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Original Article

Pgk1 and *Hprt* gene activity in the peri-implantation mouse embryo is influenced by the parental origin of the X-chromosome

KARIN STURM[#], MONICA LAFFERTY and PATRICK P.L. TAM*

Embryology Unit, Children's Medical Research Institute, Wentworthville, Australia

ABSTRACT The activity of two X-linked genes, *Pgk1* and *Hprt*, that are localized on X-chromosomes of different parental origins in the XX mouse embryo was analyzed by the quantification of allele-specific transcripts. For the *Pgk1* gene, the maternal allele-specific transcripts were consistently more abundant than the paternal transcripts in the blastocyst and the late gastrula. For the *Hprt* gene, the $Hprt^b$ allele was preferentially expressed in the blastocysts when it is present on the maternal X-chromosome. However, this skewed expression of the maternal allele was not observed in the reciprocal situation when the $Hprt^a$ allele was on the maternal X-chromosome. Like the *Pgk1* locus, significantly more maternal *Hprt* transcripts were found in the gastrula-stage embryos irrespective of their genotypes. One possible interpretation of these results is that, in the XX mouse embryos, the genetic loci on maternal X-chromosome may be transcriptionally more active than their paternal counterparts during peri-implantation development.

KEY WORDS: *X-chromosome, allele-specific transcripts, parent-specific effects, mouse embryo*

Introduction

During pre-implantation development of the female mouse embryo, both X chromosomes are transcriptionally active. However, an analysis of the transcriptional activity of two X-linked genes, *Pgk1* and *Hprt,* reveals that the amount of transcript (mRNA copies per embryo) encoded by the alleles on the maternally derived X-chromosome is about 3-10 fold more than that encoded by the alleles on the paternal X-chromosome at the 16 cell to blastocyst stages (Singer-Sam *et al.,* 1992; Latham and Rambhatla, 1995). Parent-specific influences on X-linked gene activity have been studied in uniparental mouse embryos where both X-chromosomes are of the same parental origin. Significant differences are found between the level of mRNA encoded by a set of X-linked genes *(Hprt, Pgk1, Prps1* and *Pdha1)* in embryos that have only the paternal genome (androgenones) or the maternal genome (gynogenones and parthenogenones). The maternally derived alleles are expressed at a higher level than the paternally derived alleles in the uniparental embryos (Latham and Rambhatla, 1995). Parent-specific effects on X chromosome activity have also been observed in postnatal female mice. The activity of enzymes such as G6PD and PGK1 that are encoded by alleles on the maternal X-chromosome is generally higher than that of the paternal alleles in tissues of the adult mice (Forrester and Ansell, 1985; Peters and Ball, 1990). There is also preferential expression of maternal X-linked trait such as the coat pattern in *brindled (Atp7a)* mice (Falconer *et al.,* 1982), the coat color variegation in the t(1,X)Ct mice with an X-autosome translocation (Cattanach and Perez, 1970), and the vibrissal pattern of the *Tabby* mice (Kindred, 1961). In summary, these findings suggest that the maternal alleles of the X-linked genes may generally be more active than the paternal alleles.

Parent-specific effects also impact on the progression of the inactivation of the X-chromosome. In the female mouse embryo, inactivation of the X-chromosome (X-inactivation) takes place during the immediate post-implantation period (5.3 to 6.3 days *post coitum*). The occurrence of X-inactivation in embryonic cells can be visualized cytogenetically by the asynchronous replication of the X-chromosomes. In the situation where the X-chromosomes can be distinguished by the presence or the absence of a Robertsonian

Abbreviations used in this paper: cDNA, complimentary deoxyribonucleic acid; dATP, 2'-Deoxyadenosine 5'-triphosphate; dCTP, 2'-Deoxycytosine 5'-triphosphate; dGTP, 2'-Deoxyguanosine 5'-triphosphate; dNTP, Deoxymucleoside 5'-triphosphate; G6PD, glucose-6-phosphate dehydrogenase; mRNA, messenger rinbonucleic acid; PBS, phosphate-buffered saline; PGK1, 3-phosphoglycerate kinase 1; RT-PCR, reverse transciptase polymerase chain reaction; RNase, ribonuclease; Tris, tris (hydroxymethyl)-aminomethane.

