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Parental imprinting studied by allele-specific primer extension after PCR: Paternal X chromosome-linked genes are transcribed prior to preferential paternal X chromosome inactivation

(quantitative PCR/RNA/development/*Hprt* gene/*Pgk-1* gene)

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ABSTRACT **The preferential inactivation of the paternal X chromosome in extraembryonic cells during early mouse development is an example of parental imprinting, but it has not been studied at the transcriptional level because standard methods of measuring RNA levels do not allow detection of allele-specific RNAs in individual early embryos. We sought to determine whether the paternal allele of the X chromosome-linked gene for 3-phosphoglycerate kinase 1 (*Pgk-1*), which is located very near the center of X chromosome inactivation, is transcribed prior to differentiation of extraembryonic lineages. Previous reports indicated that in heterozygous embryos there is a delay in the appearance of the phosphoglycerate kinase 1 allozyme encoded by the paternal X chromosome until 2 days after the appearance of the corresponding maternal allozyme. We report results obtained by use of a reverse transcription/PCR-based method which allows the quantitative measurement of allele-specific RNA. The assay is sensitive enough for the quantitative analysis in single embryos of allele-specific transcripts differing by only one nucleotide. We have used this assay to analyze mouse embryos heterozygous at the *Pgk-1* and *Hprt* [hypoxanthine (guanine) phosphoribosyltransferase] loci, and we find that individual 8-cell and blastocyst embryos express both *Hprt* and *Pgk-1* paternal transcripts, as do pooled 2- to 4-cell embryos. These results are discussed in view of the apparent temporal delay in paternal expression of the *Pgk-1* gene at the enzyme level.**

In the first 24–48 hr after fertilization, the mouse embryo undergoes massive changes in RNA and protein contents (1, 2). As part of this reorganization, maternal RNAs are thought to become highly degraded, including the mRNA transcribed from *Hprt*, the hypoxanthine (guanine) phosphoribosyltransferase (HPRT) gene (1), and expression of embryonic genes is believed to begin. Whether paternal X chromosome-linked genes are expressed at this time is relevant to models of parental imprinting because at the morula–blastocyst stage (3.5–4.5 days of development) the paternally derived X chromosome undergoes preferential inactivation in extraembryonic tissues, as evidenced both by cytological studies (3) and by analysis of electrophoretic variants of X-linked 3-phosphoglycerate kinase 1 (PGK-1) (4, 5). Previous studies have shown two paternally derived X-linked enzymes, α-galactosidase and HPRT, to be present prior to preferential paternal X chromosome inactivation (6–9). However, a different picture was obtained from studies on the X-linked *Pgk-1* gene, which lies close to the X chromosome controlling element (*Xce*) (10, 11), in the region of the center of X chromosome inactivation (12). No zygotic expression of the paternal PGK-1 allozyme was seen until day 6 or 7, 2 days after detection of the allozyme encoded by the maternal X chromosome (13–15). This led to the idea that PGK-1 expression from the paternal X chromosome is delayed until the developmental stage at which X chromosome inactivation occurs (14), as may be true for the X-linked *Xist* gene, which also maps in the vicinity of the X inactivation center (16, 17). The notion that the X inactivation center could influence chromatin structure and/or expression of the nearby *Pgk-1* locus is rendered more likely by the observation that *Xce* alleles affect, up to 2-fold, the levels of PGK-1 in the oocyte (18), although there is no effect of *Xce* on the temporal delay in the appearance of paternal PGK enzyme.

