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Timing of paternal *Pgk-1* expression in embryos of transgenic mice

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Summary

In mouse development, the paternal allele of the X-linked gene *Pgk-1* initiates expression on day 6, two days later than the maternal allele, which is activated on day 4. The different timing of expression of the maternal and paternal alleles may be determined by (i) imprinting of the chromosome region in which the gene resides, but not aimed specifically at the *Pgk-1* gene; (ii) gene specific imprinting, acting on *Pgk-1* irrespective of the chromosomal localization of the gene; (iii) an interplay between embryo cell differentiation, timing of X-inactivation and *Pgk-1* expression, without the involvement of imprinting at the *Pgk-1* locus itself (Fundele, R., Illmensee, K., Jagerbauer, E. M., Fehlau, M. and Krietsch, W. K. (1987) *Differentiation* 35, 31–36). Our findings in transgenic mouse lines, carrying *Pgk-1* on autosomes, indicate the importance of the X chromosomal location for the delayed expression of the paternal *Pgk-1* allele, and are in agreement with the first of the explanations listed above. We propose that the late activation of the paternal *Pgk-1* locus is a consequence of imprinting targeted at, and centered around, the X chromosome controlling element (*Xce*).

Key words: phosphoglycerate kinase-1, transgene, chromosome imprinting, mouse.

Introduction

The maternal and paternal alleles of the X-linked gene phosphoglycerate kinase-1 (*Pgk-1*) are activated at different times in the early mouse embryo. The product of the maternal allele ($Pgk\text{-}1^m$) appears on the 4th day of gestation (day of vaginal plug=day 1 of gestation), whereas the product of the paternal allele ($Pgk\text{-}1^p$) is first detected on day 6 (Papaioannou *et al.* 1981; Krietsch *et al.* 1982; Krietsch *et al.* 1986). Delayed onset of expression is not a general property of paternally transmitted alleles. For example, parental origin has no influence on the timing of expression of the autosomal gene glucose phosphate isomerase-1 (*Gpi-1*): the products of both parental alleles of *Gpi-1* are present by day 4 (Brinster, 1973; Chapman *et al.* 1971; Duboule and Burki, 1985; Gilbert and Solter, 1985; West and Green, 1983). Nor can the late activation of $Pgk\text{-}1^p$ be attributed to delayed activation of the paternal X (X^p) *in toto*: embryo-derived products of the X-linked gene hypoxanthine guanine phosphoribosyl transferase (*Hprt*) appear at the 8-cell stage (Epstein *et al.* 1978; Kratzer and Gartler, 1978; Monk and Harper, 1978), and isozyme analysis of $Hprt^a/Hprt^b$ heterozygotes has unequivocally established that the paternal allele is expressed at that time (Chapman, 1986). Also, quantitative analysis of the X-encoded α-galactosidase has shown the appearance of a bimodal distribution of activities by the 8-cell stage, indicating simultaneous transcription of two *versus* one X chromosome in the female and male embryos, respectively (Adler *et al.* 1977). Although X^p is inactivated in the trophectoderm on day 4, and in the primitive endoderm on day 5, the cells of the inner cell mass (ICM) and later, primitive ectoderm, retain two active X chromosomes up to day 6 (Monk and Harper, 1979; Takagi, 1983). The absence of $PGK\text{-}1^p$ between days 4 and 6 thus remains unexplained.

The epigenetic modification of homologous genes or chromosomes, leading to differences in their expression pattern or behavior depending on maternal or paternal inheritance, is referred to as imprinting. Grouped under this heading are diverse phenomena: preferential loss or inactivation of chromosomes (Crouse, 1960; Brown and Chandra, 1973; Lyon and Rastan, 1984), abnormal phenotypes associated with uniparental disomy (Lyon and Glenister, 1977; Cattanach and Kirk, 1985), different survival of carriers of heterozygous deletions (Johnson, 1975), and different methylation of transgenes depending on maternal or paternal inheritance (Reik *et al.* 1987; Sapienza *et al.* 1987; Swain *et al.* 1987). The combined effect of genome imprinting can account for the impossibility of producing viable parthenogenetic or androgenetic individuals in the mouse or in other mammals (McGrath and Solter, 1984; Surani *et al.* 1986). On the basis of the methylation

differences between male and female gametes, and differences in transgene methylation depending on parental origin, methylation was proposed as an epigenetic modifier of gene activity in genome imprinting (Monk *et al.* 1987; Sanford *et al.* 1987; Sapienza *et al.* 1989; Reik, 1989). In the cases of autosome imprinting in the mouse, there is no reason to believe that the imprint affects large uninterrupted regions of the chromosome (Winking and Silver, 1984). On the other hand, imprinting associated with preferential loss/inactivation of chromosomes affects the entire (X) chromosome; this effect on the X is believed to be achieved through imprinting of a small subregion of the chromosome, distinct from the centromere (Crouse, 1960; Russell, 1963; Johnston and Cattanach, 1981; Lyon and Rastan, 1984; Rastan and Robertson, 1985). It remains to be determined if X chromosome and autosome imprinting in the mouse have different molecular mechanisms.

The delayed activation of *Pgk-1*^{p} cannot be assigned unequivocally to any of the categories of imprinting described above. Although it may seem akin to the cases of mouse autosome imprinting (mainly because of the limited area affected by the imprint), it also has features in common with the imprint causing preferential X^p-inactivation in the extraembryonic membranes in the mouse; these include coincident timing of the two imprints and proximity of *Pgk-1* to the presumed target of X^p imprinting (see Discussion). As a step towards elucidating the reasons for the delayed activation of *Pgk-1*^{p}, we set out to determine if this is due to imprinting directed specifically at the gene itself, or is a consequence of imprinting of other elements near the *Pgk-1* locus, which then act in *cis* to prevent the activation of the gene prior to day 6. To this end, we produced transgenic mice which carry the *Pgk-1* gene at novel chromosome sites. Relocation of the gene would be expected to have no effect on the timing of paternal *Pgk-1* activation, if the late activation of the paternal allele is due to imprinting of the body or promoter region of the *Pgk-1* gene. Conversely, if the late activation of *Pgk-1*^{p} is a consequence of imprinting of a *cis*-acting element near the *Pgk-1* locus, relocation of the gene should allow it to escape from the influence of this element, and to be expressed at the same time as *Pgk-1*^{m}.

An interesting hypothesis on the relationship between *Pgk-1* expression and X-inactivation was proposed by Fundele *et al.* (1987). According to this model, *Pgk-1* expression (maternal or paternal) is initiated only after one of the X chromosomes is inactivated; it follows that *Pgk-1* expression begins on day 4 in the trophectoderm, but not before day 6 in the primitive ectoderm. This postulate, together with the fact that the paternal X is preferentially inactivated in the trophectoderm and the primitive endoderm, would be sufficient to account for the late appearance of the paternal *Pgk-1* product in mouse embryos. A test of this hypothesis would be to determine if a paternally transmitted *Pgk-1* gene, located in a different chromosome region (e.g. on an autosome) would be expressed in the primitive ectoderm prior to day 6. If a particular stage of cell differentiation is a precondition for *Pgk-1* expression, and X-inactivation is a cytological marker of this stage of differentiation (Monk and Harper, 1979; Monk, 1981; Sugawara *et al.* 1985), inner cell mass or primitive ectoderm cells should not contain any paternal (or embryo-derived maternal) *Pgk-1* products prior to day 6, irrespective of the localization of the *Pgk-1* gene.