***Address for reprints:** Embryology Unit, Children's Medical Research Institute, Locked Bag 23, Wentworthville, NSW 2145 Australia. FAX: 61 2 9687 2120. e-mail: patrict@mail.usyd.edu.au

[#]Present address: Pediatric Immunology, Department Research, Kantonsspital Basel, CH4031 Basel, Switzerland.

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translocation, the paternally derived X-chromosome tends to be the one showing asynchronous replication (Takagi *et al.,* 1982). A similar observation was made when the activity of the X-chromosome was studied by the expression of an X-linked *lacZ* transgene. There are more cells in the peri-implantation stage embryos that display an inactive paternally derived X-chromosome during the progression of X-inactivation (Tam *et al.,* 1994). These findings have been taken to indicate that the paternal X-chromosome inactivates sooner than the maternal X-chromosome during deve-lopment. The preferential inactivation of the paternally derived X-chromosome, as revealed by the onset of asynchronous replication, also occurs when XX embryonic stem cells differentiate *in vitro* (Tada *et al.,* 1993). Therefore, the behavior of the X-chromosome during inactivation strongly suggests that X-chromosome activity is subject to parent-specific imprinting. The differential marking of the chromosome has been postulated to be the result of the modification in the chromatin configuration of the male and female genome in the germ-line cells (Hansen *et al.,* 1996; Jamieson *et al.,* 1996; Heard *et al.,* 1997).

The disparity between the activity of the alleles of the X-linked genes in the pre-implantation mouse embryos and the postnatal mice suggests that there are strong parental influences on the expression of X-linked genes before and after X-inactivation is accomplished. However, it has yet to be established that similar parental effect on the transcriptional activity of X-linked genes is present in mouse embryos when X-inactivation is in progress during the immediate post-implantation period (Tan *et al.,* 1993; Tam *et al.,* 1994). In the present study, we have determined the relative levels of expression of the paternal and maternal alleles of two X-linked genes in individual blastocysts and gastrula-stage embryos. Our results have shown that the maternal allele of the *Pgk1* gene and the *Hprt* gene, in certain combination of genetic backgrounds, is more actively transcribed than the paternal counterpart in the peri-implantation embryos.

Results

The relative efficiency of the SNuPE assay for different Pgk1 and Hprt alleles

The quantity of allele-specific transcripts was determined by the single nucleotide primer extension (SNuPE) assay (Singer-Sam *et al.,* 1992). The allele-specific transcripts are distinguishable by their sequence polymorphism. The two *Pgk1* alleles show a single base mismatch of A versus C at position 489 of the cDNA and the *Hprt* alleles by a mismatch of C versus G at position 91 of the cDNA. During primer extension, $dCTP^{32}$ labeled the $Pgk1^a$ and the $Hprt^a$ RT-PCR products, $dATP^{32}$ labeled the $Pgk1^b$ products and $dGTP^{32}$ labeled the $Hprt^b$ products. The quantity of the SNuPE products was determined by measuring the volume of the band resolved after electrophoresis using phosphoimaging techniques. The relative amount of the two allele-specific transcripts was computed respectively as percentage of a/(a+b) and b/(a+b), where a= volume of the *a*-allele band, b= volume of the *b*-allele band and a+b= sum of the volume of *a*- and *b*-band. Our assay was able to detect a minor component at about 1% of the total transcript. Calibration curves were constructed for the SNuPE assay of *Pgk1* and *Hprt* RNA isolated from adult tail tissues of XPHA *($Pgk1^a$ $Hprt^a$)* and H253 *($Pgk1^b$ $Hprt^b$)* mice. Both calibrations showed a linear correlation between the percentage values of the band volume and the relative amount of XPHA and H253 RNA in the reaction mix.

