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Genetics

X chromosome reactivation in oocytes of *Mus caroli*

(glucose-6-phosphate dehydrogenase/meiosis)

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ABSTRACT **Mature mammalian oocytes have both of their X chromosomes active, while somatic cells from the same individual have one of their X chromosomes in an inactive state. We asked whether the X chromosomes of the germ cells never undergo inactivation in their ontogeny or whether inactivation of an X chromosome does occur but is followed by a subsequent reactivation event. Our approach has used an electrophoretic polymorphism for the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) in the mouse species *Mus caroli*. G6PD is dimeric, and a heterodimer is produced in cells from heterozygous females if and only if both X chromosomes are active. Ovaries from heterozygous fetuses at different gestational ages were dissected and either studied cytologically or pressed between microscope slides to obtain germ cell-rich and germ cell-poor preparations. No heterodimer band was detected on the 10th day of development in germ cell-rich preparations. On subsequent days, an increasingly intense heterodimer band was detected, which, by the 13th day, was approximately twice as intense as the corresponding homodimer bands. Consideration of (*i*) the G6PD activity per germ cell and per somatic cell and (*ii*) the percentage of germ cells in the germ cell-rich preparations indicated that a heterodimer band should have been visible on the 10th day had both X chromosomes been active. Cytological examinations showed that the earliest germ cells enter meiotic prophase on the eleventh day. These results demonstrate that oogonia have a single active X chromosome and that the inactive X chromosome is reactivated at or, more likely, shortly before entry into meiotic prophase.**

The Lyon hypothesis (1), which states that only one X chromosome is active in the somatic cells of female mammals, is well supported by experimental data (2). Both X chromosomes are apparently active in the preimplantation embryo (3–6), and random inactivation of one X chromosome occurs in the fetal tissues soon after implantation (7, 8). The inactive state of the X chromosome is then stably inherited in somatic cells (9–12). The state of X-chromosome activity in the germ line is not yet clear. Based on gene product studies for several X-linked genes (13–16), mature oocytes have two active X chromosomes, but this could result either from failure of inactivation in the germ line or from reactivation of a previously inactive X chromosome.

The question of germ line X chromosome activity has been investigated previously. Cytologically, Ohno (17) reported the presence of a chromatin body in migrating primordial germ cells of the mouse but not in oogonia in the ovaries of the rabbit and human (18, 19). Gartler *et al.* (20) has recently observed a heterochromatic chromosome in oogonial mitoses in the mouse. Biochemically, Gartler *et al.* (21) presented evidence that both X chromosomes are active in germ cells from human ovaries at 13 weeks, but not at 12 weeks, of gestation. Using the same approach, however, Migeon and Jelalian (22) reported that both X chromosomes were active at 8 weeks, which is around the time when the earliest germ cells enter meiosis (18, 23). By using gene-dosage approaches, which compare the activity levels of X-linked enzymes in cells having two X chromosomes with those in cells having a single X chromosome, a dosage change has been reported in the mouse after gonad differentiation (24). The same time period was also reported for the appearance of the paternal isozyme in germ cells heterozygous for the T(X; 16) translocation (25).

The use of an electrophoretic variant of a dimeric enzyme provides a qualitative means to determine whether two X chromosomes are functional in the same cell. Because our germ cell preparations contained somatic cells as well, the relative activity levels in the different cell types were measured to determine unequivocally the functional state of the two X chromosomes in germ cells. Hitherto, an electrophoretic variant for an X-linked dimeric enzyme had been unavailable in the laboratory mouse. Recently, we have discovered and characterized an electrophoretic variant for X-linked glucose-6-phosphate dehydrogenase (G6PD; D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) in the related mouse species *Mus caroli* (unpublished result). We have used this polymorphism to examine the functional state of X chromosomes in germ cells during the early stages of oogenesis. Along with an electrophoretic examination, we have made cytological observations on germ cell progression. Our results show that X-chromosome inactivation does occur in the germ line and that reactivation of the inactive X chromosome occurs about the time the germ cells enter meiosis to produce oocytes that have two active X chromosomes.