Until recently, it has been impossible to measure specific mRNA levels of X-linked genes to ascertain how soon after fertilization the paternal X chromosome begins to be expressed. We report here that reverse transcription of RNA followed by PCR (RT-PCR) (19) combined with allele-specific detection can be used to assay staged preimplantation embryos for the presence of paternally derived X-linked *Pgk-1* and *Hprt* transcripts. Using a single nucleotide primer extension (SNuPE) assay (20) that allows sensitive quantitative measurement of the relative amounts of allelic transcripts differing by one nucleotide in less than 1 ng of total RNA (21), we have probed for the presence of paternal transcripts of the *Pgk-1* and *Hprt* genes in 2- to 4-cell, 8-cell, and blastocyst female mouse embryos. We find, using embryos heterozygous at the *Pgk-1* and *Hprt* loci, that both *Pgk-1* and *Hprt* RNAs are transcribed from the paternal X chromosome in all cases.

MATERIALS AND METHODS

Genetic Crosses and Embryo Dissection. Female embryos heterozygous for *Hprt* and *Pgk-1* were obtained from reciprocal crosses of either congenic females (*Hprt^a Pgk-1^a/Hprt^a Pgk-1^a*) crossed with (C3H × C57BL/6) F_1 males (*Hprt^b Pgk-1^b*/Y) or Ha/ICR females (*Hprt^b Pgk-1^b/Hprt^b Pgk-1^b*) crossed with (C3H × C57BL/6Ros) F_1 males (*Hprt^a Pgk-1^a*/Y). The *Hprt^a* and *Pgk-1^a* alleles were originally identified in wild-derived mice and transferred to both the C3H/HeHa and C57BL/6 inbred strain backgrounds by more than 12 generations of backcross matings (22). Females were treated with 5 international units of pregnant mares' serum, injected 48 hr later with 5 international units of human chorionic gonadotropin, and mated with males on the afternoon of the same day. Embryos were flushed from fallopian tubes on the afternoon of the second day (2- to 4-cell stage), the morning of the third day (8-cell stage), and from the uterus the morning

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Abbreviations: PGK-1, X chromosome-linked 3-phosphoglycerate kinase; HPRT, hypoxanthine (guanine) phosphoribosyltransferase; SNuPE, single nucleotide primer extension; RT-PCR, reverse transcription followed by PCR.

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Table 1. Primers used for RT-PCR

Primer	Sequence	Product size, bp	Ref(s).
PGK442	GTAAAGGCCATTCCACCACCAA	309	24
PGK750	AGCTGAGCCGGCCAAAATTGAT		
HPRT41	GGCTTCCTCCTCAGACCGCTTT	702	26, 27
HPRT742	AGGCTTTGTATTTGGCTTTTCC		

of the fourth day after mating (blastocyst stage). The embryos were flushed with sterile phosphate-buffered saline (0.01 M sodium phosphate, pH 7.2/0.16 M NaCl) with bovine serum albumin at 1 mg/ml. The embryos were separated from cellular debris by repeated transfer in small volumes to culture dishes with 1 ml of fresh buffer. Embryos were then added individually or in pools of 5 (blastocyst stage), 10 (8-cell stage), or 50 (2- to 4-cell stage) to 25 μl of RNAzol (Cinna Biotecz Laboratories, Friendswood, TX) within 30 min of collection, and stored at −70°C for up to 2 weeks prior to RNA purification.

RNA Purification and RT-PCR. Additional RNAzol was added to the samples to bring them to a final volume of 100 μl, and RNA purification was carried out as described (21). Briefly, chloroform (10 μl) was added, and the solution was placed on ice for 5 min after mixing with a Vortex mixer. After centrifugation for 15 min at 4°C in a Microfuge, the aqueous phase was transferred to a fresh Eppendorf tube, and an equal volume of isopropyl alcohol was added together with 20 μg of glycogen as a carrier. After 15 min at 4°C, the samples were centrifuged as above. The precipitate was stored at −70°C in 75% (vol/vol) ethanol.