The efficiency of the amplification of the allele-specific template in the SNuPE assay was tested by comparing the band intensity of the products obtained in the reaction using a mixture of equal quantities of RNA (5 ng each) from the tails of XPHA/Y *($Pgk1^a$ $Hprt^a$* /Y) and H253/Y *($Pgk1^b$ $Hprt^b$ lacZ* /Y) mice. For the *Pgk1* assays (n= 26), equal amounts of *a*- and *b*-allele RNA gave mean values of 60.4 (±2.2)% for $Pgk1^a$ and 39.6 (±2.2)% for $Pgk1^b$. For the *Hprt* assays (n= 20), the values were 56.0 (±2.5)% for $Hprt^a$ and 44.0 (±2.5)% for $Hprt^b$. This apparently skewed ratio of allele-specific products could be attributed to the more efficient incorporation of the dCTP than dATP or dGTP during primer extension (Singer-Sam *et al.,* 1992). The SNuPE assay data were therefore corrected for the different efficiency of nucleotide incorporation (correction factors: $Pgk1^a$= 0.83, $Pgk1^b$=1.20, $Hprt^a$=0.90, $Hprt^b$= 1.11).

Further validation of the allele-specificity of the SNuPE assay was performed on XY blastocysts which only express the X-linked allele from the maternally derived X-chromosome. Four XY blastocysts each from mice of the XPHAxH253 and the H253xXPHA mating were found to express exclusively the maternal allele-specific *Pgk1* and *Hprt* transcripts. The X-chromosome of the H253 mice contains a *HMG-lacZ* transgene at band A6 position close to the *Hprt* locus (Tan *et al.,* 1995). However, similarly exclusive expression of the maternal allele-specific *Pgk1* and *Hprt* transcripts was found in five XY blastocysts (obtained from the B6D2F1xXPHA mating) which have no lacZ transgene on the X-chromosome. The yolk sac endoderm of 7.5-day gastrula embryo, where the paternal X-chromosome is preferentially inactivated during normal development, was also tested. The yolk sac endoderm of 6 XX gastrulae of the H253xXPHA mating was found to preferentially express the maternal allele-specific Pgk1 and *Hprt* mRNAs which constituted about 94 (±4.5)% and 93 (±3.6)% of the total transcripts respectively.

Preponderance of transcripts from the maternal alleles in the blastocyst and the late gastrula

Table 1 summarizes the relative amount of transcripts encoded by the paternal and the maternal alleles of *Pgk1* and *Hprt* genes in the blastocysts prior to the onset of X-inactivation and in the gastrula-stage embryos during X-inactivation. The data obtained from the analysis of the newborn in which X-inactivation is completed were included for comparison.

For the *Pgk1* gene, there were significantly more transcripts of the maternal alleles in the blastocyst. The preponderance of maternal *Pgk1* transcript was found in the blastocysts obtained from both the H352xXPHA mating, in which 77% of the total transcripts was maternally encoded, and the XPHAxH253 mating where over 99% was maternally encoded (Table 1). This suggests that the Pgk1 allele associated with the maternal X-chromosome is likely to be more actively transcribed and the enhanced activity is not related to any specific allelic polymorphism. For the *Hprt* gene, the maternally encoded transcripts constituted almost all of the *Hprt* mRNA in the blastocysts of the H253xXPHA mating. However, the situation was different in the blastocysts of the reciprocal mating, where approximately equal amount of allele-specific transcripts was present (Table 1). To test if the transgene on the X-chromosome of the H253 mice may influence *Hprt* and *Pgk1* expression, four blastocysts obtained from female B6D2F1 mice (of same genetic background as the H253 mice) that were mated with the XPHA mice were analyzed. In these blastocysts, the maternally encoded *Hprt* mRNA constituted more than 99% of the total *Hprt* transcripts, which is similar that in the

H235xXPHA blastocysts. These findings indicate that the difference in the relative level of maternally encoded Hp*rt* transcripts between the blastocysts of the reciprocal crosses of H253 and XPHA mice is not associated with the presence of the *lacZ* transgene but may be related to other differences in the genetic background of the B6D2F1/H253 mice and the XPHA mice.

