X-Linked Gene Expression in the Virginia Opossum: Differences Between the Paternally Derived *Gpd* and *Pgk-A* Loci

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ABSTRACT

Expression of X-linked glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase-A (PGK-A) in the Virginia opossum (*Didelphis virginiana*) was studied electrophoretically in animals from natural populations and those produced through controlled laboratory crosses. Blood from most of the wild animals exhibited a common single-banded phenotype for both enzymes. Rare variant animals, regardless of sex, exhibited single-banded phenotypes different in mobility from the common mobility class of the respective enzyme. The laboratory crosses confirmed the allelic basis for the common and rare phenotypes. Transmission of PGK-A phenotypes followed the pattern of determinate (nonrandom) inactivation of the paternally derived *Pgk-A* allele, and transmission of G6PD also was consistent with this pattern. A survey of tissue-specific expression of G6PD phenotypes of heterozygous females revealed, in almost all tissues, three-banded patterns skewed in favor of the allele that was expressed in blood cells. Three-banded patterns were never observed in males or in putatively homozygous females. These patterns suggest simultaneous, but unequal, expression of the maternally and paternally derived *Gpd* alleles within individual cells (*i.e.*, partial paternal allele expression). The absence of such partial expression was noted in a parallel survey of females heterozygous at the *Pgk-A* locus. Thus, it appears that *Gpd* and *Pgk-A* are X-linked in *D. virginiana* and subject to preferential paternal allele inactivation, but that dosage compensation may not be complete for all paternally derived X-linked genes. The data establish the similarity between the American and Australian marsupial patterns of X-linked gene regulation and, thus, support the hypothesis that this form of dosage compensation was present in the early marsupial lineage that gave rise to these modern marsupial divisions. In addition, the data provide the first documentation of the differential expression of two X-linked genes in a single marsupial species. Because of its combination of X-linked variation, high fecundity, and short generation time, *D. virginiana* is a unique model for pursuing questions about marsupial gene regulation that have been difficult to approach through studies of Australian species.

THE achievement of X-linked gene dosage compensation in a variety of higher eukaryote taxa has served as a focal point for studies concerning the cyclic activation and deactivation of gene loci. Such studies historically have involved *Drosophila* (LUCCHESI 1983) and eutherian mammals (LYON 1983), organisms that accomplish dosage compensation in distinctly dissimilar ways. In the former, genes on the single X chromosome of males are approximately twice as active as those on each of the two X chromosomes of females. However, in female eutherians there occurs early in embryogenesis a "random" inactivation of one or the other of the two X chromosomes in each cell (reviewed by LYON 1974, 1983; GARTLER and RIGGS 1983). This results in the presence of only a single active X chromosome in the cells of both sexes. Unfortunately, the enormous evolutionary distance between these taxa confounds interpretation of these differences in the context of fundamental gene regulatory mechanisms.

Among mammals, the eutherian/metatherian (marsupial) dichotomy provides another, more subtle distinction in strategies of X-linked gene regulation. In contrast to the random X-inactivation pattern in cells of eutherian females, dosage compensation in all Australian metatherians examined [members of the families Macropodidae (kangaroos and related forms), Phalangeridae (Australian possums), and Dasyuridae (Australian marsupial "cats" and "mice")] is thought to result from the exclusive, determinate (nonrandom) inactivation of genes on the paternally derived X chromosome (reviewed by GRAVES 1983; VANDEBERG *et al.* 1983; 1986). Additionally, examination of cultured cells cloned from females heterozygous for X-linked genes revealed that the paternal X chromosome is only partially inactive in some cell types of certain metatherian species (COOPER *et al.* 1977a; VANDEBERG, COOPER and JURGELSKI 1979). In eutherians, only a single X chromosome is normally active in all cell types except dictyate oocytes and certain early embryonic cells (reviewed by EPSTEIN 1983; VANDEBERG 1983).

The mechanism of dosage compensation in American marsupials (Didelphidae and Caenolestidae) has not been documented. However, existing data suggest that it follows the Australian marsupial pattern of paternal *X* inactivation. Rare electrophoretic variants of thyroxine-binding protein (T_4BP) (DAVIS and JURGELSKI 1973), glucose-6-phosphate dehydrogenase (G6PD) (VANDEBERG, COOPER and JURGELSKI 1979; VANDEBERG *et al.* 1983), and phosphoglycerate kinase-A (PGK-A) (VANDEBERG *et al.* 1983) have been discovered in sera and hemolysates of the Virginia opossum (*Didelphis virginiana*). In each case, regardless of sex, the rare variant phenotypes consisted of a single electrophoretic band of mobility different from that of the common form; no individuals displaying multiple bands in blood were detected. These patterns are inconsistent with either autosomal codominant or *X*-linked random inactivation models of inheritance. Furthermore, the loci encoding G6PD (*Gpd* locus) and PGK-A (*Pgk-A* locus) have been found to be *X*-linked in all eutherian and metatherian species examined, and an *X*-linked thyroxine-binding globulin has been identified in humans (DAIGER *et al.* 1981) and baboons (LOCKWOOD *et al.* 1984). According to the mammalian *X*-chromosome conservation hypothesis (OHNO 1967, 1969), all mammalian *X* chromosomes are orthologous and highly conserved; hence, genes known to be *X*-linked in one or a few species will likely be found to be *X*-linked in all other mammalian species as well. This hypothesis has been repeatedly corroborated in many eutherian and metatherian species (COOPER *et al.* 1977b; OHNO 1983; WILLARD 1983), and there is no reason to believe it does not hold equally for *D. virginiana*.

In this paper we present data from natural populations and from controlled laboratory crosses that indicate that *Gpd* and *Pgk-A* are indeed *X*-linked in *D. virginiana*, and that they behave in a manner consistent with the model of determinate inactivation of the paternally derived *X* chromosome in somatic cells. Additionally, we show that partial expression of the paternal *Gpd* allele, but not of the paternal *Pgk-A* allele, is detectable in almost all tissues examined, and that the degree of partial expression is tissue specific. These observations constitute the first evidence of paternal *X*-chromosome inactivation in any non-Australian marsupial species, establish the occurrence of locus-specific expression of *X*-linked genes within a single marsupial species, and indicate that the extent and locus specificity of partial expression of *X*-linked genes vary among marsupial taxa.