In the present report, we describe our findings on *Pgk-1* expression in transgenic mouse lines carrying copies of the *Pgk-1*^{a} allele on autosomes. These findings are discussed in relation to the models for delayed paternal *Pgk-1* activation, outlined above. The results presented here indicate the importance of the position of the *Pgk-1* gene on the X chromosome for the delayed activation of the paternal *Pgk-1* allele.

Materials and methods

Mice

Non-inbred CD-1 mice and male (C57Bl/6J×DBA/2J)F_1 mice (B6D2F_1) were purchased from Charles River. Both the CD-1 and the B6D2F_1 mice carry the *Pgk-1*^{b} allele on their X chromosomes. CD-1 females mated with B6D2F_1 males were used as donors of fertilized eggs. CD-1 females, mated with vasectomized CD-1 males, were used as pseudopregnant recipients of the injected eggs. For some of the isozyme assays, *Pgk-1*^{b}/ *Pgk-1*^{a} heterozygous embryos were produced from crosses of CD-1 females with male mice, carrying the *Pgk-1*^{a} (and *Hprt*^{a}) alleles on B6 background (these mice were kindly provided by Dr Verne Chapman).

Embryo microinjections

These were performed as described by Gordon *et al.* (1980). Eggs were removed from oviduct ampullae on the day of the vaginal plug appearance and eggs were freed from adhering cumulus cells by treatment with 300 $\mu g\,ml^{-1}$ hyaluronidase (Sigma) in M2 medium (Hogan *et al.* 1986). The eggs were washed in M2 medium and transferred to microdrop cultures under paraffin oil in a humidified CO_2 incubator. Injections were performed using a Leitz inverted microscope, with a Leitz micromanipulator. The injected construct pCA1a contains the complete *Pgk-1*^{a} gene, including 5 kb of 5′ and 2.8 kb of 3′ flanking sequences, in the pSP64 vector. The plasmid was linearized by *Sal*I digestion (there is a single *Sal*I site in the polylinker of the pCA1a plasmid), and the DNA was extracted with phenol/chloroform, precipitated and redissolved in 10 mM Tris, pH 7.5, 1 mM EDTA, at a concentration of 4 $\mu g\,ml^{-1}$. Healthy injected eggs were transferred to pseudopregnant recipients and allowed to develop to term.

DNA analysis of the transgenic mice

Spleen DNA was extracted by the method of Blin and Stafford (1976). For Southern analysis, DNA was digested overnight under conditions recommended by the restriction enzyme supplier. The digested DNA was loaded on 0.7–0.8% agarose gels at 10–20 μg per lane, and the gel was run overnight at 0.7 $V\,cm^{-1}$. The DNA was blotted to nitrocellulose filters (Schleicher and Schuell) by the method of Southern (Southern, 1975). The filters were baked for 3 h at 80°C, and prehybridized for 4 h at 65°C in 6×SSC (1×SSC=0.15 M sodium chloride, 0.015 M sodium citrate), 10×Denhardt's solution (1×Denhardt's=0.02% Ficoll,

0.02 % bovine serum albumin, 0.02 % polyvinylpyrrolidone), 50 mM sodium phosphate pH 7.0, 1 % glycine, and 500 $\mu g\,ml^{-1}$ denatured, sheared salmon sperm DNA. Hybridization was performed in 6×SSC, 2×Denhardt's solution, 20 mM sodium phosphate pH 7.0, 10 % dextran sulfate, 100 $\mu g\,ml^{-1}$ denatured sheared salmon sperm DNA, and 10^6 cts $min^{-1}\,ml^{-1}$ probe, at 65 °C, for 18 h. Several probes were used in the Southern blot analysis of the transgenic lines. Probe A (Fig. 1) is a 680 bp *Eco*RI–*Pvu*II fragment from the 5′ region of the gene, spanning the first exon and adjacent portions of the promoter and the first intron; probe B is a 1.5 kb *Bgl*II fragment from the 3′ region of the gene, spanning exons 9, 10 and 11; probe C is a750 bp *Eco*RI–*Pst*I fragment of pBR322, containing a portion of the β-lactamase gene. This gene is also present in the pSP64 vector, but the *Eco*RI and the *Pst*I sites have been abolished. The probe was ^{32}P-labeled by nick-translation to a specific activity of $0.5–2\times10^8$ cts $min^{-1}\,\mu g^{-1}$ DNA, or by the random-primer method (Feinberg and Vogelstein, 1983) to a specific activity of $1–2\times10^9$ cts $min^{-1}\,\mu g^{-1}$ DNA.

Quantitation of transgene copy number in transgenic line 55 was done by slot-blot hybridization of appropriate dilutions of sample and control DNAs. DNA from heterozygous transgenic mice was serially diluted (1:2, 1:4, etc.) with DNA from a nontransgenic mouse, and 1 μg of the diluted DNA was slot-blotted. As a control, the 750 bp *Eco*RI–*Pst*I fragment was added to nontransgenic mouse DNA in a ratio corresponding to 1, 2, 4, etc. copies per genome. With an estimate of mouse haploid genome size of 2.7×10^9 bp, and fragment size of 750 bp, for a single copy/cell, this ratio was calculated to be 0.140 pg fragment DNA per 1 μg of genomic DNA. The blots were hybridized with the ^{32}P-labeled *Eco*RI–*Pst*I fragment as described above, with omission of dextran sulfate.

In situ *hybridization*

Chromosome slides were prepared from cultures of dissociated newborn mouse liver cells. The slides were banded by the trypsin–Giemsa method (Wang and Fedoroff, 1972). Suitable spreads were photographed and their coordinates recorded. The slides were washed free of immersion oil in three changes of xylene, and baked for 5 h at 80 °C prior to hybridization. The slides were denatured for two minutes at 70 °C in 70 % formamide (Fluka), 2×SSC, and dehydrated through a series of 70 %, 95 %, 95 %, and 100 % ethanol. The hybridization probe was the entire pSP64 vector, ^{3}H-labeled by the random-primer method (Feinberg and Vogelstein, 1983) to a specific activity of 3.5×10^8 cts $min^{-1}\,\mu g^{-1}$ DNA. The hybridization solution contained 50 % formamide, 2×SSC, 1×Denhardt's solution, 10 mM sodium phosphate pH 7.0, 4 mg ml^{-1} *E. coli* tRNA (Boehringer-Mannheim), 2 mg ml^{-1} denatured salmon sperm DNA (Sigma), and 250 ng ml^{-1} labeled DNA. After application of the hybridization solution, the slides were covered with parafilm and incubated at 37 °C in a humid chamber for 18 h. After hybridization, the slides were washed twice in 2×SSC at 55 °C for 20 min each, once in 0.1×SSC at 65 °C for 30 min, rinsed in 0.1×SSC, dehydrated and dipped in Kodak NTB2 emulsion. The slides were developed after two weeks, stained with Giemsa (Fisher), and re-examined under the microscope for localization of silver grains. Assignments are based on the mouse chromosome band nomenclature of Nesbitt and Francke (1973).