Gastrula-stage embryos were collected from 7.5-day pregnant mice for the quantification of *Pgk1* and *Hprt* allele-specific transcripts. To avoid any spurious results due to the contamination by tissues that express an excess amount of maternally encoded transcripts, the trophoblast tissues, the ectoplacental cone and the extraembryonic (yolk sac and amnion) membranes were completely removed. Only the embryonic portion of the conceptus, which contains the embryo proper, was analyzed by the SNuPE assay.

For the *Pgk1* gene, the maternally encoded transcript was significantly more abundant than the paternal counterpart in the gastrula embryo of both types of mating (Table 1). The XPHA and H253 mice used in this study differ not only in their polymorphic X-linked gene loci but also the *Xce* haplotype. The XPHA mice carry the Xce^c allele and the H253 the Xce^a allele. It is well established that when different *Xce* haplotypes are present on the X-chromosomes of the same cells, the chromosome with the dominant *Xce* (i.e., the Xce^c of the XPHA X-chromosome) tends to remain active and thus results in an apparently non-random pattern of X-inactivation (Cattanach, 1975; Nielsen and Chapman, 1977; Johnston and Cattanach, 1981). Whether the *Xce* haplotype may also influence the rate of X-inactivation is not known. However, since consistently more maternally encoded *Pgk1* transcript was found in embryos of the reciprocal genotypes (Table 1), this may indicate that the preference for maternal *Pgk1* allele transcription is independent of any *Xce* effect.

To test if the *HMG-lacZ* transgene on the H253 derived X-chromosome may influence *Pgk1* activity, embryos obtained from the H253xXPHA and the B6D2xXPHA crosses were compared. Similar preponderance of the maternal allele-specific transcripts was found in both types of gastrula embryos (4 embryos from B6D2F1xXPHA mating were analyzed for Pgk1 transcript: [Xm]= 85.9±11.0% and [Xp]= 14.1±11.0%). This result indicates that the presence of *lacZ* transgene in the maternal X-chromosome has no effect on *Pgk1* activity.

A similar parent-specific effect is found for the expression of the *Hprt* gene. Gastrula-stage embryos derived from the reciprocal mating of XPHA and H253 mice consistently displayed significantly more abundant transcripts that were encoded by the maternal allele

TABLE 1

RELATIVE LEVELS OF ALLELE-SPECIFIC TRANSCRIPTS IN FEMALE BLASTOCYSTS AND LATE-PRIMITIVE-STREAK STAGE GASTRULA EMBRYOS, AND IN THE NEWBORN FEMALE MICE

Mating	Genotype#								N	Relative amount of allele-specific transcripts: mean (SE) %			
										Pgk1		*Hprt*	
	Xm-loci				Xp-loci					[Xm]	[Xp]	[Xm]	[Xp]
	Xce	*Pgk1*	*Hprt*	*lacZ*	*Xce*	*Pgk1*	*Hprt*	*lacZ*					
Blastocyst													
XPHA x H253	*c*	*a*	*a*	-	*a*	*b*	*b*	*+*	11⁼	77.7(7.1)*	22.3(5.8)	51.1(5.1)	48.9(4.6)
H253 x XPHA	*a*	*b*	*b*	+	*c*	*a*	*a*	-	12(nd)	>99*	<1	>99*	<1
Late gastrula													
XPHA x H253	*c*	*a*	*a*	-	*a*	*b*	*b*	*+*	6	71.7(1.6)*	28.3(1.6)	65.6(7.3)*	34.3(7.3)
H253 x XPHA	*a*	*b*	*b*	+	*c*	*a*	*a*	-	6	73.5(6.1)*	26.5(6.1)	68.2(8.2)*	31.8(8.2)
Newborn													
XPHA x H253	*c*	*a*	*a*	-	*a*	*b*	*b*	+	3(nd)	60.5	39.4	43.6	56.3
H253 x XPHA	*a*	*b*	*b*	+	*c*	*a*	*a*	-	10	53.9(3.2)	46.0(3.2)	69.2(5.7)*	30.7(4.4)