MATERIALS AND METHODS

***Didelphis virginiana* breeding colony:** The colony was initiated with adults and pouch young from natural populations in Texas, Florida, and Wisconsin (see RESULTS). The colony is maintained in a temperature (22–25°) and humidity (55–65% RH) controlled building under a natural lighting regimen (filtered skylight). Several different caging schemes have been utilized successfully: (1) individual, interconnectable stainless steel mesh cages (90 × 56 × 56 cm; h × l × w) each with an external aluminum nest box; (2) larger (70 × 150 × 95 cm), galvanized steel mesh cages with two or more external nest boxes for housing male/female pairs or females rearing litters; and (3) group cages (walled pens with concrete floors varying in size from 11.5 to 34.5 m^2 floor area) with internal nest boxes for housing single males with multiple females.

The animals are supplied water and a pelleted fox diet ("maintenance" grade; Milk Specialty Products, New Holstein, Wisconsin) *ad libitum*. The diet is augmented once weekly with liver and twice weekly with fresh fruit or vegetables. A liquid vitamin supplement (Vi-Daylin Plus Iron, Ross Laboratories, Columbus, Ohio) is added to the food daily (1.0 ml/100 g food).

We have successfully utilized several approaches for breeding *D. virginiana* in our colony (*e.g.*, method of RODGER and BEDFORD 1982, for lactating females; method of JURGELSKI and PORTER 1974, for nonlactating females), but we have now adopted a group caging approach exclusively for all routine breeding protocols. This method involves continual access of one male and several females in the group cages. The females are monitored twice weekly for the presence of pouch young beginning 15 days after exposure to males (the gestation period of *D. virginiana* is 14–15 days). Upon the discovery of pouch young, the female and her litter are removed to a separate cage and maintained there until the litter is weaned (approximately 100 days).

Biochemical methods: Routine G6PD and PGK-A typings of wild *D. virginiana* and of those produced within the colony were performed by electrophoretic analysis of blood clots or ear pinna samples. Blood was drawn via cardiac puncture and allowed to clot at room temperature. Clots were separated from serum and stored at −80° until used. Ear pinna samples were obtained by excision with surgical scissors of a small (approximately 0.25 cm^2) piece of tissue from the top of the pinna. The ear samples were generally used immediately, but could be stored for several months at −80° without adverse effect on the electrophoretic pattern. Additional tissues were collected by dissection of animals after sacrifice (T-61 euthanasia solution; American Hoechst Corporation, Somerville, New Jersey) or spontaneous death.

Samples (clot or tissue) were homogenized in an ice cold "homogenizing solution" containing deionized water, DL-dithiothreitol (5%, w:v), and $NADP^+$ (0.5%, w:v) using a motor-driven Teflon/glass homogenizer. The ratio of sample to homogenizing solution was varied according to tissue and specific goal (*i.e.*, routine typing or examination of gene expression). Homogenates were centrifuged at 23,000 × *g* for 20 min (at 4°) to obtain clear supernatant "extracts" for electrophoresis.

PGK-A phenotypes were visualized on starch gels after electrophoresis and staining according to the method of VANDEBERG, COOPER and CLOSE (1976). G6PD electrophoresis was performed on a vertical polyacrylamide slab gel system consisting of a 0.38 M Tris-HCl buffered running gel (pH 8.9) of 7.5% acrylamide (1:27, bis:acrylamide), with a 3.75% acrylamide (also 1:27) stacking gel buffered with 61 mM Tris-HCl (pH 6.7). The electrode buffer was 98 mM Tris, 3.8 mM glycine (pH 8.2). $NADP^+$ was added to the buffer in the cathodal electrode chamber to a final concentration of 0.13 mM. Sample extracts were mixed in equal parts with a 40% sucrose solution before their application to the sample wells. Electrophoresis was conducted at ap-

TABLE 1

Frequency of rare phenotypes of glucose-6-phosphate dehydrogenase (G6PD) and phosphoglycerate kinase-A (PGK-A) in blood samples of *D. virginiana* from several locations in North America

Location	No. examined				Rare phenotypes observed[a]	
			UN[b]	T[c]	G6PD	PGK-A
Texas	194	293	29	516	0	4 (0.008)[d]
Florida	105	119[e]	0	224	7 (0.031)[f]	0
Wisconsin	72	45	54	171	0	0
North Carolina	81	44	4	129	1 (0.008)[g]	0
Arkansas/Missouri	18	27	0	45	0	0
Maryland	5	4	0	9	0	0
Pennsylvania	5	4	0	9	1 (0.111)[g]	0
Virginia	4	3	0	7	0	0
Total	484	539	87	1110	9 (0.008)	4 (0.004)

[a] Number of individuals with rare phenotypes observed (numbers in parentheses are relative frequencies). G6PD[b] and PGK-A[b] were the common phenotypes in all populations.
[b] Sex unknown.
[c] Total number of animals examined.
[d] Three were PGK-A[c], one was PGK-A[a], all were males.
[e] One male was not examined for G6PD.
[f] All were G6PD[a]; six were females, one was a male.
[g] G6PD[a] female.

proximately 21 V/cm for 6–8 hr at 4°. G6PD activity was visualized by soaking the gel in the dark at 37° in a 0.1 M Tris-HCl buffer (pH 8.0) containing Na_2-D-glucose-6-phosphate (1.0 mg/ml), $NADP^+$ (0.08 mg/ml), MTT tetrazolium (0.1 mg/ml), and phenazine methosulfate (0.025 mg/ml).