RT-PCR was carried out as previously described (19, 21, 23), with primers designed to span introns (24–26). Each RNA sample was divided in half and reverse transcribed in 20 μl of RT mix [10 mM Tris·HCl, pH 8.3/50 mM KCl/5 mM $MgCl_2$/1 mM dNTPs/RNasin (Promega) at 2 units/ml] containing Moloney murine leukemia virus reverse transcriptase (BRL) at 2.5 units/μl and either the *Pgk-1* or *Hprt* downstream primer (1 μM) (see Table 1). The tubes were incubated at 42°C for 15 min and 99°C for 5 min and equilibrated at 50°C; then 80 μl of PCR mix containing the appropriate upstream primer was added to each tube, and amplification was carried out for 40–45 cycles. Conditions of PCR were, for *Pgk-1*, 95°C for 1 min (first cycle, 2 min), 59°C for 1 min; for *Hprt*, 95°C for 1 min (first cycle, 4 min), 49°C for 2 min, 72°C for 1 min. The $MgCl_2$ concentration was 2 mM and 1 mM for PCR with *Pgk-1* and *Hprt* primers, respectively.

The amplified products were extracted with phenol, precipitated with ethanol, and gel-purified by use of Gelase (Epicentre Technologies, Madison, WI) (21). After precipitation with ethanol and resuspension in 10 mM Tris·HCl, pH 8/1 mM EDTA, the samples were assayed by the SNuPE assay described below.

Quantitative SNuPE Assay. The quantitative SNuPE assay was done as previously described (21). In the case of samples amplified for *Pgk-1* cDNA, the SNuPE assay was done with [^{32}P]dCTP or [^{32}P]dATP; for amplified products of *Hprt* transcripts [^{32}P]dCTP or [^{32}P]dGTP was used. Each 10-μl reaction mix contained (in addition to [^{32}P]dNTPs) 2–5 mM $MgCl_2$, 10 mM Tris·HCl at pH 8.3, 50 mM KCl, 0.001% gelatin, the appropriate SNuPE primer (1 μM), approximately 5 ng of amplified PCR product, and 0.75 unit of *Taq* DNA polymerase (Cetus). The SNuPE primers used are given in Table 2. Samples were incubated at 95°C for 1 min, 42°C for 2 min, and 72°C for 1–2 min for one cycle in an Ericomp thermal cycler. After denaturing polyacrylamide gel electrophoresis, the ratio of the paternal to the maternal allele was determined as described (21).

Table 2. Primers used for quantitative SNuPE

Primer	Sequence	Mismatch detected	Base position	Ref.
PGK471	TCCGAGCCTCACTGTCCA	A vs. C	489	28
HPRT109	CGCTGGGACTGCGGGTCG	C vs. G	91	27

RESULTS

Quantitative SNuPE Assay for Allele-Specific RNA. To assay for the relative amount of each allelic transcript, we used the PCR-based quantitative SNuPE assay (20, 21). As outlined in Fig. 1, after RNA isolation the first step involves the amplification by RT-PCR of both alleles of each transcript with primers flanking the allelic differences. After gel purification of the amplified products, the relative contribution of each allelic transcript to the amplified product is assayed by extension by a single nucleotide of a primer whose 3′ end is just 5′ to the position of the allelic difference. In two separate reactions, the [^{32}P]dNTP is added that corresponds to the appropriate base for each of the two alleles present. After denaturing gel electrophoresis, the ratio of the radioactivity in the products of the two reactions is determined.

The coding regions of the *Pgk-1*^{a} and *Pgk-1*^{b} alleles differ by only a single base, a C-to-A transversion at position 490 of the cDNA sequence, resulting in a Thr to Lys change at amino acid position 155 of PGK-1 (28). The *Hprt*^{a} and *Hprt*^{b} alleles differ at four positions in the cDNA sequence, including a G-to-C transversion at position 91 that results in a change from Ala to Pro (27). It had previously been shown, by comparison of incorporation of [^{32}P]dCTP to that of [^{32}P]dATP in the SNuPE assay of amplified products, that *Pgk-1*^{a} transcripts can be accurately measured relative to *Pgk-1*^{b} transcripts, even when the minor component is 0.1% of the total (21). To extend these findings to the accurate measurement of *Pgk-1*^{b} templates in an excess of *Pgk-1*^{a} templates, and of the *a* and *b* allelic variants of *Hprt*, reconstruction experiments were done as shown in Fig. 2. It