Genotypes for *Xce*, *a*=Xce^a, *c*=Xce^c, for *Pgk1* and *Hprt*, *a* = $Pgk1^a$and $Hprt^a$, *b*= $Pgk1^b$ and $Hprt^b$, and for lacZ, + = presence and - = absence of the *HMG-lacZ* transgene. Xm, Xp = maternal and paternal X-linked loci. [Xm]= Xm-transcripts and [Xp] = Xp-transcript. N = number of specimens (embryos or newborn) analyzed. Every sample was analyzed for both the *Pgk1* and *Hprt* transcript, except for the blastocyst of the XPHA x H253 mating(⁼) where one of the 11 embryos was analyzed only for the *Pgk1* transcript. SE, standard error. nd = SE not determined due to small sample size or when the amount Xm-allele specific transcripts is more than 99% of the total. *The relative amount of Xm-transcripts is significantly different from the 50:50 ratio (χ2-test, at P<0.05) and there are more Xm-transcripts than the Xp-transcripts detected by the SNuPE assay (Wilcoxon sign-rank test, P<0.05). Data obtained from less than 4 samples were not tested for statistical significance.

(Table 1). However, the difference in the relative level of allele-specific transcripts could not be detected in the gastrula embryo derived from the B6D2xXPHA mating (4 embryos were analyzed: [Xm]= 50.2±12.5%, [Xp]= 49.8±12.5%). Whether this may implicate any modulation by the activity of the *lacZ* transgene of the expression of the *Hprt* locus is not known at this time.

In the newborn of the H253xXPHA mating, there were significantly more maternal than paternal Hp*rt* transcripts but a similar amount of allele-specific *Pgk1* transcripts was found (Table 1). In the newborn derived from the reciprocal mating, there was a hint of more maternally encoded *Pgk1* transcripts but the relative amount of allele-specific *Hprt* transcripts was not different (Table 1). However, the number of samples analyzed was too few to warrant any definitive conclusion. Nevertheless, these results show that there is no consistent parent-specific effect on the transcription of the two X-linked genes in the newborn mice.

Discussion

Results of this study show that there is a significant parent-specific influence on the expression of the X-linked *Pgk1* and *Hprt* genes in the gastrula during the progression of X-inactivation. It has been shown, by monitoring the relative proportion of embryonic cells that express an X-linked *lacZ* transgene, that there are more cells displaying an active maternal X (Xm)-chromosome than the paternal X (Xp)-chromosome in the peri-implantation embryo (Tam *et al.,* 1994). Such disparity is not seen after X-inactivation is concluded when an equal number of cells is found to display either an active paternal or maternal X-chromosome (Tan *et al.,* 1993). One possible interpretation of this finding is that after the embryonic cells have initiated the process of X-inactivation, the paternal X-chromosome is silenced at a faster pace than the maternal counterpart. This interpretation is further supported by cytogenetic findings that the Xp-chromosome is more frequently found to be late-replicating than the Xm- chromosome before the conclusion of X-inactivation (Takagi *et al.,* 1982). The expeditious inactivation of the Xp-chromosome may account for the apparently more abundant maternally encoded transcripts when the embryonic cell population is analyzed in its entirety. Although direct evidence is not available, it is reasonable to expect that the activity of the *Xce* allele may lead to variations in the rate of the inactivation of X chromosomes with different haplotypes. If this does happen, then with a certain combination of *Xce* haplotypes, there will be a skewed rate of X-inactivation resulting in the presence of more cells in the gastrula embryo that display an active Xp-chromosome. This may mask the parent-specific effect on the level of transcription such that the maternal bias could be either diminished or reversed to a paternal bias. However, our data show that there is a consistent preponderance of maternal allele-specific *Pgk1* and *Hprt* transcripts irrespective of the combination of *Xce* haplotypes. This finding therefore argues against that the presence of more maternally encoded transcripts is a direct consequence of the variations in the progression of X-inactivation, but supports the notion that this may be the outcome of parent-specific regulation of gene activity. Previous studies have shown that by assaying the activity of the X-encoded PGK1 enzyme, the different combinations of *Xce* haplotypes have no significant impact on the preferential inactivation of the Xp-chromosome in the yolk sac endoderm (also shown in the present study), or the parent-specific levels of enzyme activity in the fetus and the adult blood (Bucher *et al.,* 1985; Forrester and Ansell, 1985).

