West, Frels, Chapman & Papaioannou, 1977). This analysis was extended to enzymically separated yolk-sac mesodermal and endodermal layers. An unbalanced mosaicism of X-chromosome expression ($X^m > X^p$) was observed in the mesodermal layer but only the maternal PGK-1 was observed in yolk-sac endoderm. West et al. concluded that the preferential expression of

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the maternal X chromosome in mouse yolk sac was probably the consequence of nonrandom X-chromosome inactivation in the primitive endoderm, the progenitor of the yolk-sac endoderm.

The preferential expression of $X^m$ in primitive endoderm derivatives prompted us to examine X-chromosome expression in a trophectoderm derivative since cell commitment to the trophectoderm precedes primitive endoderm formation (Gardner & Papaioannou, 1975).

We used electrophoretic variation for PGK-1 to study X-chromosome expression in mural trophoblast, a trophectoderm derivative, at 9-5 days post coitum. At this stage, mural trophoblast is composed mainly of primary and secondary giant cells. The primary giant cells are derived from the mural trophectoderm of the blastocyst and the secondary giant cells are derived later from the polar trophoblast. Our findings suggest that only the maternally derived X chromosome is expressed in the trophectoderm and its derivative tissues. We conclude that random X-chromosome inactivation does not occur in the earliest cell type to differentiate in mouse embryogenesis.

MATERIALS AND METHODS

Mouse stocks and matings

PGK-1A mice were derived from wild-trapped mice from Denmark as described by Nielsen & Chapman (1977). C3H/HeHa, DBA/Ha and random-bred Ha/ICR, all PGK-1B, were from colonies maintained in the Molecular Biology Department or at the West Seneca Production Facility of Roswell Park Memorial Institute. Various matings were constructed to produce female conceptuses heterozygous for both the X-linked PGK-1 and the autosomally-coded glucose phosphate isomerase (GPI). Conceptuses were sexed by PGK-1 phenotype, the double-banded heterozygous females being easily distinguishable from the single-banded hemizygous males and homozygous females.

Sample preparation

Females were bled and conceptuses dissected at 9-5 days post coitum. PGK-1 phenotypes of the pregnant females were verified by electrophoretic analysis of hemolysates. Watchmaker's forceps were used to tear the mural trophoblast tissue and the yolk sac away from the ectoplacental cone on the isolated conceptuses. Parietal endoderm was removed from the mural trophoblast and discarded. Tearing of the allantois separated the fetus from the placenta. Amnion was removed from the fetus and discarded. Fetus, mural trophoblast, yolk sac and placenta were washed twice in 0.16 M-NaCl, coded, and frozen in approximately 5 μl of H$_2$O for 8–20 h before the electrophoretic analysis.

Kidneys isolated from PGK-1A and PGK-1B mice were used in the PGK-1 activity assay and in the gel sensitivity experiments. Kidneys were homogenized to 10 % weight/volume in H$_2$O. Homogenates were centrifuged at 35000 g for
30 min. Supernatants were diluted 13-fold to approximate PGK-1 activity levels found in isolated 9-5-day mural trophoblasts and analyzed on starch gels.

Mural trophoblasts from $P_{gk-1b}^b/P_{gk-1b}^b \times P_{gk-1a}^a/Y$ matings were sexed by PGK-1 phenotype and heterozygous female conceptuses pooled in two separate experiments. In the first experiment, trophoblasts from six female conceptuses were homogenized in 50 $\mu$l H$_2$O, and in the second experiment, mural trophoblasts from four female conceptuses were homogenized in 20 $\mu$l. These pooled samples were assayed for PGK-1 activity, and analyzed for PGK-1 phenotype on starch gels.

**Embryo transfers**

Embryos of 2½ days (8-cell) were flushed from the Fallopian tubes with phosphate-buffered medium of Whittingham & Wales (1969). The embryos were transferred into the lumen of the Fallopian tubes of pseudopregnant day 0 (day of vaginal plug) recipients (Ha/ICR) according to the method of Tarkowski (1959). These recipients were killed approximately 8 days following transfer and the conceptuses dissected into fetus, trophoblast and yolk-sac tissues as described by Frels, Rossant & Chapman (1979).