PGK-1 enzyme assay

This assay was performed as described by Bücher *et al.* (1980), with the modifications described by Monk (1987). Extracts from brain, thymus, lung, heart, liver, kidney, spleen and testis were prepared in a buffer consisting of 20 ml triethanolamine 50 mM (pH 7.6), 20 ml glycerol, 6 mg 1,4-dithioerythritol and 10 mg BSA fraction V. Day-3 embryos were flushed from the oviducts, day-4 and early day-5 embryos were flushed from the uterus, and collected in 2–4 μl of PB1.PVP medium (Monk, 1987) with a fine capillary pipette. Inner cell masses of day 4 embryos were isolated after treatment with the calcium ionophore A23187 (Sigma) and short pronase (Sigma) digestion, as described by Surani *et al.* (1978). The embryos and ICMs were transferred to 5 μl micropipettes, both ends of which were flame sealed. The embryos were stored at −70 °C prior to analysis. PGK-1 isozyme separation was carried out on cellulose acetate strips (Sartorius) in a Sartophor gel tank at 240 V for 90 min. The electrophoresis buffer contained 20 mM sodium barbital, pH 8.8, 10 mM sodium citrate, 5 mM magnesium sulfate, 2 mM EDTA, 0.1 mg ml^{-1} dithioerythritol. For the enzyme assays, approx. 0.5 μl of tissue or embryo extract was transferred to the cellulose acetate strips with an applicator. The staining reaction was performed as described by Monk (1987).

Results

The pCA1a construct that we used for embryo microinjection contained an insert of approximately 26 kb, including the entire *Pgk-1* gene, plus 5 kb of 5′ and 2.8 kb of 3′ flanking sequences, in the pSP64 vector (Adra, 1988; Boer *et al.* 1990). The 5′ *Bam*HI subfragment of this construct, approximately 21 kb in size, was isolated from genomic DNA of a female AT10-C3H/HeHa mouse, carrying the *Pgk-1*^{a} allele. This *Bam*HI fragment is polymorphic, and has a size of 17 kb in mice with the *Pgk-1*^{b} genotype (Adra *et al.* 1988). This *Bam*HI fragment contains the first 8 exons of the *Pgk-1* gene (Adra *et al.* 1987). The amino acid polymorphism causing the different electrophoretic mobility of the PGK-1A and PGK-1B isozymes was localized to position 156 (Potten *et al.* 1989), encoded by the fifth exon of the gene (Michelson *et al.* 1985; Mori *et al.* 1986; Boer *et al.* 1990). The 5 kb *Bam*HI–*Eco*RI fragment of the pCA1a insert, containing the last three exons of the *Pgk-1* gene, is derived from the 3′ portion of the *Pgk-1*^{b} allele. The restriction map of the pCA1a construct shown in Fig. 1 is from Adra (1988) and Boer *et al.* (1990). Previous experiments have shown that 0.9 kb of 5′ flanking sequences are sufficient for the expression of the genomic *Pgk-1* clone upon transfection of HeLa cells (Adra *et al.* 1987). Later, it was found that only 450 bp of 5′ flanking sequences and 220 bp of 3′ flanking sequences are sufficient to restore expression to the B13 pseudogene (pseudogene II – Potten *et al.* 1989), which represents a promoterless but potentially functional cDNA copy of *Pgk-1*^{b} (Boer *et al.* 1990). The 500 bp fragment immediately 5′ of the gene contains a number of sequence motifs usually present in the promoters of constitutively expressed housekeeping genes (Adra *et al.* 1987). This fragment directed efficient transcription of a linked *lacZ* gene, after transfection into mouse fibroblasts or embryonal carcinoma cells (Adra, 1988). Transfection of the pCA1a construct into HeLa cells resulted in the expression of the PGK-1A form of the enzyme (Adra,

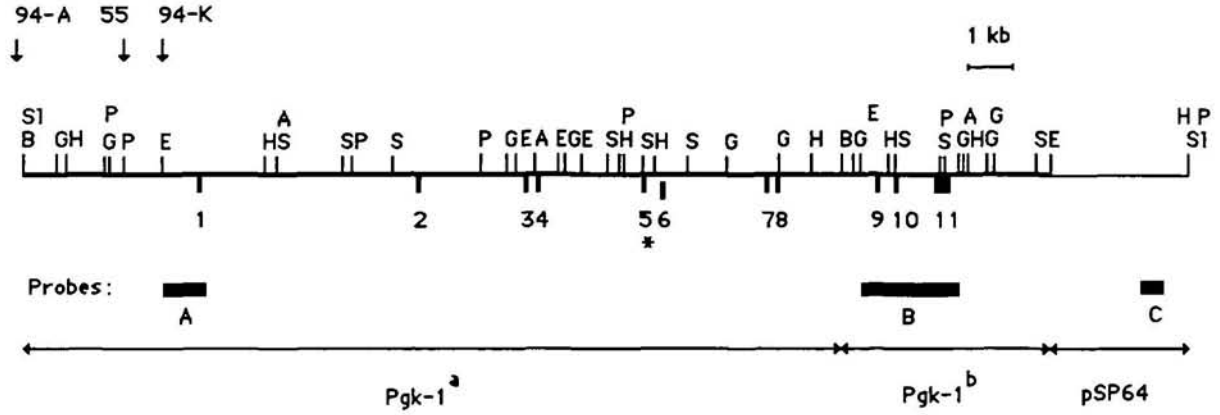

Fig. 1. Restriction map of the pCA1a clone (from Adra, 1988 and Boer *et al.* 1990). Black bars under the map indicate the position of the exons; these positions should be considered approximate. Exon 5, marked with an asterisk, encodes the amino acid polymorphism which causes the different electrophoretic mobility of PGK-1A and PGK-1B. The portions of the pCA1a clone isolated from *Pgk-1*^{a} and *Pgk-1*^{b} mice are indicated under the map. The vertical arrows at the 5′ end of the clone mark the most distal intact restriction site present at the 5′ end of the three transgene loci: 55, 94-A and 94-K. The heavy lines below the restriction map indicate the position of the pCA1a subclones, which were used as probes in the Southern analysis of the transgenic lines. Restriction sites: B, *Bam*HI; P, *Pst*I; G, *Bgl*II; H, *Hin*dIII; E, *Eco*RI; S, *Sst*I; A, *Apa*I; Sl, *Sal*I.