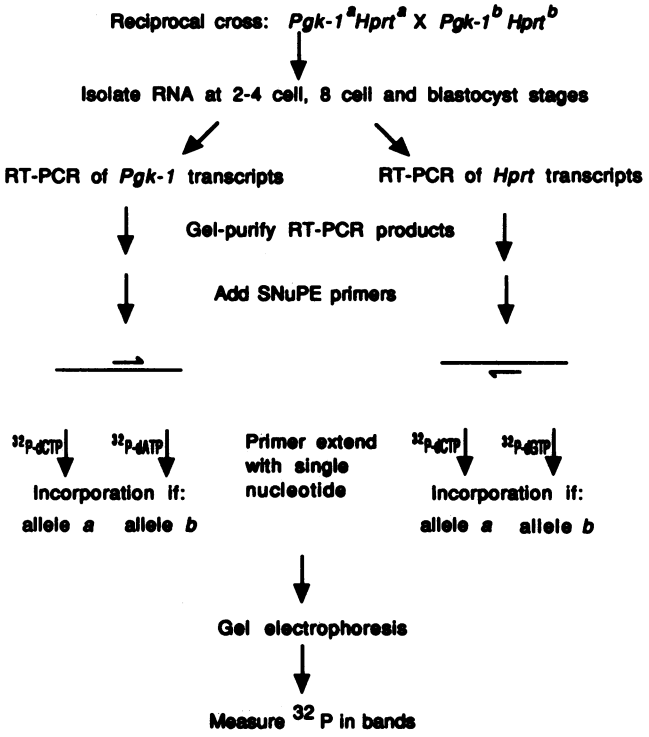

FIG. 1. Outline of experiment. Details of the crosses and of the quantitative RT-PCR SNuPE assay are given in *Materials and Methods* and *Results*. The 5′ to 3′ orientation of the *Pgk-1* and *Hprt* SNuPE primers is indicated by the direction of the arrows.

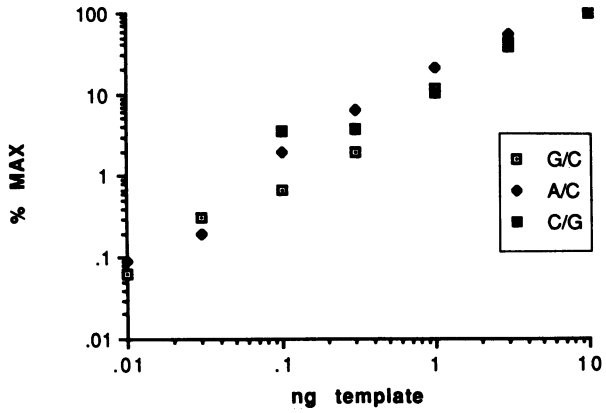

FIG. 2. Reconstruction experiment showing quantitative detection of G/C, A/C, and C/G nucleotide differences. Increasing amounts of the template being measured were added together with 10 ng of the corresponding template differing by one base pair. For the A/C nucleotide difference the templates used were *Pgk-1*^{a} and *Pgk-1*^{b} 309-base-pair amplified products. For the C/G and G/C nucleotide difference the templates used were PCR products of *a* and *b* variants of the *Hprt* gene. In each case, background was subtracted, then values were normalized to the signal obtained when 10 ng of the variable template was added (% max).

can be seen that quantitative measurements can be obtained for C/G, G/C, and A/C nucleotide differences, even when the minor component is present as 0.01 of the total.