**PGK assay**

Kinetic assays were performed spectrophotometrically on kidney homogenates and embryonic tissues. The reaction mix contained 0.1 M Tris-HCl buffer pH 7.6, 7 mM MgSO$_4$, 0.5 mM EDTA, 7.2 mM phosphoglycerate, 1.2 mM ATP, 0.12 mM NADH and 7.7 units glyceraldehyde-3-phosphate dehydrogenase. Reactions were followed at 340 nm and were linear for at least 20 min at 37 °C.

**Tissue analysis**

Electrophoretic phenotypes were determined using 12 % starch gels (90 x 70 x 3 mm). Thawed samples were absorbed on to 2 x 4 mm Whatman No. 3 paper wicks and inserted into the gels. Electrophoresis was conducted using a Tris-citrate buffer system at pH 7.0 for approximately 3 h at 150 V (8 mA/gel). PGK-1 migrates anodically and GPI migrates cathodically in this buffer system. After electrophoresis, gels were stained for PGK-1 and GPI. The staining procedure for PGK-1 was a modification of that used by West et al. (1977), with a NADH concentration of 0.38 mM. Samples were scored on a 1- to 5- scale described previously by West et al. (1977) (see Table 2). GPI was stained according to Carter & Parr (1967).
RESULTS

Validity of assumptions

The use of PGK-1 electrophoretic variants as an indicator of X-chromosome expression rests on two assumptions. First, that PGK-1 can be used as a marker for entire X-chromosome function. And second, that the relative histochemical staining intensity of an electrophoretic form corresponds to the proportion of cells with that X chromosome active.

The validity of the first assumption is suggested by the coordinate expression of three X-chromosome-linked genes, PGK-1, hypoxanthine-guanine phosphoribosyl transferase, and glucose-6-phosphate dehydrogenase, in *M. musculus × M. caroli* F₁ hybrid tissue culture clones derived from single cells (Chapman & Shows, 1976).

The validity of the second assumption is suggested by two results. First, PGK-1A and PGK-1B stained in proportion to the percentage of each allelic form in mixtures of kidney homogenates analyzed by starch-gel electrophoresis (West et al. 1977). Second, no significant differences were observed in specific activity measurements between PGK-1A and PGK-1B allelic forms (Table 1).

**PGK-1 activity levels and gel sensitivity**

PGK-1 total activity and specific activity levels for mural trophoblast, yolk sac, placenta and fetus of 9-5-day conceptuses (Table 1b), and for pooled and homogenized mural trophoblasts (Table 1c) are shown in Table 1. Supernatants from 10% kidney homogenates from PGK-1A and PGK-1B mice were diluted 13-fold to approximate mural trophoblast PGK-1 activity levels. These diluted PGK-1A and PGK-1B samples were mixed in various proportions and analyzed on the starch-gel electrophoretic system to determine the percentage of each allelic form that could be detected in a mixture. Both PGK-1A and PGK-1B can be detected when they comprise only 10% of the activity in a mixture of the two forms (Table 2).

**Nonrandom X-chromosome expression in mural trophoblast**

The expression of PGK-1 electrophoretic phenotype was examined in the fetus and the mural trophoblast of 9-5-day conceptuses from a *Pgk-1⁺/Pgk-1⁺ Pgk-1⁺/Y* mating (Fig. 1). Heterozygous PGK-1AB female conceptuses had a mean fetus score of 2.4. Only PGK-1B (score of 1) was observed in the mural trophoblasts of the 31 female conceptuses tested. Male conceptuses had a uniform expression of PGK-1B in both fetus and mural trophoblast tissue. These findings indicate that only Xᵐ is expressed in the mural trophoblasts of these female conceptuses.

Mural trophoblasts from several female conceptuses (four and six) were pooled and analyzed in two experiments in order to increase the sensitivity of the test for possible paternal PGK-1A expression. Only PGK-1B (Xᵐ) expres-
**Table 1.** Mean PGK-1 activity levels and mean specific activity levels for (a) 10% kidney supernatants of PGK-1A and PGK-1B, (b) fetus, mural trophoblast, yolk sac, and placenta of 9-5-day conceptuses, and (c) pooled 9-5-day mural trophoblasts

Protein was determined by the method of Lowrey, Rosebrough, Farr & Randall (1951).