1988). For simplicity, we will refer to this construct as *Pgk-1*^{a} gene, although the non-polymorphic 3′ portion of the construct was derived from a *Pgk-1*^{b} allele.

Transgene structure and chromosomal localization

Transgenic founder 55 was a male offspring of a CD-1×B6D2F$_1$ cross. The transgene was present in 8 copies per cell, as estimated by slot-blot hybridization of transgenic and control DNA (Fig. 2A,B). The detection of single repeat units with all three of our probes indicates that there are no gross rearrangements in the transgene cluster. The size of the restriction fragments spanning the junction of the tandem units indicated head-to-tail arrangement of the repeats. Southern analysis with probe A indicated that the integration into host DNA had occurred through breakage at a site distal to the *Pst*I site in the 5′ most member of the cluster (Fig. 1). The presence of single junction fragments, detected with probes A and C, and the approximately equal number of copies transmitted to all offspring, indicated that the transgene cluster had integrated at a single chromosomal site.

In situ hybridization of ^{3}H-labeled vector DNA to banded metaphase chromosome spreads from a presumed homozygous offspring (homozygosity was inferred from the intensity of the junction fragment), showed that this transgene had integrated in, or adjacent to, the A3 region of chromosome 17: of 251 grains found over chromosomes in 109 analyzed spreads, 142 (57%) were over chromosome 17, and 135 (54%) were in the A1–C region of the chromosome, with a pronounced peak at band A3 (Fig. 3).

The male transgenic founder 94 transmitted to its progeny two transgene patterns, both of which segregated as autosomal or pseudo-autosomal loci. These patterns, which we termed 94-K and 94-A, differed in the number of tandem repeats and in the restriction

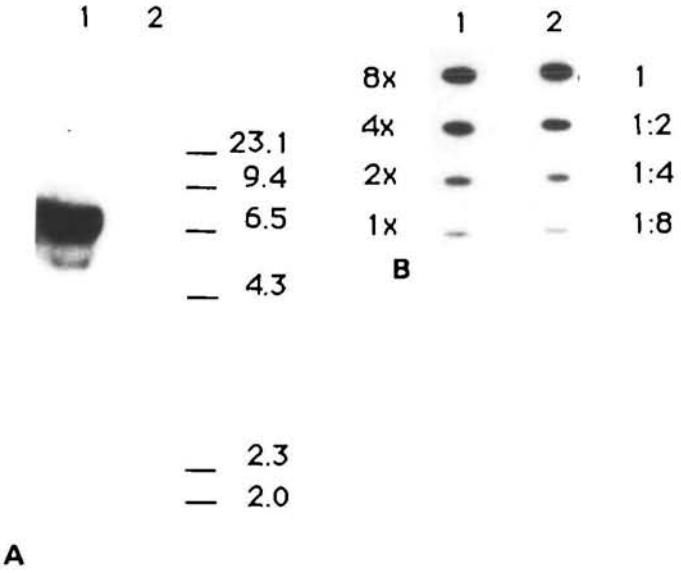

Fig. 2. (A) Southern blot of *Eco*RI digested spleen DNA from an F$_1$ offspring of transgenic founder 55 (lane 1) and a negative CD-1×B6D2F$_1$ mouse (lane 2). The probe is a 750 bp *Eco*RI–*Pst*I fragment of the β-lactamase gene of pBR322 (probe C). The numbers to the right indicate the positions of the relative molecular mass markers (*Hin*dIII cut λ DNA). (B) Slot blots of transgenic and control DNAs, probed with the 750 bp *Eco*RI–*Pst*I fragment of the β-lactamase gene (probe C). Increasing concentrations of *Eco*RI–*Pst*I fragment DNA, mixed with non-transgenic mouse DNA are shown in column 1; 1×corresponds to one copy of this sequence per cell. Decreasing concentrations of heterozygous transgenic DNA, diluted with non-transgenic DNA at the indicated ratio, are shown in column 2.

Fig. 3. Distribution of grains after *in situ* hybridization of ^{3}H-labeled pSP64 to banded chromosome slides of a presumed homozygous transgenic offspring. A single peak of grains, centered in band A3 of chromosome 17, marks the integration site of the transgene.

map in the 5′ end of the cluster. An illustration of these differences is shown in Fig. 4. Pattern 94-K is associated with novel fragments (i.e. fragments not predicted from the restriction map of the endogenous gene or the injected clone) detected with probe A after *Kpn*I, *Pst*I, *Bgl*II, *Hin*dIII, *Apa*I, *Sac*I, and *Bam*HI digestion, but not with *Eco*RI digestion; this indicates that the transgene cluster is joined to chromosomal DNA at its 5′ end somewhere between the *Eco*RI and the *Pst*I sites closest to the first exon (Fig. 1). By comparing the intensity of the fragments on Southern blots of line 94-K with those of line 55, line 94-A and nontransgenic controls, we estimate that mice with the 94-K pattern contain 4–5 copies of the transgene. Pattern 94-A is associated with a different set of restriction fragments detected with probe A. Novel fragments are seen after *Kpn*I and *Apa*I digestion, but not after *Pst*I, *Bgl*II, *Hin*dIII, or *Sac*I digestion. In addition to the novel *Bam*HI fragment, unique to pattern 94-K, probe A detects a large *Bam*HI fragment common to patterns 94-A and 94-K. This common *Bam*HI fragment is most likely derived through loss of the 3′ *Bam*HI site, and thus must be present on a non-functional copy of the gene, lacking the last three exons. Probe B detects no fragments other than those predicted from the restriction map of the gene in either 94-A or 94-K mice. We estimate that 94-A mice have two copies of the transgene (see e.g. *Apa*I and *Bam*HI blots in Fig. 4), at least one of which is uninterrupted throughout its entirety (as determined by hybridization with probes A, B and C), and contains the complete 5′ flanking region of the clone, up to, and including, the 5′ most *Bam*HI site (Fig. 1). The different number of transgene copies and the different restriction map of the 5′ region of the transgene clusters would seem to suggest that they represent two different transgene loci. However, we have not found any transgenic F_1 animals combining patterns 94-A and 94-K (0/33), nor have we been able to produce such mice by breeding 94-A with 94-K progeny (0/30). These findings are more easily explained by assuming that 94-A and 94-K represent a single integration event, followed by rearrangement in the 5′ end of the cluster, resulting in two different restriction patterns. The common *Bam*HI fragment