RESULTS

G6PD and PGK-A phenotypes; variation in natural populations: In the course of identifying and purchasing animals for the establishment of the breeding colony, blood samples (N = 1063) or ear pinna biopsies (N = 47) from 1110 wild *D. virginiana* were examined for G6PD and PGK-A phenotypes. We found that variation in these enzymes was rare and unequally distributed among populations (Table 1). Because most of the blood and ear samples were obtained from commercial animal dealers with several source populations within a given state, precise ascertainment of trapping localities within states was not possible.

Two electrophoretic mobility classes of G6PD and three classes of PGK-A were detected in blood clot extracts (because clots consist primarily of a mixed population of blood cells and various blood proteins, we hereinafter refer to clot samples as blood samples). The G6PD classes were designated G6PD[a] and G6PD[b] for the more and less anodally migrating types, respectively. The PGK-A phenotypes were designated PGK-A[a], PGK-A[b], and PGK-A[c] in order of decreasing anodal mobility. Each of the three PGK-A phenotype classes resolved as a single band of activity on starch gels in both blood and ear pinna extracts. Examples of ear pinna PGK-A patterns (identical to blood patterns) are shown in Figure 1. The blood G6PD phenotypes also were single banded in most individuals, as were most of the ear pinna G6PD patterns. (A weak subband migrating anodal to the major G6PD band can be seen in several samples in Figures 2 and 4. This subband should not be mistaken for an allelic isozyme of the major G6PD component.) However, females exhibiting the rare G6PD[a] phenotype in blood extracts exhibited three G6PD bands in ear pinna extracts (Figure 2A, Channel 3). In addition, blood of one (but only one) female exhibiting the G6PD[a] phenotype under the normal staining protocol (15–20 min incubation) was seen to display, on gels stained exhaustively (45–60 min incubation), a three-banded phenotype similar to that seen in ear pinna, but with the two less anodally migrating bands staining relatively less intensely than those in the ear pinna pattern. Based on the low frequency of the G6PD[a] phenotype in nature, these females almost certainly were Gpd^a/Gpd^b heterozygotes (genotype designates allele with greater expression/allele with lesser expression). Because G6PD is a dimeric enzyme, the three-banded (presumed A_2 homodimer, AB heterodimer, and B_2 homodimer) pattern suggests the simultaneous, but unequal, expression of both the maternally and paternally derived *Gpd* genes within individual cells. We have designated this pattern as the G6PD[a(b)] phenotype (the parenthetical designation for b indicates that B_2 is the weaker of the homodimer bands). No females exhibiting the alternative G6PD[b(a)] phenotype in ear pinna or blood extracts were detected in our study. However, only 47 of the 1110 animals screened were typed via ear biopsy (only two of the nine G6PD[a] individuals were detected through ear biopsy samples). Furthermore, any Gpd^b/Gpd^a heterozygote females that might otherwise have exhibited weak A_2

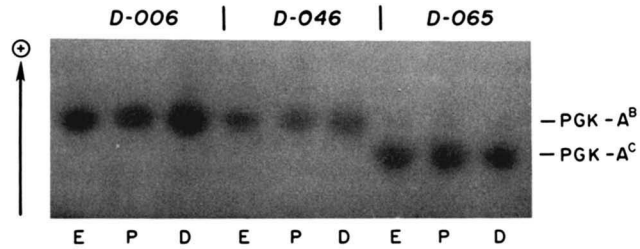

FIGURE 1.—Starch-gel zymogram of PGK-A patterns in esophagus (E), ear pinna (P), and diaphragm (D) extracts from homozygous (D-006) and heterozygous (D-046 and D-065) females of the pedigree shown in Figure 3A. D-046 and D-065 are reciprocal heterozygotes (*Pgk-A*^{b}/*Pgk-A*^{c} and *Pgk-A*^{c}/*Pgk-A*^{b}, respectively).

and AB bands in exhaustively stained blood samples would have been indistinguishable from *Gpd*^{b}/*Gpd*^{b} females or *Gpd*^{b}/*Y* males because of interfering subband activity superimposed on the A_2 and AB zones on heavily stained gels (see Figure 2A, channels 1 and 2).

Phenotypes in a family segregating for PGK-A^b and PGK-A^c: A three generation pedigree of a family segregating PGK-A phenotypes is shown in Figure 3A. The male PGK-A^c variant (D-001) was mated to an unrelated, phenotypically normal (PGK-A^b) female. One of five progeny (sex undetermined) was lost at 2 weeks postpartum, but one male and three females (presumed heterozygotes) survived to adulthood. Blood and ear tissue extracts of each member of this family were analyzed for PGK-A expression. The sire expressed the same single-banded PGK-A^c phenotype in blood and ear pinna, whereas the dam and all four offspring uniformly exhibited the single-banded PGK-A^b phenotype in each of these tissues. One of three grandprogeny (D-065), produced by a daughter (D-044) and an unrelated PGK-A^b male (D-053), exhibited the PGK-A^c phenotype in both tissues.

G6PD phenotypes in wild variant females and in families segregating for G6PD^a and G6PD^b: Three females exhibiting the rare, three-banded G6PD$^{a(b)}$ phenotype in ear pinna extracts were acquired. Blood samples of one of these females (D-054) exhibited the

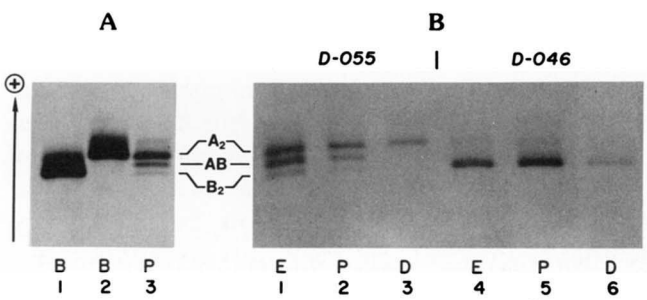

FIGURE 2.—Polyacrylamide gel zymograms of G6PD phenotypes in extracts of blood (B), ear pinna (P), esophagus (E), and diaphragm (D) of homozygous and heterozygous *D. virginiana*. A, Channel 1 = D-046 (homozygous; *Gpd*^{b}/*Gpd*^{b}), channel 2 = D-054 (heterozygous; *Gpd*^{a}/*Gpd*^{b}), channel 3 = D-055 (heterozygous; *Gpd*^{a}/*Gpd*^{b}). B, Channels 1–3 = D-055 (heterozygous; *Gpd*^{a}/*Gpd*^{b}), channels 4–6 = D-046 (homozygous; *Gpd*^{b}/*Gpd*^{b}).