<table>
<thead>
<tr>
<th>Sample</th>
<th>n*</th>
<th>PGK-1 activity (nmole/2 μl)</th>
<th>PGK-1 specific activity (μmole/mg protein/min)</th>
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<td>(a) 10% kidney supernatants</td>
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<td>PGK-1A</td>
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<td>49.8 ± 1.9†</td>
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<td>10</td>
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<td>(b) Conceptus tissues</td>
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<tr>
<td>Fetus</td>
<td>7</td>
<td>13.5 ± 1.7</td>
<td>0.56 ± 0.05</td>
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<td>Mural trophoblast</td>
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<td>Yolk sac</td>
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<td>5.7 ± 1.1</td>
<td>0.54 ± 0.04</td>
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<td>Placenta</td>
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<td>9.3 ± 0.9</td>
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<td>(c) Pooled mural trophoblasts</td>
<td>2</td>
<td>22.8 ± 4.7</td>
<td>0.47 ± 0.07</td>
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* Number of samples. † Mean ± S.E.
† Pool of three yolk sacs. § Activity applied to gels.

**Table 2. Gel sensitivity**

PGK-1 scores and average scores for mixtures of PGK-1B and PGK-1A kidney supernatants. Each mixture contains the same amount of PGK-1 activity as an average 9-5-day mural trophoblast. Scores are based on a scale of 1-5. A score of 1 is given if only PGK-1B is seen, 2 if PGK-1B is greater than PGK-1A, 3 if both PGK-1A and PGK-1B are expressed in approximately equal proportions, 4 if the PGK-1A is greater than PGK-1B, and 5 if only PGK-1A is seen.

<table>
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<tr>
<th>Mixture</th>
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PGK-1 phenotypes from PGK-1B/PGK-1B × PGK-1A mating

Fig. 1. Distributions and mean scores of PGK-1 phenotypes for mural trophoblast (top panels) and fetus (bottom panels) from 9.5-day conceptuses. Conceptuses were from a PGK-1B/PGK-1B × PGK-1A mating and consisted of heterozygous PGK-1A/PGK-1B females (panels on left) and hemizygous PGK-1B males (panels on right).

Expression was observed in these experiments as well, even after the enzyme bands had greatly overstained. This limits possible X<sup>m</sup> expression to less than 0.5% in a mural trophoblast tissue.

Only two heterozygous female conceptuses were analyzed in the reciprocal F<sub>1</sub>mating. Both showed a score of 5 (PGK-1A) in their mural trophoblasts. These findings also suggest that the X chromosome expressed in the mural trophoblasts of heterozygous females is independent of the PGK-1 alleles. The results of reciprocal backcross matings (below) provide additional evidence for this conclusion.

Expression of only the maternal PGK-1 allele in the mural trophoblasts of these F<sub>1</sub> females could be due to nonrandom X-chromosome expression, selection of mural trophoblast cells expressing the maternal X chromosome, or contamination of the mural trophoblast by enzyme from maternal tissues. The mural trophoblast abuts directly against maternal decidual tissue and it would be the most likely tissue to show cell selection by the maternal environment, or contamination by enzyme from maternal tissues.
Maternally derived X chromosome in the mouse

Cell selection

Reciprocal backcross matings were constructed to test for cell selection and maternal enzyme contamination in the mural trophoblast. Heterozygous PGK-1AB females were mated to PGK-1A and to PGK-1B males. The mural trophoblasts of PGK-1AB conceptuses should express the PGK-1A and PGK-1B allelic forms in approximately equal proportions, i.e. a score of 3, if cell selection or maternal tissue contamination were significant factors determining the mural trophoblast phenotype. Conversely, selective inactivation of Xp in the mural trophoblasts of heterozygous females from the reciprocal backcross matings would result in PGK-1 expression similar to that seen in the F₁ matings.

Results from the backcross matings are shown in Fig. 2: Fig. 2(a), the backcross to PGK-1A males and Fig. 2(b), the backcross to the PGK-1B males. The mean score for distributions of the heterozygous PGK-1AB fetuses are the same (3-5) in both matings. However, the distributions of the mural trophoblast scores of these heterozygous conceptuses are completely opposite. Mural trophoblasts from the backcross to the PGK-1A male (Fig. 2a) have a mean score of 1-3 (15 of 19 have a score of 1), whereas mural trophoblasts from the PGK-1B male backcross have a mean score of 4-7 (25 of 35 have a score of 5). Thus the mural trophoblasts of heterozygous conceptuses do not show random expression of the PGK-1 alleles in the reciprocal backcross matings as would be expected if maternal selection or uterine contamination were exerting a significant effect in determining X-chromosome expression. These findings suggest a selective inactivation of the paternally derived X chromosome in the mural trophoblast.