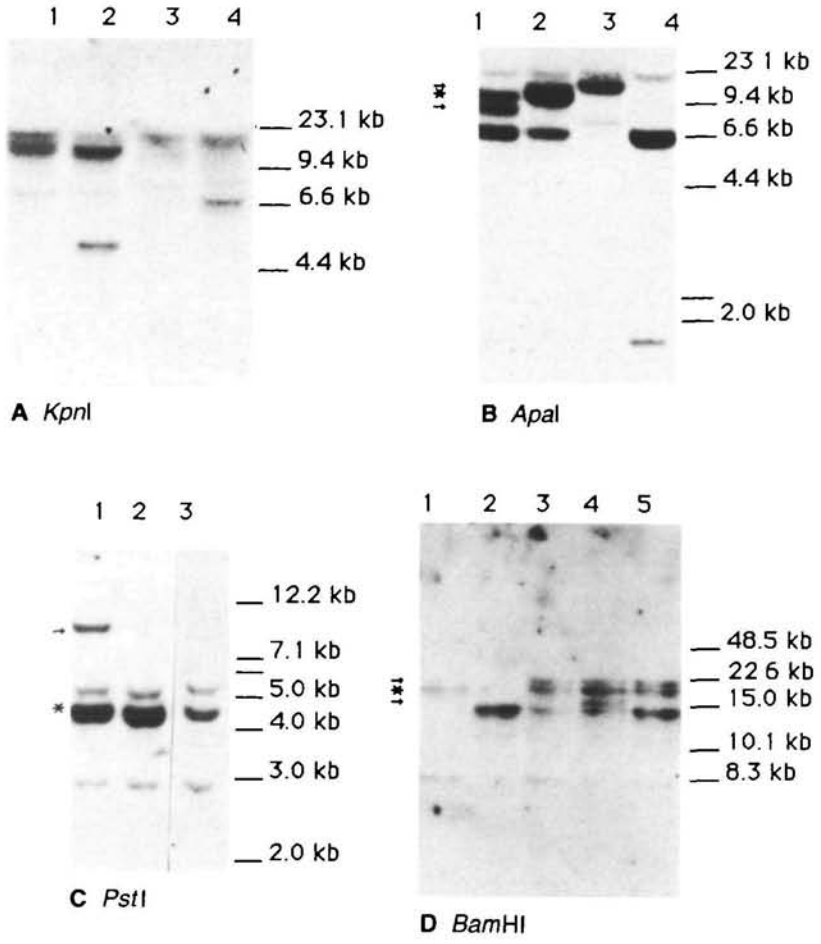

Fig. 4. Southern analysis of spleen DNA of heterozygous transgenic offspring of lines 94-A and 94-K, hybridized with probe A. Asterisks mark the position of fragments derived from the tandem transgene repeats; arrows indicate the position of novel restriction fragments in the transgenic lines. In addition to the fragments derived from the transgene and the endogenous X-linked gene, probe A detects additional fragments, which represent pseudogenes. (A) *Kpn*I digest. Lane 1, 94-A DNA; lane 2, 94-K DNA; lane 3, DNA from a nontransgenic *Pgk-1*^{a} mouse; lane 4, DNA from a nontransgenic *Pgk-1*^{b} mouse. Numbers to the right indicate the position of *Hin*dIII cut λ markers; (B) *Apa*I digest. Lane 1, 94-A DNA; lane 2, 94-K DNA; lane 3, nontransgenic *Pgk-1*^{a} DNA; lane 4, nontransgenic *Pgk-1*^{b} DNA. The 94-A DNA (lane 1) is from a female mouse; each of the fragments derived from the transgene has about half the intensity of the endogenous X gene fragment, indicating that there are two copies of the transgene in this line. The novel fragment in the 94-K line (lane 2) migrates close to the transgene repeat fragment and is not clearly visible as a separate band on this gel. Relative molecular mass markers are as in A. (C) *Pst*I digest. Lane 1, 94-K DNA; lane 2, 94-A DNA; lane 3, DNA from a control *Pgk-1*^{b} mouse. Relative molecular mass markers – 1 kb ladder; not all marker bands are shown. (D) *Bam*HI digest. Lane 1 – non-transgenic *Pgk-1*^{a} DNA; lane 2, nontransgenic *Pgk-1*^{b} DNA; lanes 3 and 5, male and female 94-A DNA; lane 4, 94-K DNA.The relative intensity of the transgene and endogenous fragments in 94-A DNA (lanes 3 and 5) indicates the presence of two copies of the transgene in this line. The numbers to the right indicate the positions of the high relative molecular mass markers (BRL); not all marker bands are shown.

then would represent the common 3′ junction fragment of these two patterns. The absence of F_2 progeny combining the two patterns would be attributed to the disruption of a vital endogenous gene as a result of the integration. The same factor would account for the absence of transgenic progeny homozygous for the 94-A or 94-K patterns.

Preliminary results from *in situ* hybridization indicate that 94-K is localized in the proximal region of chromosome 11. In 77 analyzable spreads, a total of 185 grains were found over chromosomes. Of these, 20 (11%) were in the A1–B1 region of chromosome 11, with a peak at bands A3–4 (not shown).

Although 94-A and 94-K are likely to be integrations in a single locus, in this report, they will be considered separately, for the following reasons. (1) 94-A and 94-K have different numbers of transgene copies; this may be associated with different susceptibility to imprinting and is also of importance when considering the ability of the enzyme assay to detect early expression of both higher than normal and physiological levels of PGK-1. (2) 94-A and 94-K retain different portions of the 5′

region of the gene in the 5′ end of the cluster; if imprinting modifies this region of the *Pgk-1* gene, deletions of some portions may alter its sensitivity to imprinting. (3) 94-A and 94-K are joined to different chromosomal sequences at the 5′ end of the transgene cluster, and these may affect differently the expression of the transgene.

Transgene expression

To determine if the injected construct is expressed in a normal pattern *in vivo*, we analyzed, by cellulose acetate electrophoresis, extracts from various organs of transgenic offspring. The X chromosomes of these mice carry the CD-1-derived $Pgk\text{-}1^b$ allele and thus allow easy detection of the transgene PGK-1A product. Results from the isozyme assays are shown in Fig. 5 and summarized in Table 1. The fact that expression of the transgene was found in all tested tissues is in accordance with the expectations for an ubiquitously expressed 'housekeeping' gene, and indicates that the injected construct contains the *cis*-regulatory elements required for the efficient expression of the gene *in vivo*. It also shows that the inclusion of vector sequences in the injected construct does not interfere significantly with the expression of the transgene *in vivo*. In all three lines, the level of PGK-1A activity roughly correlated with the number of transgene copies.