Design of Experiment. Fig. 1 shows a schematic diagram of the experiments done. Reciprocal crosses were done between homozygous female mice and hemizygous males (*Hprt*^{a} *Pgk-1*^{a}/*Hprt*^{a} *Pgk-1*^{a} × *Hprt*^{b} *Pgk-1*^{b}/Y or *Hprt*^{b} *Pgk-1*^{b}/*Hprt*^{b} *Pgk-1*^{b} × *Hprt*^{a} *Pgk-1*^{a}/Y). Embryos were collected either individually or in pools, without separate determination of genotypic sex. Thus, individual samples were either heterozygous females or hemizygous males containing the maternal X chromosome, while pooled samples were expected to include both male and female embryos. Embryos were collected at the 2- to 4-cell, 8-cell, and blastocyst stages, and their RNAs were analyzed for paternal and maternal transcripts by RT-PCR followed by allele-specific primer extension of the amplified products as described above. After gel electrophoresis, a comparison of the radioactively labeled primers revealed the relative amount of each transcript initially present.

2- to 4-Cell and 8-Cell Embryos. Fig. 3*A* shows the results for three individual embryos assayed for *Hprt*^{a} and *Hprt*^{b} transcripts at the 8-cell stage. Two of the three embryos have no significant paternal transcripts (lane 4 vs. lane 3; lane 5 vs. lane 6); it is most likely that these embryos are males, even though we had no means of positively verifying the sex of each embryo at the RNA level. For the third embryo, shown in the two left lanes, the paternal transcript is clearly present (lane 2 vs. lane 1). Fig. 3*B* shows the results obtained for two individual 8-cell embryos (four left lanes) and one pool of 49 2- to 4-cell embryos (two right lanes) assayed for *Pgk-1* transcripts. One embryo has no detectable paternal *Pgk-1* RNA (lane 1 vs. lane 2); the other clearly shows its presence (lane 3 vs. lane 4). In addition, lanes 5 and 6 show the presence of paternal *Pgk-1* transcripts at the 2- to 4-cell stage.

The pooled 2- to 4-cell embryos were assayed for *Hprt* and *Pgk-1* transcripts in triplicate, and it was found that paternal transcripts were present as 10–20% of the total in each case. Thus, both *Hprt* and *Pgk-1* paternal transcripts are present even at this early stage, as had been suggested for *Hprt* by previous isozyme studies (9).

Individual Embryos at the 8-Cell Stage. Results obtained with individual 8-cell embryos of both reciprocal crosses are summarized in Fig. 4. It is apparent that there are two classes of embryos, those without *Hprt* or *Pgk-1* paternal RNA and those with paternal RNA from both of these genes. The lack

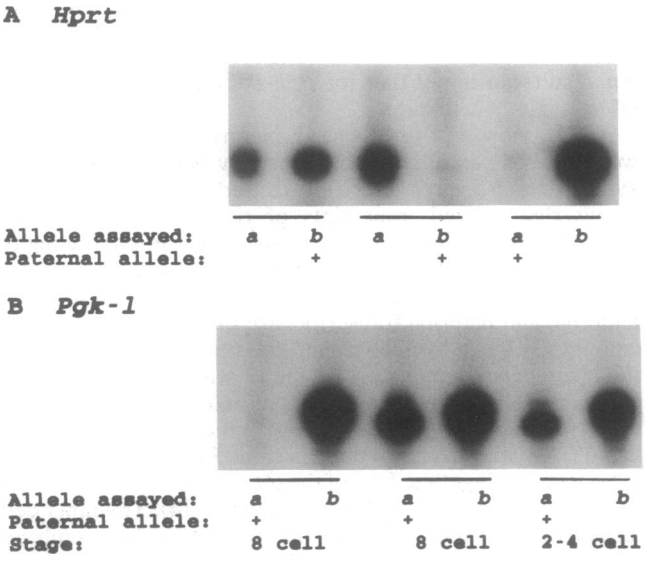

FIG. 3. Quantitative RT-PCR SNuPE assay of embryos for paternal and maternal *Hprt* and *Pgk-1* transcripts. (*A*) Assay of three individual 8-cell embryos for *Hprt*^{a} and *Hprt*^{b} transcripts. The first two embryos were derived from crosses between females homozygous for the *a* allele and males hemizygous for the *b* allele; the third embryo was derived from a reciprocal cross. (*B*) Assay of two individual 8-cell embryos and a pool of 2- to 4-cell embryos for *Pgk-1*^{a} and *Pgk-1*^{b} transcripts. In each case embryos were derived from crosses between females homozygous for the *b* allele and males hemizygous for the *a* allele. Unsexed female (XX) and male (XY) embryos were assayed; for each sample, the paternal allele potentially contributed by the male is indicated by a +.