The yolk sacs of the heterozygous conceptuses in Fig. 2 have a mean PGK-1 score of 1-8 in matings to the PGK-1A males (Fig. 2a), and a mean PGK-1 score of 4-2 in matings to the PGK-1B males (Fig. 2b). Thus, the unbalanced mosaic pattern (Xᵐ > Xᵖ) observed in the yolk sac of these 9-5-day conceptuses, agrees with previously published data of West et al. (1977) for 13-day embryos. An unbalanced PGK-1 mosaic pattern of Xᵐ > Xᵖ was also observed for placenta. However, definitive conclusions concerning the significance of preferential X-chromosome expression in this tissue is difficult because the placenta is composed of a mixture of cell types.

All conceptuses derived from the F₁ and from the backcross matings were heterozygous for the autosomal locus GPI. Where the GPI phenotypes could be reliably scored (875 of 991 or 88% of the conceptus tissues), equal expression of the two alleles was observed. This suggests that paternal, as well as maternal, autosomes are functioning in all conceptus tissues studied.
Fig. 2. Distributions and mean PGK-1 scores for placentas, yolk sacs, mural trophoblasts, and fetuses of PGK-1A, PGK-1A/PGK-1B, and PGK-1B conceptuses from the backcross matings. In Fig. 2(a), the phenotypic PGK-1A conceptuses consists of hemizygous \(Pgk-1^a/\ Y\) males and homozygous \(Pgk-1^a/Pgk-1^a\) females, the PGK-1A/PGK-1B conceptuses are heterozygous females, and the PGK-1B conceptuses are hemizygous \(Pgk-1^b/\ Y\) males. The one mural trophoblast with a score of 5 is a misclassification. Scoring misclassifications occurred when the electrophoretic migration of a single-banded phenotype was intermediate to PGK-1A and PGK-1B on the same gel. Scoring in these cases may result in the assignment of the incorrect PGK-1 phenotype to that particular sample. Retesting the sample was not always possible after the sample was uncoded and recorded because of the small amount of tissue. Scoring misclassifications occurred only 1% of the time. Phenotype groups are the same in Fig. 2(b), except the homozygous females are PGK-1B/PGK-1B.
Maternally derived X chromosome in the mouse

Embryo transfers

Embryo transfers were conducted as a more stringent test of uterine selection on the mural trophoblast. Embryos of 2½ days (8-cell) from a $P_gk-1^a/P_gk-1^a \times P_gk-1^b/Y$ mating were transferred to the Fallopian tubes of pseudopregnant $P_gk-1^b$ recipients.

Kelly (1977) has shown that trophectoderm differentiation has not occurred by the 8-cell stage in the mouse. Thus trophectoderm differentiation and development of the transferred PGK-1AB female and PGK-1A male embryos occurs in a PGK-1B recipient female. If cell selection were responsible for $X^m$ expression in the mural trophoblast, we would expect expression of PGK-1B in the mural trophoblasts of the transferred PGK-1AB females. On the other hand, expression of the X chromosome of the genetic mother would result in expression of PGK-1A in these transferred females. Tissue contamination by the uterine environment would result in expression of PGK-1B in the mural trophoblasts of the transferred PGK-1A males.

Results for the embryo transfers are shown in Fig. 3. Heterozygous PGK-1AB female conceptuses have a mean fetus score of 3-2. However, 14 of 16 trophoblast scores are 5 (PGK-1A), and 2 of 16 show a score of 4 (PGK-1A > PGK-1B). Male conceptuses (PGK-1A) from the embryo transfers show only PGK-1A

Fig. 3. Distributions and mean PGK-1 scores for yolk sacs, trophoblasts, and fetuses from embryo transfer conceptuses. PGK-1A males are in the panels on the left, and PGK-1A/PGK-1B females in the panels on the right.
(score of 5) in their trophoblasts and yolk sacs. Thus, the X chromosome from the genetic mother (PGK-1A) is expressed in the trophoblast, regardless of having differentiated and developed in a PGK-1B maternal environment.