To determine if the paternally transmitted *Pgk-1* transgene is active on day 4, or its expression is delayed up to day 6, we performed isozyme analyses of day-3, -4, -5, and -6 mouse embryos. These embryos were produced by crossing CD-1 females with homozygous line 55 males, or with heterozygous 94-A and 94-K males. All assays were done on pooled embryos. In all three crosses, the PGK-1A product of the transgene was detected on day 4. A PGK-1 assay on embryos from crosses with line 55 (having the highest transgene copy number) and 94-A (having the lowest transgene copy number) is shown in Fig. 6A and B. As a control, we followed the expression of the paternal $Pgk\text{-}1^a$ gene in crosses between CD-1 females and male mice carrying a $Pgk\text{-}1^a$ allele on their X chromosome. In this cross, in five separate assays with different embryos, the paternal *Pgk-1* product was first detected on day 7 (Fig. 6B). This is one day later than the time of activation reported by Papaioannou *et al.* (1981), Krietsch *et al.* (1982) and Krietsch *et al.* (1986). By measuring PGK-1 activity in individual embryos, Krietsch *et al.* (1982) found that on day 6 $PGK\text{-}1^p$ represents about 17% of the total PGK-1 activity of the embryo (or about 1/5 of the activity of the maternal enzyme). On the basis of this estimate, and assuming a 50:50 ratio between males and females in the progeny, we should expect the paternal PGK-1 to be about 10% of the maternal PGK-1 activity in our pooled embryo samples. To determine if such a contribution of PGK-1 would be detected by the assay, we mixed PGK-1B with increasingly higher dilutions of PGK-1A. As shown in Fig. 6C, the PGK-1A product could easily be detected at 2% of its original concentration (equal to that of

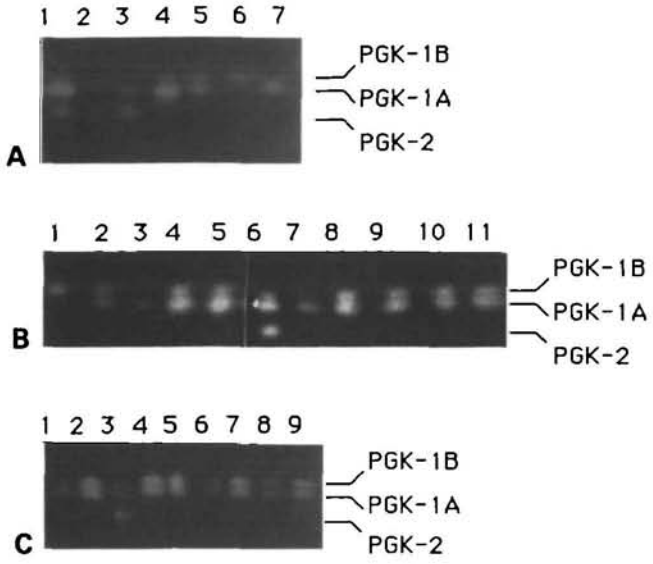

Fig. 5. PGK-1 enzyme assay of transgenic and control adult mice. Approx. 0.5 μl of each extract was used for the electrophoresis.The position of the PGK-1A and PGK-1B isozymes is indicated on the right. (A) PGK-1 expression in organs of adult transgenic mice of line 55: Lane 1, testis of an F_1 transgenic offspring; lane 2, testis of a nontransgenic $Pgk\text{-}1^b$ mouse; lane 3, testis of a nontransgenic $Pgk\text{-}1^a$ mouse; lane 4, liver of an F_1 transgenic offspring; lane 5, liver of a nontransgenic $Pgk\text{-}1^a/Pgk\text{-}1^b$ mouse; lane 6, liver of a nontransgenic $Pgk\text{-}1^b$ mouse; lane 7, liver of a nontransgenic $Pgk\text{-}1^a$ mouse. The most anodal band, visible in testis extracts, represents the activity of the autosomal, testis-specific PGK-2 (encoded by a gene on chromosome 17). (B) *Pgk-1* expression in progeny of 94-K mice. Lane 1, kidney of a nontransgenic $Pgk\text{-}1^b$ mouse; lane 2, kidney of a nontransgenic $Pgk\text{-}1^a/Pgk\text{-}1^b$ mouse; lane 3, kidney of a nontransgenic $Pgk\text{-}1^a$ mouse; lanes 4–8, extracts from 94-K organs and tissues: lane 4, muscle; lane 5, kidney; lane 6, testis; lane 7, lung; lane 8, heart; lane 9, thymus; lane 10, eye; lane 11, brain. (C) Progeny of 94-A mice. Lane 1, spleen; lane 2, muscle; lane 3, testis; lane 4, liver; lane 5, kidney; lane 6, lung; lane 7, heart; lane 8, thymus; lane 9, brain.

Table 1. *PGK-1A expression in transgenic mice*

Mouse line	Brain	Eye	Thymus	Lung	Heart	Spleen	Kidney	Liver	Testis	Muscle
55	+	N.T.	+	+	+	+	+	+	+	N.T.
94-A	+	N.T.	+	+	+	+	+	+	+	+
94-K	+	+	+	+	+	+	+	+	+	+

N.T.=Not tested.

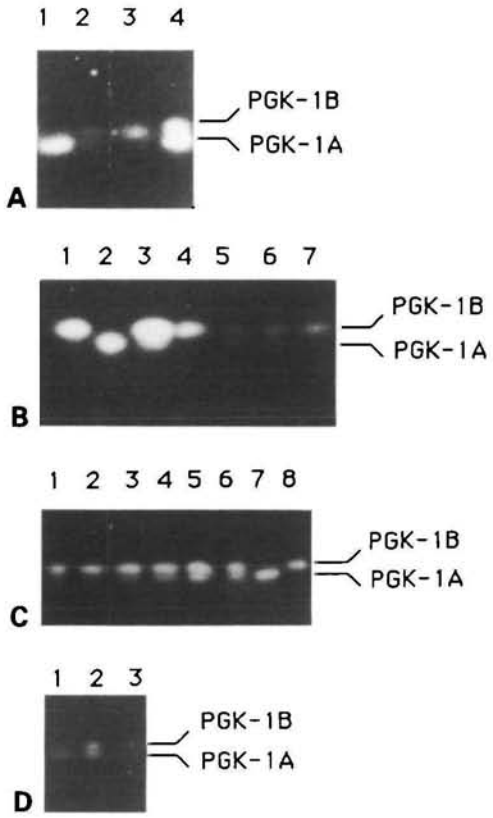

Fig. 6. PGK-1 assay of transgenic and control mouse embryos. (A) Pgk-1 expression in transgenic embryos from a cross between CD-1 females and male mice of line 55, homozygous for the transgene. Lane 1, whole, day-5 transgenic embryos (6 embryos, collected in 5 μl of medium); lane 2, whole, day-4 transgenic embryos (13 blastocysts, collected in 4 μl of medium); lane 3, whole, day-3 embryos (13 embryos collected in 4 μl of medium); lane 4, spleen extract from a *Pgk-1*^{a}/*Pgk-1*^{b} mouse. (B) *Pgk-1* expression in embryos from a cross between CD-1 females and heterozygous 94-A males. Lanes 1 and 2, kidney extracts from control nontransgenic *Pgk-1*^{b} and *Pgk-1*^{a} mice; lane 3, day-7 embryos of a cross between CD-1 females (*Pgk-1*^{b}/*Pgk-1*^{b}) and nontransgenic *Pgk-1*^{a} males (11 embryos collected in 6 μl of medium); lane 4, day-6 embryos of the same cross (11 embryos collected in 4 μl of medium); lane 5, day-5 embryos of a cross between CD-1 females and 94-A males (26 embryos in 4 μl); lane 6, day-4 embryos of the same cross (55 embryos in 4 μl); lane 7, day-3 embryos of the same cross (35 in 3 μl). (C) Enzyme assay of mixtures of PGK-1B with progressively higher dilutions of PGK-1A. The starting concentration was chosen to approximate the activity of PGK-1B detected in day-6 embryos of the control *Pgk-1*^{b}/*Pgk-1*^{b}×*Pgk-1*^{a} cross. The PGK-1A:PGK-1B ratios are: lane 1, 0.025:1, lane 2, 0.075:1; lane 3, 0.1:1; lane 4, 0.2:1; lane 5, 0.3:1; lane 6, 0.4:1; lanes 7 and 8, PGK-1A and PGK-1B controls. (D) *Pgk-1* expression in the inner cell mass of day-4 transgenic embryos: lane 1, ICMs of late day-4 embryos from a cross between CD-1 females and homozygous line 55 males (14 ICMs collected in 1 μl of medium); lane 2, thymus extract of a *Pgk-1*^{a}/*Pgk-1*^{b} heterozygote; lane 3, whole, day-4 embryos, same cross as in 1 (13 blastocysts collected in 4 μl of medium).