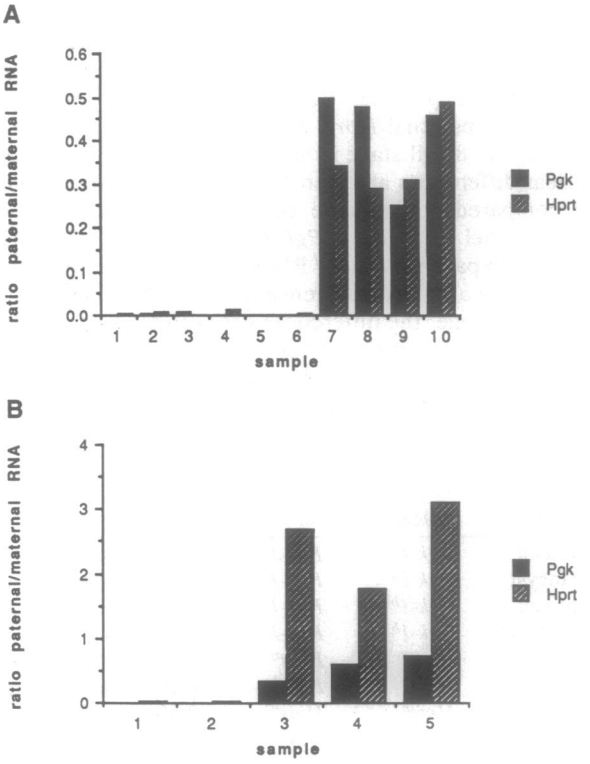

FIG. 4. Ratio of paternal to maternal transcripts in individual 8-cell embryos. (*A*) Female *Pgk-1*^{b} *Hprt*^{b}/*Pgk-1*^{b} *Hprt*^{b} × male *Pgk-1*^{a} *Hprt*^{a}/Y. (*B*) Female *Pgk-1*^{a} *Hprt*^{a}/*Pgk-1*^{a} *Hprt*^{a} × male *Pgk-1*^{b} *Hprt*^{b}/Y.

of signal from the paternal allele in ≈½ of the embryos provides a negative control, as male embryos would not have any paternal X-linked genes. Thus there is no significant contamination of the embryos by any external source of RNA. Contamination by genomic DNA can be ruled out because primers for both genes span introns, and control experiments (not shown) confirm that DNA gives no signal for either RNA assayed.

The finding of a clear *Pgk-1* paternal signal correlated with *Hprt* transcripts shows that spliced paternal *Pgk-1* RNA is indeed present in female embryos. Thus, in contrast to previous work on enzyme expression, our experiments demonstrate that *Pgk-1* transcripts are present prior to preferential paternal X chromosome inactivation (see *Discussion*).

Summary of Results with Individual and Pooled Embryos. Table 3 summarizes results obtained with all embryos, (individual and pooled) of both reciprocal crosses, at the 8-cell and blastocyst stages. It can be seen that paternal *Pgk-1* and *Hprt* transcripts are present in all cases. The paternal/maternal ratio decreases by the blastocyst stage in all cases but one (male $Hprt^a$/female $Hprt^b$). Using Wilcoxen rank-sum tests for statistical significance, we found that for male $Hprt^b$/female $Hprt^a$, the paternal-to-maternal ratio in blastocysts decreases to 30% of the 8-cell stage value ($P = 0.019$), while for $Pgk\text{-}1^a/^b$ and $Pgk\text{-}1^b/^a$, the blastocyst values are 17% ($P = 0.002$) and 18% ($P = 0.051$) of the 8-cell stage values, respectively. This reduction in relative paternal transcripts is expected by the blastocyst stage because of inactivation of the paternal X chromosome in extraembryonic cells.