Our results show that in the XX blastocysts the major fraction of the *Pgk1* transcripts is encoded by the allele on the maternally derived X-chromosome (Singer-Sam *et al.,* 1992; Latham and Rambhatla, 1995). However, unlike the situation of the *Pgk1* alleles, the maternal bias is only observed when the $Hprt^b$ allele, but not the $Hprt^a$ allele, is carried on the Xm chromosome. A non-equivalent expression pattern of the *Hprt* gene has also been observed in the embryos derived from the reciprocal mating of mice carrying polymorphic *Hprt* alleles (Latham and Rambhatla, 1995). The level of expression of the X-linked loci in the blastocysts is unlikely to be influenced by the *Xce* haplotype before the onset of X-inactivation, but may be influenced by other factors associated with the specific genetic background. In the newborn, the activity of the maternal *Pgk1* and *Hprt* allele varies with the genotype combination but it does not seem to follow the expected effect of the *Xce* haplotype or any parent-specific pattern. To further resolve the relative impact of *Xce,* allelic polymorphism and parent-specific effects on gene activity, it may be necessary to study the expression of the X-linked genes in the presence of other permutations of the polymorphic X-linked alleles and the *Xce* haplotypes.

Our recent study on X0 mouse embryos has revealed that the parental origin of the monosomic X-chromosome has a significant impact on embryonic development (Jamieson *et al.,* 1998). Xp0 embryos display developmental retardation during gastrulation and early organogenesis but Xm0 embryos develop normally. The discovery that the retardation of Xp0 embryo is associated with poorly developed ectoplacental tissues raises the possibility that a dysfunctional placenta rather than an embryonic defect may be the causal factor for the Xp0 phenotype. An important implication of the findings of the present study on the parent-specific effects on X-linked gene activity on the pathology of the X0 embryos is that the embryos that are monosomic for the Xp chromosome may be inflicted with a deficiency of certain X-linked gene products that are critical for tissue growth during gastrulation and early organogenesis. Although the Xp chromosome may remain active in the extraembryonic tissues of the Xp0 conceptus (Jamieson *et al.,* 1998), the overall activity of the X-linked genes may be inadequate to restore the full activity or gene dosage normally provided by the Xm chromosome.

Materials and Methods

Mice

Two strains of mice were used in this study: H253 and XPHA. The H253 mice carry on their X-chromosome a *lacZ* transgene under the control of the 5' regulatory elements of the mouse *3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG)* gene (Tam and Tan, 1992) on the C57BL/6xDBA/2 (=B6D2) F1 hybrid background. The XPHA mice were derived from the mating of one male C57BL/6Ros. $Hprt^a.Pgk1^a$ congenic mice (PGK mice, gift of the late Dr Verne Chapman) with the H253 mice and the F1 mice were back-crossed to the male PGK mice. The offspring were screened for homozygosity of the $Pgk1^a$ and $Hprt^a$ alleles during the establishment of the breeding colony of XPHA mice which are homozygous for $Pgk1^a$, $Hprt^a$ and Xce^c alleles.

Embryos and newborn were obtained from mice of two types of mating. First, female XPHA mice *($Pgk1^a$ $Hprt^a$/ $Pgk1^a$ $Hprt^a$)* were mated to H253 males *($Pgk1^b$, $Hprt^b$ lacZ* /Y) to produce female offspring of the $Pgk1^a$ $Hprt^a$ / $Pgk1^b$, $Hprt^b$ *lacZ* genotype. Second, the reciprocal mating of female H253 and male XPHA mice was set up to produce female offspring of the $Pgk1^b$, $Hprt^b$ *lacZ* / $Pgk1^a$ $Hprt^a$ genotype. The female embryos obtained from mice in these two mating types differ with regard to the parental origin of the *Pgk1* and *Hprt* alleles.

Blastocysts were collected from 3.5-day pregnant mice by flushing the uteri with PB1 medium. The zone pellucida was removed by treatment with

acid Tyrode's solution. The zone-free embryos were washed thrice in phosphate-buffered saline (calcium and magnesium free, Ca-Mg free PBS). Individual embryos were transferred to 20 μl of RNase inhibitor solution (40 U/μl, Boehringer-Mannheim) in micro-centrifuge tubes. The embryos were lysed by incubating in this solution for 30 min at 37°C and 5 min at 90°C. All lysate and mRNA samples were stored at -80°C.