PGK-1B), indicating that the absence of PGK-1A in day 6 embryos is not due to low sensitivity of the assay. A more likely explanation is that, in this cross, very few of the female embryos activate the paternal allele on day 6. This explanation is consistent with previous findings that, while PGK-1^p is first detected on day 6, not all of the female embryos contain detectable levels of the paternal enzyme on day 6 (Papaioannou *et al.* 1981; Krietsch *et al.* 1982).

To determine whether ICM cells are capable of expressing the *Pgk-1* gene on day 4, or acquire this ability only on day 6, we isolated ICMs of day-4 embryos from a cross between CD-1 females and homozygous male progeny of transgenic line 55. At this stage, the ICM has not yet differentiated into primitive endoderm and primitive ectoderm. Trophectoderm cells were lysed by the calcium ionophore A23187, and were removed by brief pronase digestion. In our hands, this treatment resulted in the production of ICMs that occasionally retained a few trophectoderm cells (viewed under phase contrast these cells appeared as attached to, and protruding from, the compact ICMs). Thus in the present report, we consider this procedure to be highly enriching for ICM cells, rather than completely removing the trophectoderm cells. Cellulose acetate electrophoresis of extracts prepared from ICMs of day-4 embryos showed distinct expression of the paternally transmitted *Pgk-1*^{a} transgene. A comparison of the relative intensity of the PGK-1A and PGK-1B bands in whole embryos and ICMs at day 4, showed no dilution of the PGK-1A activity, which would have been expected if the sole source of this activity were the few remaining adhering trophectoderm cells. On the contrary, the relative intensity of the PGK-1A band in the ICM samples appeared stronger than that of the PGK-1A band in whole embryo extracts. The markedly higher relative intensity of the PGK-1A band in the ICM extracts shown in Fig. 6D, may be in part due to the slightly more advanced age of the embryos (late day 4), from which they were isolated. We conclude that ICM cells of day-4 mouse embryos are capable of expressing the embryonic *Pgk-1* genes.

Discussion

In choosing to inject a complete genomic clone of the *Pgk-1* gene, with flanking sequences exceeding the span of the known regulatory region of the gene (as defined in transfection assays), we sought to achieve a faithful reproduction of the normal expression pattern of *Pgk-1*, and to make this expression independent of the transgene integration site. In particular, we wanted to avoid position effects which are commonly seen with minigene constructs, which could interfere with the expression of the transgene in early embryos, so as to make it uninformative or misleading. This expectation was borne out by our finding that the transgenes are expressed in all tissues tested, as is the endogenous X-linked gene. The level of expression correlated with the number of transgene copies (Fig. 5). Because of the

large size of the gene and the presence of numerous restriction sites in the insert, it proved difficult to separate the vector sequences from the insert of the *Pgk-1* clone. Retention of vector sequences has been shown to result in low level or lack of expression of some, but not all, transgenes (Hammer *et al.* 1987). However, as the results described in the previous section indicate, there was no evidence of such influence in the adult mice (all lines expressed the transgene at a level corresponding to the number of transgene copies), in the whole day-4 to -6 embryos (transgene *Pgk-1* was activated on day 4, showing no delay, in comparison with the maternal X-linked gene), or in the ICM of day-4 embryos. Most likely, the overwhelmingly larger size of the insert, with retention of the introns and large 5′ and 3′ flanking fragments, was sufficient to counter the negative effect of the plasmid sequences (Brinster *et al.* 1988).

The expression of the paternally transmitted *Pgk-1*^{a} transgene in day-4 mouse embryos indicates that the late activation of the X-linked *Pgk-1*^{p} is not due to gene-specific imprinting which modifies the *Pgk-1* gene during male gametogenesis, irrespective of the chromosomal localization of the gene. This disproves the first of the models outlined in the introduction, as a possible explanation for the late onset of expression of the paternal X-linked *Pgk-1* gene.

The present study did not address the question of whether the maternal and paternal alleles of the transgenes begin expression simultaneously. Thus it remains possible that the maternally transmitted autosomal transgenes are still expressed earlier than their paternal alleles. If this were the case, it would imply that there are two types of imprinting affecting the *Pgk-1* gene: (1) a gene-specific imprinting, operating independently of chromosome position, which allows the paternal allele to be activated on day 4, and the maternal allele – a few days earlier; (2) imprinting of the X chromosome region around the *Pgk-1* locus, which delays expression of the maternal allele till day 4, and of the paternal allele till day 6. If further experiments provide evidence for the existence of such dual imprinting of *Pgk-1*, the conclusions reached in the previous paragraph will be applicable only to the factors preventing paternal *Pgk-1* activation between days 4 and 6.

The model for delayed *Pgk-1*^{p} expression proposed by Fundele *et al.* (1987) is based on quantitative measurements of PGK-1 activity in whole embryos and ICMs of day-4 and -5 embryos. Because of the disproportionately low activity of PGK-1 found in the ICM of day-5 embryos, the authors concluded that the *Pgk-1* gene is not expressed in this tissue prior to day 6, the only activity detected there being the product of prezygotic *Pgk-1* transcripts. Fundele *et al.* (1987) proposed that in each of the early embryonic tissues, *Pgk-1* is activated only following inactivation of one of the X chromosomes. The authors do not specify the nature of the postulated relationship between X-inactivation and *Pgk-1* activation. However, their conclusion that *Pgk-1* expression (both maternal and paternal) is delayed up to day 6 in the ICMs of all embryos, 50 % of which would be expected to be male, implies that *Pgk-1* expression cannot be triggered by X-inactivation *per se*. Rather, timing of *Pgk-1* expression would have to be determined by a particular stage of differentiation, reached by primitive ectoderm cells of both male and female embryos by day 6; X-inactivation would be only an event marking the attainment of this differentiation stage by XX embryos (Monk and Harper, 1978). In this interpretation, the model of Fundele *et al.* (1987) is inconsistent with our finding of expression of the *Pgk-1* transgene in the ICM of day-4 embryos, two days before X-inactivation would take place in these cells. The paternal transmission of the *Pgk-1* transgene eliminates any uncertainty as to the prezygotic *versus* embryonic origin of the PGK-1A activity.