Additionally, a clear trend is seen for higher relative levels of paternal *Hprt* transcripts than of *Pgk-1*. There is a difference between the relative level of paternal $Hprt^b$ transcripts vs. $Pgk\text{-}1^b$ transcripts at the 8-cell and blastocyst stages (P = 0.002 and 0.052, respectively), as well as a difference between relative levels of paternal $Hprt^a$ and $Pgk\text{-}1^a$ transcripts at the blastocyst stage ($P = 0.005$). In these cases the relative level of paternal RNA for *Hprt* ranges from 3- to 6-fold higher than that for paternal *Pgk-1* RNA. Only when relative amounts of paternal $Hprt^a$ and $Pgk\text{-}1^a$ transcripts are compared at the 8-cell stage is no difference seen ($P = 0.556$).

Clear differences are seen as well when reciprocal alleles are compared at the same stage. At the 8-cell stage, the relative level of paternal $Pgk\text{-}1^b$ RNA seems only slightly higher than paternal $Pgk\text{-}1^a$ RNA ($P = 0.09$), while the same cross shows a 10-fold difference for *Hprt* ($P = 0.002$). At the blastocyst stage, the differences between the two alleles are not statistically significant for *Pgk-1*, perhaps because the values are low for both alleles, but for *Hprt* the 2-fold difference seen is significant ($P = 0.028$).

Table 3. Ratio of paternal to maternal transcripts

Stage	Paternal allele	Maternal allele	Ratio	*n*
Eight-cell	$Pgk\text{-}1^a$	$Pgk\text{-}1^b$	0.32 ± 0.05	11 (6)
Blastocyst	$Pgk\text{-}1^a$	$Pgk\text{-}1^b$	0.054 ± 0.01	6 (2)
Eight-cell	$Pgk\text{-}1^b$	$Pgk\text{-}1^a$	0.66 ± 0.19	8 (3)
Blastocyst	$Pgk\text{-}1^b$	$Pgk\text{-}1^a$	0.12 ± 0.10	4 (2)
Eight-cell	$Hprt^a$	$Hprt^b$	0.32 ± 0.03	7 (4)
Blastocyst	$Hprt^a$	$Hprt^b$	0.30 ± 0.04	6 (2)
Eight-cell	$Hprt^b$	$Hprt^a$	2.30 ± 0.21	8 (3)
Blastocyst	$Hprt^b$	$Hprt^a$	0.67 ± 0.05	3 (1)

Ratios are mean ± SEM; means shown were calculated from values obtained for pooled samples and individual female embryos. The number of individual embryos is shown in parentheses. The values for each pooled sample were doubled because ½ the embryos were assumed to be males.

DISCUSSION

We have demonstrated by SNuPE assay of individual embryos that paternally derived RNA for two X-linked genes, *Hprt* and *Pgk-1*, is present in 2- to 4-cell, 8-cell, and blastocyst mouse embryos. Approximately half of the individual embryos assayed contained no significant paternal transcripts from these genes, as expected for male embryos (Fig. 4). The concomitant assay for *Pgk-1* and *Hprt* transcripts in the same sample provided a positive control, as paternal *Hprt* enzyme is known to be present by the 8-cell stage.

Models favored prior to this work were based on an apparent delay in expression of paternally derived PGK-1 until about the time of random X chromosome inactivation in the inner cell mass (13, 15). The models suggested a limited spread of a chromatin modification from the X inactivation center to the nearby *Pgk-1* locus but not to the more distant genes for α-galactosidase and HPRT. Our work rules out the possibility that a paternal imprint completely prevents transcription of the *Pgk-1* gene. However, we cannot conclude that there is no position effect, as paternal *Pgk-1* expression is generally lower than that of *Hprt*, which is more distal to the center of X chromosome inactivation (see Table 3).