Estimate of contamination

Mural trophoblast tissues could be potentially contaminated by enzyme from either fetal or uterine tissues or maternal blood cells. Contamination of mural trophoblast by fetal tissues was tested in the heterozygous conceptuses from the \( F_1 \) matings (Fig. 1). Mural trophoblast scores in these matings were uniformly single-banded indicating no significant contamination of the mural trophoblast by fetal tissues.

A direct estimate of maternal tissue contamination of trophoblast samples (Fig. 2) is provided by the mural trophoblast scores of the PGK-1A and the PGK-1B conceptuses in the heterozygous uterine environments of the backcross matings. Fourteen of 192 (7.3%) of the PGK-1A and PGK-1B conceptuses have a double-banded phenotype in their mural trophoblasts, providing an estimate that maternal tissue contamination occurs less than 8% of the time. Furthermore, when contamination does occur, it contributes only a minor proportion of the PGK-1 phenotype seen in all mural trophoblasts. Thus, expression of the PGK-1 phenotype is intrinsic to the mural trophoblast cells.

**DISCUSSION**

Results of these studies show nonrandom expression of maternal PGK-1 in the mural trophoblast of the mouse at 9.5 days post coitum. This nonrandom expression could be a result of nonrandom X-chromosome inactivation, or a result of selection of X\(^m\)-active cells in the mural trophoblast.

We tested the possibility of the uterine environment exerting selection pressure on the mural trophoblast cells by doing backcross matings and embryo transfers. Mural trophoblasts of heterozygous conceptuses from reciprocal backcross matings expressed either a single-banded maternal PGK-1 or a predominately maternal, X\(^m\) > X\(^p\) PGK-1 phenotype. The frequency of embryos expressing some paternal PGK-1 (26%) was greater than the number predicted by maternal tissue contamination alone (8%). This could represent some selection for cells expressing the paternal X chromosome but the frequency is far lower than the balanced expression we had predicted for selection. Sample contamination during the electrophoretic assay may account for part of the double-banded expression in these samples. This could occur if mural trophoblast samples from heterozygous conceptuses were adjacent to samples that contained the paternal PGK-1 during the electrophoretic analysis. This occurred frequently with backcross matings where the coded fetal and extra-embryonic membrane samples were analyzed in the same gel. All fourteen mural trophoblast samples showing some expression of X\(^p\) were located next to potential sources of contamination.
during the electrophoretic assay. Unfortunately, trophoblast samples available for reassay were insufficient to verify this possibility.

A more stringent test of selection was provided by the embryo transfer experiments. PGK-1AB heterozygous embryos from PGK-1A mothers were transferred to a uterus of a host female that was PGK-1B. Even in these circumstances the trophoblast of the heterozygous female conceptuses expressed the PGK-1A of the genetic mother and not the PGK-1B of the host. These findings indicate that the phenotype of the uterine environment has no effect upon X-chromosome expression in mural trophoblast. Taken together these findings and the results from reciprocal backcrosses suggest that the expression of $X^m$ in trophoblast is not a consequence of selecting against cells that express an X chromosome that is genetically different from the maternal genome.

Pooling experiments indicated that less than 0.5% of mural trophoblast expresses detectable $X^p$ PGK-1 phenotype. We conclude that most, if not all, cells of the mural trophoblast express $X^m$. A companion study by Frels et al. (1979) showed similar results for the chorionic ectoderm, another trophectoderm derivative. These findings and those of West et al. (1977) on the yolk-sac endoderm suggest that $X^m$ is preferentially active in the first two cell types that differentiate during mouse development, the trophectoderm and primitive endoderm.

These data indicate that $X^m$ and $X^p$ are different from each other in early embryos and that this difference results in inactivation of $X^p$ in trophectoderm and primitive endoderm. Random inactivation in the fetus suggests that the $X^m$ and the $X^p$ difference disappears or is not recognized in the progenitor cells of the fetus.

Injection chimera experiments by Gardner & Lyon (1971) indicate that at least some of the cells of the inner cell mass have not undergone X-chromosome inactivation at 4-5 days. Previously, Takagi (1974) demonstrated asynchronous replication as early as 40 cells in mouse embryos. More recently, studies of asynchronously replicating X chromosomes in 3-5-day mouse blastocysts have shown that the asynchronously replicating X was $X^p$ (Takagi, Wake & Sasaki, 1978). These findings coupled with our results support the notion that X-chromosome inactivation may accompany differentiation in the mouse trophectoderm.

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REFERENCES


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