The finding that ICM cells have the capacity to express the *Pgk-1* gene on day 4 (this report), and the measured low activity of PGK-1 in ICM cells before day 6 (Fundele *et al.* 1987) can be accommodated by two alternative explanations. (1) The maternal X-linked *Pgk-1* gene is expressed throughout the embryo beginning on day 4; the relatively low activity of PGK-1 in the ICM is due to production of smaller quantities of the enzyme by these cells. (2) Neither of the X-linked *Pgk-1* genes is expressed in the ICM prior to day 6; the inactivity of these genes, however, is due to their particular chromosome location, and not to non-permissive cellular conditions.

The view that ICM cells do express the *Pgk-1* gene before day 6 is in agreement with the measurements of PGK-1 activity in isolated ICMs, reported by Papaioannou *et al.* (1981). These measurements indicated a sharp increase in PGK-1 activity in both whole embryos and dissected ICMs, between days 5 and 6. Since PGK-1 translated from prezygotic maternal mRNAs declines to very low levels by day 4, the increase in enzyme activity is best explained by expression of the *Pgk-1* gene in the embryo, and in the ICM in particular, prior to day 6. Interestingly, the percentage of the total PGK-1 activity attributable to the ICM was found to be higher than the percentage of embryo cells included in the ICM at that stage (50 % and 20 % respectively, for day-5 embryos – Papaioannou *et al.* 1981; Handyside and Hunter, 1986). This finding differs markedly from the measurements of Fundele *et al.* (1987), but is in accordance with our observation that transgenic PGK-1A activity was relatively higher in ICMs, than in whole, day-4 embryos.

The conclusion that ICM cells are expressing the *Pgk-1* gene before X-inactivation is also corroborated by the finding of PGK-1 activity in embryonic stem (ES) cells (Martin, 1981; Evans and Kaufman, 1981; Axelrod, 1984). In terms of their differentiation stage, developmental potential and antigenic properties, the ES cells and the related pluripotential embryonal carcinoma (EC) cells are believed to correspond to primitive ectoderm cells of approx. day-6 embryos (Martin, 1981; Evans and Kaufman, 1981; Robertson and Bradley, 1986), before X-inactivation has taken

place. The expression of *Pgk-1* by these cells, and particularly the expression of both copies of the gene in ES cells with two active X chromosomes (demonstrated in $Pgk\text{-}1^a/Pgk\text{-}1^b$ heterozygotes) (Martin and Lock, 1983), is inconsistent with the central postulate of the model of Fundele *et al.* (1987), which makes X-inactivation a prerequisite for *Pgk-1* expression.

On the basis of these data and considerations, we favor the view that $Pgk\text{-}1^m$ is expressed in the ICM cells as early as day 4; the absence of the $Pgk\text{-}1^p$ product before day 6 must be attributed to factors indigenous to the X chromosome region in which the gene resides. Our data shed no light on the nature of these factors.

The late expression of the paternal *Pgk-1* has an intriguing parallel in the behavior of the paternal X chromosome in early mouse development. In the first embryonic tissues to differentiate, namely, the trophectoderm on day 4 and the primitive endoderm on day 5, X^p is preferentially or exclusively inactivated (Takagi and Sasaki, 1975; West *et al.* 1977; Frels *et al.* 1979; Frels and Chapman, 1980; Papaioannou and West, 1981; Takagi *et al.* 1982). The differential features allowing X^p to be singled out for inactivation appear to fade out by day 6, when X-inactivation takes place in the embryonic ectoderm: in this tissue, both X chromosomes are equally likely to become inactivated (West *et al.* 1977; Takagi, 1983). The removal of the imprint from the X chromosome thus coincides with the onset of expression of the paternal *Pgk-1* allele. The molecular nature of the X chromosome imprint is unknown, but its effect is likely to be mediated through the X chromosome region which initiates the process of X-inactivation, termed X-inactivation center (Russell, 1963; Grumbach *et al.* 1963; Russell and Montgomery, 1970; Therman *et al.* 1974; Takagi, 1980) or X chromosome controlling element (*Xce*) (Cattanach and Isaacson, 1967; Cattanach, 1975; Johnston and Cattanach, 1981). The *Pgk-1* gene is very closely linked to *Xce*, with a genetic distance between the two loci of about 1–4 cM (Cattanach and Papworth, 1981; Krietsch *et al.* 1986; Lyon, 1989). The close proximity of *Pgk-1* to the locus controlling X-inactivation, and the coincident timing of expression of $Pgk\text{-}1^p$ and randomization of the process of X-inactivation, suggest the possibility that *Pgk-1* and *Xce* are coordinately regulated. This regulation may be envisaged as a localized, limited-spread X-inactivation of the paternal X, initiated at the *Xce*, and encompassing *Pgk-1*, but not the more distant *Hprt* and α-galactosidase genes; while it lasts, this chromatin modification would prevent the paternal *Pgk-1* allele from being expressed, and would make the paternal X the exclusive choice for inactivation. If this model is correct, other loci in the chromosome region between *Pgk-1* and *Xce* would be expected to be similarly influenced by the X-inactivation center. It would be interesting to determine if a reporter gene, inserted in this area by targeted mutagenesis, would show delayed expression upon paternal inheritance. The rapid advance in the cloning and analysis of genes surrounding *Xce* may identify other endogenous genes in this region, which are expressed prior to day 6 and allow determination of the time of activation of the paternal allele.

In line 55 the transgene had integrated in a chromosomal region known to be subject to imprinting. (The proximal region of chromosome 11, where the 94-K transgene was provisionally assigned is also subject to imprinting; however, this line will not be discussed here, pending verification of the assignment.) Mice inheriting both copies of the proximal region of chromosome 17 from the father, have decreased viability compared to normal mice or mice inheriting both copies of this region from the mother (Lyon and Glenister, 1977). Another manifestation of the imprinting of this region is the heterozygous lethality of the hairpin-tail (T^{hp}) deletion (Bennett, 1975; Silver *et al.* 1979) when inherited from the mother, but not from the father (Johnson, 1975). It was proposed that a gene (or genes) in this region of chromosome 17, are inactivated or suppressed subsequent to passage of the chromosome through the male germ line (McLaren, 1979). Winking and Silver (1984) further defined the target of imprinting within the T^{hp} deletion as a small segment of 17, designated T-associated maternal effect (*Tme*) locus. *Tme* is located in the A3 band of chromosome 17 (Lyon, 1989), the same region to which the *Pgk-1* transgene was mapped by *in situ* hybridization. The strong expression of the paternally transmitted *Pgk-1* transgene reinforces the conclusion, that if the effect of the imprint is to inactivate genes on 17^p, the imprint must be carried by a rather restricted area in the A3 region of the chromosome, leaving closely linked genes unaffected (Winking and Silver, 1984).

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