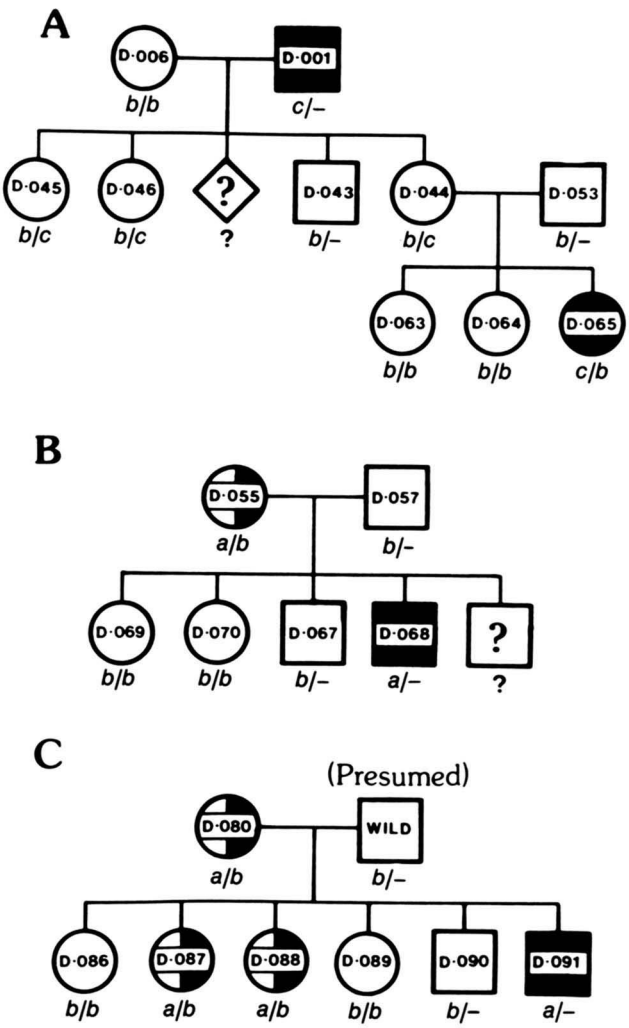

FIGURE 3.—A, Pedigree of a *D. virginiana* family segregating for PGK-A. Circles represent females; squares represent males; diamond indicates early infant death (sex and PGK-A phenotype undetermined). Phenotypes are indicated by shading of symbol: closed = PGK-A^a; open = PGK-A^b. Presumed genotypes (maternal allele/paternal allele) are listed beneath each symbol (dash indicates hemizygous condition). B and C, Pedigrees of *D. virginiana* families segregating for G6PD. Closed symbols = G6PD^a; open symbols = G6PD^b; half-closed symbols = G6PD$^{a/(b)}$.

single-banded G6PD^a phenotype. The second female (D-080) displayed a strong A_2 band and very weak AB and B_2 bands in blood. Blood from the third female (D-055) was not examined.

Female D-055 was mated with a phenotypically normal (G6PD^b in ear pinna and blood extracts) male and produced five progeny (Figure 3B). One male died in infancy, but two females and two males reached adulthood. The two female offspring (D-069 and D-070) each displayed identical single-banded G6PD^b phenotypes in ear and blood samples, and one male (D-067) also showed the G6PD^b phenotype in ear pinna (blood from this animal was not examined). The other male (D-068) exhibited the single-banded G6PD^a phenotype in both tissue types.

Female D-054 died without reproducing in our

colony, but D-080 arrived bearing a litter of six pouch young sired by an unknown male (presumed G6PD^b on the basis of rarity of the G6PD^a phenotype in natural populations). Ear pinna extracts from the six offspring exhibited the following phenotypes: G6PD^b in two females and one male, G6PD^a in one male, G6PD$^{a(b)}$ in two females (Figure 3C). Blood samples have not been collected from these animals.

The most parsimonious interpretation (but see DISCUSSION for alternatives) of the foregoing results is that *Gpd* and *Pgk-A* are *X*-linked and subject to determinate *X*-chromosome inactivation, but that in ear pinna cells (and in blood cells of some individuals) the *Gpd* allele on the inactive *X* chromosome is partially expressed (the presence of an inactive *X* chromosome has not been documented cytologically in any American marsupial; but to facilitate discussion, we assume that one is present in the adult somatic cells of *D. virginiana* females, and that the unexpressed or partially expressed *Pgk-A* and *Gpd* alleles mark this chromosome). The PGK-A pedigree (Figure 3A) indicates that it was the paternally derived *Pgk-A* allele that was inactive. Under the assumption that the sire in the second G6PD pedigree (Figure 3C) was, in fact, G6PD^b, the combined data from the two G6PD families indicate that it was the paternally derived allele that was inactive (or less active) at the *Gpd* locus as well.

G6PD and PGK-A phenotypes in other tissues: Based on the paternal *X*-inactivation hypothesis, we undertook a survey of tissue-specific expression of *Gpd* and *Pgk-A* alleles in heterozygous females. G6PD and PGK-A phenotypes in tissue extracts (Table 2) were examined on gels stained exhaustively to detect the presence of minute levels of enzyme activity attributable to partial activity of the paternal allele. Presumed homozygous females and hemizygous males served as controls for the presence of nonallelic variation (subbanding).