Late-primitive-streak stage (gastrula) embryos were collected from 7.5-day pregnant mice. The ectoplacental cone was isolated and transferred to 50 μl of lysis buffer, incubated for 60 min at 55°C and 10 min at 95°C, and the lysate was stored at -20°C for sexing of the embryo. The visceral yolk sac was then separated from the embryonic portion of the conceptus by cutting with finely polished metal needles. The yolk sac was incubated in a solution of 0.05% trypsin and 0.25% pancreatin in Ca-Mg free PBS for 20-30 min at 4°C. The endodermal layer was separated from the mesodermal tissues by dissecting with fine glass needles. The endoderm was then transferred to 0.3 ml TRIZOL reagent (Gibco-BRL) and stored at -80°C. The embryonic portion of the conceptus left after the removal of all the extraembryonic tissues were transferred to 0.3 ml TRIZOL reagent (Gibco-BRL) and mRNA was isolated according to the protocol supplied by the manufacturer. Tail tissues were collected from newborn female mice and treated with TRIZOL reagent to extract the RNA. All the extracts were stored at -80°C.

Sexing the specimens

It is critical for the present study that only female embryos and newborn were analyzed for allele-specific transcriptional activity. The sex of the embryo and newborn was determined by the PCR-based co-amplification of the *Sry* and *Hprt* sequences in a single reaction using primers previously described (Jamieson *et al.,* 1997). DNA of females yields only the *Hprt* specific PCR product, while that of male gives both the *Sry* and *Hprt* products.

Ten μl of blastocyst or ectoplacental cone lysate and purified tail DNA were added to 40 μl PCR mix [pH8.3, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 0.1 μM each of the four primers], to which 1 U of Tag polymerase (Boehringer-Mannheim) and 0.2 μl of TagStart antibody (Clontech) were added. PCR was carried out on an Omnigene thermal cycler (Hybaid) for one cycle at 94°C for 5 min and 40 cycles at 53°C for 40 sec, 72°C for 1 min 40 sec and 93°C for 30 sec, followed by extension at 72°C for 10 min.

RT-PCR and quantitative assay of Pgk1 and Hprt transcripts

The reverse transcription was performed using 10 μl of blastocyst lysate or 10 μl of RNA isolated from the late-gastrula embryo or newborn tail. The samples were reacted with a random hexamer mix (Boehringer-Mannheim) in 20 μl of the solution containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 1 mM dNTPs, 20 U RNase inhibitor, 50 U M-MuLV reverse transcriptase (Boehringer-Mannheim). The reaction mix was incubated for 60 min at 42°C, 10 min at 99°C and equilibrated at 50°C. Seven μl of the reaction mixture were used for each SNuPE assay. PCR amplification of the cDNA template was performed as described by Singer-Sam *et al.* (1992) except that the reaction was performed in a 50 μl volume. The RT-PCR product was purified using the Qiagen QIAEX II gel extraction kit. The products were then quantified by Hoechst-dye fluorometry using a Perkin-Elmer MPF-3L fluorescence spectrophotometer. For the SNuPE reaction, 10 ng of each RT-PCR product and 0.75 U of Tag polymerase were used. The cycling parameters for amplification are for *Hprt,* 1 min at 95°C, 2 min at 42°C and 2 min 72°C, and for *Pgk1*, 2 min at 95°C, 2 min at 42°C and 2 min at 72°C. The SNuPE primers used to detect the mismatch of A vs. C at position 489 of the *Pgk1* cDNA and of C vs. G at position 91 of the *Hprt* cDNA were the same as described by Singer-Sam *et al.* (1992). The reaction products were electrophoresed in a 15% denaturing polyacrylamide gel which after the run was vacuum-dried for 2 h at 80°C. The dried gel was exposed to phosphoimaging screens overnight, scanned by a STORM phosphoimager (Molecular Dynamics) and the signal was quantified using the ImageQuant software.

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