Particularly suggestive is the finding that for *Pgk-1* the ratio of paternal to maternal transcripts is less than one in both reciprocal crosses at the 8-cell stage, prior to X chromosome inactivation. This nonreciprocal effect is not seen for *Hprt* transcripts at the 8-cell stage; it is observed only at the blastocyst stage, when extraembryonic cells have inactivated the paternal X chromosome. The relative transcript levels we observe for *Pgk-1* at the 8-cell stage may be due to a combination of factors, and we cannot at the present time distinguish between them. These include the following: (*i*) the rates of transcription, (*ii*) the time required to process the transcript, (*iii*) the half-life of the spliced RNA, (*iv*) the levels of preovulation mRNA that persist during early cleavage, and (*v*) the relative effects of differences in chromatin structure that occur between sperm- and oocyte-derived genes. These factors may differ not only between the *Pgk-1* and *Hprt* gene products but also between alleles of the same gene. However, our results do imply that for *Pgk-1* there is some parental effect at the 8-cell stage.

At first view, our results seem inconsistent with previous work showing an absence of paternally derived PGK-1 isozymes until 6-7 days after fertilization (13, 15). Our work shows that spliced transcripts of the paternal allele are present, beginning at the 2- to 4-cell stage and continuing to the blastocyst stage. Moreover, there seems to be no large reservoir of maternal RNA for either *Pgk-1* or *Hprt*, since maternal and paternal transcripts are present in comparable amounts by the 2- to 4-cell stage. The simplest explanation of the discrepancy would be that the relative level of paternal allozyme remains below the limits of detection of the PGK-1 assay [the reported limit of allele-specific isozyme detection is 2–3% (13, 15)]. At day 4 there is some PGK remaining from the oocyte, as less than 50% of the total PGK-1 activity seems to be due to new synthesis by the embryo (14). The inner cell mass makes up only ≈20% of the total cells of the embryo (29), and the remainder of cells are extraembryonic, expressing only the maternal allozyme. In addition, we find (see Table 3) that paternal transcripts may be one third as abundant as maternal transcripts. Therefore, paternal PGK-1 activity might well approach the limits of detection of the assay ($0.5 \times 0.3 \times 0.2 = 0.03$).

While this explanation may reconcile our results for day 4 blastocysts, it does not easily explain the delay in enzyme detection until day 6 or 7 (13, 15). Pravtcheva *et al.* (15) suggest that some embryos begin expressing X-linked paternal PGK-1 on day 6, but that not all embryos do so until day 7, even though an autosomally located *Pgk-1* transgene

begins expression as early as day 4. Of interest also is a study by S. S. Tan and P. P. L. Tam (personal communication), who examined an X-linked transgene for β-galactosidase, using a cytological staining procedure that shows enzyme activity in single blastomeres. They found a chronological difference in the onset of *lacZ* expression depending on the parental origin of the transgene. Blue cells indicative of β-galactosidase activity were seen at the 4-cell stage if the transgene was maternally derived, but not until the 10- to 20-cell stage if paternally derived.

An intriguing question is how a small change in transcript level might cause a great difference at the protein level. One interesting possibility is that chromatin modifications (in DNA and/or protein) could cause changes in the transcription start site or in termination, which, in turn, could lead to altered splicing and mRNA function. At first glance this model seems unlikely, but it is known that sex determination in *Drosophila* involves use in the very early embryo of an upstream promoter and then a switch in promoter usage and splicing (30). We have assayed only for a small transcript near the middle of the gene, because it contains the only known nucleotide change between *Pgk-1*^{a} and *Pgk-1*^{b}. Eventually it may be possible, and, if *Drosophila* is a guide, necessary to investigate parental-source-specific alternative splicing and promoter usage.

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