Based on the pedigree in Figure 3A, sisters D-044, D-045, and D-046 must have been heterozygous at the *Pgk-A* locus, yet each expressed the same single-banded PGK-A^b phenotype as their mother (presumed *Pgk-A*^{b}/*Pgk-A*^{b}) in each of the 20 tissues and organs examined (Table 2).

The tissue survey of G6PD phenotypes was conducted on two wild-caught females, D-054 and D-055, presumed to be heterozygous on the basis of three arguments. First, both females exhibited the three-banded G6PD$^{a(b)}$ phenotype in ear pinna extracts. Second, the low frequency of the *Gpd*^{a} allele makes it unlikely that D-054 and D-055 were *Gpd*^{a}/*Gpd*^{a} homozygotes. Finally, D-055 transmitted both the G6PD^a and G6PD^b phenotypes to her sons (Figure 3B).

The survey revealed a broad range of G6PD phenotypes suggestive of intertissue and interindividual variation in expression of the presumed paternal *Gpd* allele (*Gpd*^{b}), as judged by the relative intensities of the A_2, AB, and B_2 bands. We did not quantify these relative intensities, but the different phenotypes could be grouped unambiguously into five distinct classes based on the relative levels of expression of the two *Gpd* alleles. In class 0, the A_2 homodimer was intensely active, but no evidence of AB or B_2 activity was detectable. In class 1, AB was clearly visible but much weaker than the A_2 homodimer, while B_2 was barely distinguishable. In class 2, the B_2 homodimer was plainly visible, but distinctly less intense than A_2, whereas the AB heterodimer was equally or just slightly less intense than A_2. In class 3, the activity of the A_2, AB, and B_2 molecules approached a 1:2:1 ratio. The ratios of A_2, AB, and B_2 activity in each of

TABLE 2

Relative expression of maternal and paternal alleles at the *Gpd* and *Pgk-A* loci in female *D. virginiana* inferred from relative activities of allelic isozymes of G6PD and PGK-A in heterozygous females

	Gpd locus[a]		*Pgk-A* locus[b]
Tissue/Organ	D-054	D-055	c
Adrenal gland	NT	1	0
Bladder	1	$\underline{1}$	0
Blood	0	1[d]	0
Brain	1	1	0
Diaphragm	0	1	0
Ear pinna	2	2	0
Esophagus	NT	3	0
Eye (lens removed)	2	NT	0
Fallopian tube	0	NT	NT
Heart	1	$\underline{1}$	0
Intestine	U	U	0
Kidney	1	1	0
Lens	A	A	0
Liver	1	$\underline{1}$	0
Lung	0	1	0
Ovary	1	1	NT
Skeletal muscle	1	1	0
Spleen	A	1	0
Stomach	U	U	0
Tongue	2	1	0
Uterus	1	$\underline{1}$	0
Vagina	1	$\underline{2}$	0

[a] Numerals refer to phenotypic classes discussed in text. The numeric values indicate increasingly similar levels of expression of the two alleles: *e.g.*, class 0 exhibits expression of only one allele, while class 3 manifests near parity of expression of both alleles. A = class A (anomalous band ratio; see text), U = uninterpretable (see text), NT = not tested. Underlined class designation indicates that despite the placement of D-054 and D-055 into a single class type, the expression of the paternal allele was not equivalent between the two females; the underlined female exhibited greater expression of the paternal allele relative to that of the maternal allele than did the other female.

[b] 0 = No detectable activity of paternal allele, NT = not tested.

[c] Combined results of siblings D-044, D-045, and D-046 except that adrenal gland, eye, and lens were examined in D-044 alone.

[d] D-055 was not tested; this result is from female D-080.

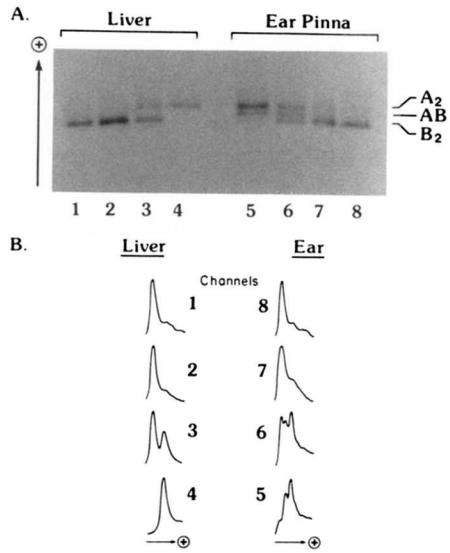

FIGURE 4.—A, Briefly stained polyacrylamide gel zymogram of G6PD patterns in liver and ear pinna of *D. virginiana*. Channels 1 and 8, Gpd^b/Y males; channels 2 and 7, Gpd^b/Gpd^b females; channels 3 and 6, 50:50 mixtures of Gpd^b/Gpd^b and Gpd^a/Gpd^b females; channels 4 and 5, Gpd^a/Gpd^b females. Note that although liver and ear pinna extracts of heterozygous females each exhibit multiple bands (A_2, AB, and B_2) on exhaustively stained gels, only A_2 is seen in liver extracts on gels stained briefly, as in this case. B, Densitometric scans of the G6PD patterns on the zymogram shown in Figure 4A. All scans were standardized vertically to an identical peak height for the tallest peak regardless of the optical density of that peak. The difference in peak heights in channel 3 is attributable to differences in levels of G6PD activity in the extracts from the two animals. Each homodimeric allelic isozyme has an anodal subband as indicated by the scans. In channels 3, 5, and 6 the subband of the B_2 homodimer is superimposed on the A_2 homodimer band.

these classes fit the patterns expected assuming random association of A and B subunits in all cells of the extracted tissue sample, but a fifth class of G6PD phenotypes, designated class A (anomalous band ratio) that did not fit such a pattern was also observed. Spleen and lens of D-054, and lens of D-055 exhibited three bands of G6PD activity, but in each case the AB heterodimer band was visibly weaker than either homodimer. The A_2 homodimer was the strongest band in each phenotype. Spleen of D-055 exhibited a normal class 1 pattern. The presence of the putative heterodimer band does indicate activity of both *Gpd* alleles with single cells in these tissue samples, but the abnormal activity ratios are inconsistent with a homogeneous activity-state cell population in these tissues (see DISCUSSION).

Nineteen of the tissues examined revealed some evidence of activity (partial) of the paternal *Gpd* allele (*i.e.*, showed a multiple-banded phenotype) in at least one female. We note that brief incubation of gels in the G6PD staining solution was sufficient to yield multiple-banded patterns in some tissues (*e.g.*, ear pinna, esophagus, heart), but not in all tissues tested (see Figure 4). For example, liver extracts from Gpd^a/Gpd^b females exhibited only a single strong A_2 band after 5–7 min incubation (thus appearing to be $G6PD^a$ and not $G6PD^{a(b)}$), but showed the AB and B_2 bands after prolonged (30–45 min) incubation. Such multiple bands were never observed in the homozygous (Gpd^b/Gpd^b) female tissues used as standards nor in ear pinna or blood extracts from males regardless of the length of incubation. Figures 2B and 1 contrast the patterns of partial expression of the paternal *Gpd* allele in three tissues from a Gpd^a/Gpd^b female with the single-banded PGK-A phenotypes seen in these same tissue-types in two reciprocal *Pgk-A* heterozygotes ($Pgk\text{-}A^b/Pgk\text{-}A^c$ and $Pgk\text{-}A^c/Pgk\text{-}A^b$).

G6PD patterns in the remaining two tissues, stomach and intestine, were uninterpretable inasmuch as the normal A_2, AB, and B_2 bands were absent. G6PD activity in these tissues consisted of one to four (depending on tissue and animal) irregularly spaced bands located anodal to the normal G6PD zone. These peculiar patterns appeared in some, but not all, males and homozygous females as well. We do not know the basis of these patterns. Examination of the retardation of relative mobilities of the normal and abnormal G6PD molecules on a series of gels of increasing polyacrylamide concentration (four gels; 5, 7, 9, and 11%; method of HEDRICK and SMITH 1968) revealed no major differences in the sizes of these molecules (data not shown); hence the normal and abnormal G6PD patterns did not represent different aggregation states of the G6PD subunits or holoenzymes.

Although it appears from these results that the paternal *Pgk-A* allele was unexpressed in all tissue types, and that the paternal *Gpd* gene was silent in blood, diaphragm, fallopian tube, and lung of D-054, it is possible that these genes were expressed in these tissues at a level below that detectable by our staining methods. In order to determine the sensitivity of our G6PD and PGK staining methods, we used the serial dilution method of KLEBE (1975) to determine the lower limit of expression of the less active (paternal) alleles relative to that of the more active (maternal) ones that would be detectable in extracts of selected tissues on our gels. Extracts of blood, kidney, brain, and intestine from $PGK\text{-}A^b$ females (both Pgk^b/Pgk^b homozygotes and Pgk^b/Pgk^c heterozygotes) were examined for extinction of the $PGK\text{-}A^b$ band in a twofold dilution series. Similarly, extracts of blood cells, esophagus, ear pinna, tongue, and diaphragm from Gpd^a/Gpd^b females were examined for extinction of the A_2, AB, and B_2 bands. The results (Table 3) indicated that activity of $PGK\text{-}A^c$ on the order of 1/64 to 1/32 of that of $PGK\text{-}A^b$ would have been detectable in tissues with high PGK-A activity such as blood, kidney, and brain. Even low activity tissues such as intestine would have exhibited two bands of activity if $PGK\text{-}A^c$ had at least 1/16 of the $PGK\text{-}A^b$ activity level. The situation for G6PD was similar to that for

TABLE 3

Activity extinction profiles for G6PD and PGK-A isozymes in serially diluted extracts of selected tissues of heterozygous females

	G6PD Isozyme[a]			PGK-A Isozyme[b]	
Tissue/Organ	A_2	AB	B_2	B	C
Blood	1/64[c]	NA[c]	NA[c]	1/64	NA
Esophagus	1/128	1/256	1/128	NT	NT
Ear pinna	1/64	1/32	1/16	NT	NT
Tongue	1/32	1/16	1/16	NT	NT
Diaphragm	1/32	1/8	1/1	NT	NT
Kidney	NT	NT	NT	1/64	NA
Brain	NT	NT	NT	1/32	NA
Intestine	NT	NT	NT	1/16	NA

Value indicates the last dilution in a twofold series of standard extracts in which activity of the particular isozyme was visible after exhaustive staining. Standard extracts were the supernatant fraction of 1:1 (weight tissue: volume buffer) homogenates. Dilutions are relative to full strength extracts (1/1 = full strength), NA = no activity detected at any dilution, NT = not tested.

[a] Genotype: *Gpd*^{a}/*Gpd*^{b}.

[b] Genotype: *Pgk-A*^{b}/*Pgk-A*^{c}.

[c] Sample from female D-054.

PGK-A. For example, in the blood extract from D-054, activities of the AB heterodimer and the B_2 homodimer would have to have been less than 1/64 of A_2 activity in order to have gone undetected.

The results of the dilution series also serve to reveal the range of variation in partial expression of the paternal *Gpd* allele among various tissues. As an extreme example, expression of the paternal *Gpd*^{b} allele was nearly equal to that of the maternal *Gpd*^{a} allele in esophagus from D-055 (the data in Table 3 indicate that A_2 and B_2 were equally active in esophagus, but it is obvious from Figure 2 that A_2 was somewhat more active than B_2; the insensitivity of the dilution method to this difference arises from the stepwise nature of the dilution procedure), while it produced no more than 1/32 as much product as *Gpd*^{a} in diaphragm of the same animal.

DISCUSSION

This paper presents the initial findings of an ongoing investigation of gene expression patterns and developmental events involved in X-chromosome dosage compensation in *D. virginiana*. The simplest interpretation consistent with the data is that X-chromosome dosage compensation in this species is the result of inactivation or reduced expression of genes on the paternally derived X chromosome; *i.e.*, paternal X-chromosome inactivation.

The transmission pattern of the PGK-A^c phenotype in the PGK-A pedigree indicates that the *Pgk-A*^{c} allele was present in the female progeny sired by D-001, but that it was not expressed. Nevertheless, one of these females subsequently passed the gene to a daughter (grandprogeny of D-001) in which it was expressed. This is the pattern of transmission expected for a paternally derived X-linked gene subject to paternal X inactivation. The complete absence of multiple-banded PGK-A phenotypes among the 1110 blood and ear samples from natural populations also is consistent with this model of X inactivation. Because the phenotype of the sire of the G6PD family in Figure 3C could be inferred only from population phenotype frequencies, the pedigree data for G6PD cannot discriminate rigorously between inactivation of the maternally or the paternally derived allele. However, no verified case of determinate maternal X-linked gene inactivation has been reported in mammals, and *Gpd* and all other X-linked genes studied in Australian marsupials exhibit inactivation of the paternally derived allele (VANDEBERG *et al.* 1983, 1986). These observations, together with the high probability that the phenotype of the sire of D-080's litter was G6PD^b, lead us to believe that the pattern of *Gpd* expression observed in this study reflects the paternal X-inactivation scheme.

An alternative model to explain the patterns of X-linked gene expression we observed is random X inactivation followed by cell selection. This process is known to occur in blood of eutherian females heterozygous for particular X-chromosome structural variants (discussed by GARTLER 1976; MIGEON 1982; MOHANDAS and SHAPIRO 1983) and could account for the patterns of *Pgk-A* and *Gpd* expression we observed (although additional explanation would be required to account for the G6PD phenotypes observed in tissues other than blood; see discussion beyond). However, in the PGK-A pedigree, this model would require selection against the paternally derived X chromosome in the female heterozygotes D-044, D-045, and D-046 and selection favoring the same chromosome in female D-065 (daughter of D-044). In general, such alternation of selective advantage of chromosomes between generations is not an expected feature of the random inactivation/cell selection model, although in this particular pedigree it could be explained by postulating a recombinational event in the germ line of D-044 or selective differences between alternative chromosomes carrying the *Pgk-A*^{b} allele. We believe the combined evidence from pedigree analyses of *D. virginiana* and all Australian species studied weighs heavily against such contrived explanations.

A slight modification of the foregoing cell selection hypothesis is random X inactivation followed by cell selection based entirely on the parental source of the X chromosomes. The data from the present investigation (and all data from Australian marsupials) are entirely consistent with this hypothesis. Nevertheless, it is well established that preferential or exclusive inactivation of paternal X chromosomes does occur in

the extraembryonic tissues of rodents and humans (reviewed by EPSTEIN 1983; VANDEBERG 1983), whereas cell selection based on parental source of the *X* chromosomes has never been shown to be a feature in the mammalian developmental process. These facts lead us to favor the paternal *X*-inactivation hypothesis over the cell selection model.

We have interpreted the presence of the three G6PD bands observed electrophoretically in extracts of ear pinna and other tissues of D-055 and other presumed heterozygous females to indicate that both alleles are expressed at the *Gpd* locus in most tissues of female *D. virginiana*. The skewed activity ratio of the A_2, AB, and B_2 bands indicates that one allele (presumed paternal) is expressed to a lesser extent than the other in most or all tissues. But variation in this expression ratio (Table 2) shows that regulation of the paternal *Gpd* allele may be tissue dependent and subject to individual variation. Because this does not seem to be the case for *Pgk-A*, it can be concluded that *Gpd* and *Pgk-A* are not regulated in a completely coordinated manner in *D. virginiana*. Although this is suspected to be the case in Australian marsupials as well (VANDEBERG *et al.* 1983), the present data provide the first conclusive demonstration of differential expression of *X*-linked genes in a single marsupial species.

Two distinct hypotheses of gene activity are consistent with the G6PD phenotypes observed in extracts from female *D. virginiana* heterozygous for *Gpd*. One postulates a homogeneous cell population in which the paternal allele is expressed at some level less than that of the maternal allele. This is the conventional, partial activity model (see VANDEBERG *et al.* 1983, 1986). The second possibility is a tissue mosaic consisting of two distinct cell populations, one expressing only the maternal allele, and the other expressing both the maternal and paternal alleles equally (of course more complex mosaics are possible as well). Such mosaicism has not been documented for *Gpd* (or other *X*-linked loci) in mammalian adult somatic tissues, but MIGEON *et al.* (1985) have reported such a situation in cells cultured from extraembryonic membranes of early first trimester human abortuses. Cells cloned from these cultures exhibited a broad range of activity states including expression of the maternal allele only, expression of the paternal allele only, and several cases of simultaneous expression (equal and unequal) of both alleles.

In the present study, most tissues exhibited G6PD dimer ratios consistent with random association of A and B subunits. The partial expression hypothesis predicts such ratios in all tissues regardless of the relative levels of expression of the maternal and paternal alleles. Under the mosaic hypothesis, only a limited set of cell population ratios would be expected to yield patterns resembling random association of subunits, but such ratios can be proposed on an *ad hoc* basis to explain any of the phenotypes observed (including the seemingly anomalous ratios noted in lens of D-054 and D-055, and spleen of D-054). Our data cannot discriminate between the partial activity and mosaic hypotheses, nor are the two models of gene expression mutually exclusive. However, cloning experiments have confirmed the activity of both *Gpd* alleles in fibroblasts derived from adult female kangaroos and failed to reveal any evidence of mosaic expression among such clones (JOHNSTON *et al.* 1978). In all cases, the clones exhibited the same phenotype (apparent heterodimer formation) as the mass cultures from which they were derived. We view the partial activity hypothesis as the most parsimonious model consistent with the G6PD phenotypes observed in *D. virginiana* and we believe it should be the model favored in the absence of contrary findings.

Simultaneous expression of maternal and paternal *Gpd* alleles has been observed in cultured cells from kangaroos (JOHNSTON *et al.* 1978; JOHNSTON and ROBINSON 1986), but the expression of both *Gpd* genes in adult tissues, as seen in *D. virginiana*, has not been observed in kangaroos or any other marsupial species. However, a similar phenomenon has been seen at the *Pgk-A* locus in some tissues of female kangaroos (*Macropus parryi, M. giganteus,* and *M. fuliginosus*) and brush-tailed possums (*Trichosurus vulpecula*) (VANDEBERG *et al.* 1983). In these species, only the maternally inherited *Pgk-A* allele was expressed in many tissues, but in some tissues (which varied between species) the paternally derived allele was also expressed at a low level. Nothing like the nearly ubiquitous expression of both *Gpd* alleles in female *D. virginiana* tissues has been noted for any *X*-linked gene in Australian species. Thus, the pattern of *X*-linked gene expression in *D. virginiana* differs from that of all Australian species studied both in the tissues and in the loci at which partial expression is observed (reviewed by GRAVES 1983; VANDEBERG *et al.* 1983, 1986). The basis for this distinction is unknown, but it could be something as simple as a chromosomal rearrangement that altered the relative proximities of the *Gpd* and *Pgk-A* loci relative to a controlling element that influences the activities of genes within its particular chromosome segment (discussed by VANDEBERG *et al.* 1986).

An important question that has remained unanswered until now is whether the evolution of the paternal *X*-inactivation pattern is a phenomenon restricted to Australian marsupials or is a basic feature of all marsupial lineages (COOPER *et al.* 1977b). The present data reveal clearly the commonality of paternal *X* inactivation in Australian and American marsupials and thereby suggest the derivation of this form

of X-linked gene regulation from their common ancestor. This interpretation, in turn, places the emergence of the random (eutherian) and deterministic (metatherian) variants of the single active X strategy at a time prior to the divergence of the Australian and American Metatheria, some 80–100 million years ago (see COOPER *et al.* 1977b; ARCHER 1984; KIRSCH 1984).

Despite the conspicuous difference of random *vs.* determinate (paternal) X inactivation in adult somatic tissues, a growing body of evidence suggests that the bases of eutherian and metatherian X-linked gene regulation systems are fundamentally quite similar (reviewed by VANDEBERG *et al.* 1986). For example, partial expression and independent regulation of eutherian X-linked genes has been noted in cases of spontaneous and induced reactivation of genes on the inactive X chromosome *in vitro* (reviewed by MILLER 1985). Such reactivation generally affects only a single locus, and the reactivated loci usually exhibit lower activity than their counterparts on the active X chromosome. Additional evidence of a piecemeal regulation of X-linked genes is the observation that at least three loci (*Xg, MIC2X,* and *Sts*) on the short arm of the human X escape inactivation (reviewed by MOHANDAS and SHAPIRO 1983; see also GRAVES 1983; GARTLER and RIGGS 1983; GOODFELLOW *et al.* 1984). Lack of inactivation of the *Sts* locus, which encodes the enzyme steroid sulfatase, has also been documented in wood lemmings (ROPERS and WIBERG 1982) and probably occurs in mice as well (KEITGES *et al.* 1985). Finally, as already mentioned, eutherian embryos regularly exhibit paternal X inactivation *in vivo* in cells of the extraembryonic membranes. Such similarities are undoubtedly a consequence of the common ancestry of all therian X-inactivation systems, and the differences can be viewed as evolutionary modifications of a common ancestral pattern of gene expression. Differences of this kind can be valuable tools in a comparative approach to research on basic molecular processes of gene regulation.

Many aspects of metatherian X inactivation remain to be characterized. Perhaps most important are the timing and pattern of X-linked gene activity during early embryogenesis which, judging from our understanding of eutherian development, is when the critical events of X inactivation take place (reviewed by GARTLER and RIGGS 1983; EPSTEIN 1983; VANDEBERG 1983). Some differences between eutherians and metatherians have already been documented. For example, during eutherian oogenesis the inactive X is switched on near the beginning of meiosis; thus, both X chromosomes are active in dictyate oocytes. Both X chromosomes also are active in female cleavage embryos, but the paternally derived X is inactivated early in the differentiation of yolk sac and other extraembryonic membranes. Later, random X inactivation takes place in those cells destined to become the cells of the embryo proper. In contrast, studies of *Gpd* expression in kangaroos (*Macropus robustus*) have shown that the paternally derived *Gpd* allele is inactive in dictyate oocytes (JOHNSTON and ROBINSON 1985) indicating that the stage of X-linked gene (X-chromosome) reactivation in this metatherian species differs from that of eutherian mammals. Moreover, 21–25-day female *M. robustus* embryos express only the maternal *Gpd* allele in somatic tissues and certain extraembryonic tissues (allantois and amnion), but express both alleles in the avascular and vascular yolk sac (JOHNSTON, ROBINSON and JOHNSTON 1985).

Progress in the characterization of gene regulation events during oogenesis and early embryogenesis in metatherians has been slow due to the difficulty of obtaining sufficient biological material from kangaroos which have long generation times, low fecundities, and limited X-linked genetic variation. With the establishment of *D. virginiana* as a tractable metatherian model possessing high fecundity, short generation time, and at least two X-linked polymorphisms, studies of these critical developmental events are now quite practical.

We believe that *D. virginiana* also will be very important in studying the molecular basis of X-linked gene regulation. For example, eutherian species have served extensively as models for examining site-specific DNA methylation patterns and sensitivities to endonuclease activity of active and inactive X-linked genes (*e.g.*, WOLF *et al.* 1984a, b; JABLONKA *et al.* 1985; RIGGS, SINGER-SAM and KEITH 1985; WOLF and MIGEON, 1985). Using *D. virginiana* it will be possible to extend such studies to test hypotheses regarding the state of the DNA among tissues exhibiting a wider range of activities (fully active, inactive, and several levels of partial activity) and among different stages of the same tissue exhibiting developmental changes in gene expression.

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