MATERIALS AND METHODS

Mice. Electrophoretic polymorphism for the X-linked enzyme G6PD has been established in *Mus caroli* (unpublished result). The alleles coding for the faster and slower electrophoretically migrating proteins are called *G6PD-A* and *G6PD-B*, respectively. *M. caroli G6PD-A* and *G6PD-B* stocks have been maintained in our laboratory by random mating for several generations. Developmental progression in *M. caroli* is similar to that in *M. musculus*; however, because of accelerated development throughout gestation, the gestational period in *M. caroli* is shorter, varying between 17 and 19 days (26). In this paper, the standard gestation was assumed to be 17 days, with the observance of a vaginal plug being taken as day 0. Standard development was based on anatomical criteria, using Theiler's description of development in *M. musculus* as a guide (27). Day 10 in *M. caroli* corresponds to day 11 1/2 in *M. musculus* and is characterized by constriction of the front limbs only. On day 11 (*M. musculus* day 12 1/2), indentation and ray formation occur in the front limbs and the gonads are barely differentiated. On day 12 (*M. musculus* day 13 1/2), the fingers on the front limbs separate, and the eye becomes round. These criteria

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Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

formed the basis for discarding fetuses that lagged. Increased litter size was achieved through superovulation with intraperitoneal injections of 4 international units each of pregnant mares' serum gonadotropin (Organon) and human chorionic gonadotropin (Ayerst, New York) in midafternoon separated by 48 hr. Heterozygous female fetuses were produced, in the majority of the cases, by mating *G6PD-B* females with *G6PD-A* males.

Germ Cell Preparations and Characterizations. Fetal ovaries were dissected from the mesonephros in saline and pooled in phosphate-buffered medium. Ovaries could be distinguished from testes easily on day 12 and with difficulty on day 11. To avoid misclassification, the sex of day 11, as well as of day 10 fetuses, was determined by the electrophoretic phenotype of fetal head homogenates. From each litter, a few ovaries were set aside for cytological examination and the remaining ovaries were pooled on a siliconized microscope slide. Under gentle pressure between two siliconized microscope slides, germ cells are preferentially released (28). The individual cells were sucked up in a drawn Pasteur pipette and transferred to a 0.4-ml Microfuge tube (Brinkman). Germ cell-poor preparations were obtained by either pressing the ovary remnants further or by sucking them in and out of a drawn Pasteur pipette. The more tightly held cells freed by these procedures were transferred to a second Microfuge tube. The cells were washed by two cycles of centrifuging in an International clinical desk-top centrifuge for 5 min, drawing off the supernatant, and resuspending in cold medium.

An aliquot of each cell suspension was loaded on a hemocytometer to determine the cell density. A second aliquot was removed to microscope slides to determine the percentage of germ cells in each preparation. The cells, without prior fixing or drying, were stained by the method of Gomori (29) for alkaline phosphatase activity, which is high in germ cells and low in somatic cells.

G6PD Activity. A third aliquot was freeze-thawed and kinetically assayed for G6PD activity. The incubation mixture was 20 mM Tris·HCl, pH 8.0/0.2% bovine serum albumin/0.1 mM NADP/0.3 mM glucose 6-phosphate/10 mM $MgCl_2$ in a 2-ml volume. The increase in fluorescence at 37°C was measured every 4–10 min in an Aminco spectrofluorometer. The assay was linear over several orders of magnitude. Control values for the activity in an equal volume of supernatant were subtracted from each experimental value.

G6PD Electrophoresis. The electrophoretic phenotype of the germ cell-rich preparations was determined by the cellulose-acetate gel method (30). The cells were concentrated in ≈ 1 μl of medium, freeze-thawed, and applied directly to the gel applicator. The electrode buffer was 25 mM Tris·glycine, pH 8.5 with NADP (0.2 mg/ml) added to the cathodal buffer. After an electrophoretic run at 200 V for 35 min, the positions of the bands were visualized by overlaying the gel with an agar mixture containing 1.2 μmol of NADP, 50 μmol of glucose 6-phosphate, and 20 μmol of $Mg(OAc)_2$ in 25 mM Tris·HCl, pH 8.0. By this procedure, bands representing 50 pmol of NADPH produced per hr could be detected.

Cytology. Ovary squashes were made by a modification of the method of Beaumont and Mandl (31). On dissection, the ovaries were transferred to 1.2% sodium citrate at room temperature for 20 min and then fixed in absolute methanol/glacial acetic acid (3:1) at 4°C overnight. The ovaries were then hydrolyzed in 1 M HCl at 37°C for 20 min and transferred to 45% acetic acid at 4°C for at least 10 min. Next, the ovaries were placed on a microscope slide in a drop of aceto-orcein and covered with a coverslip. Pressure was applied to the coverslip with the thumb over a layer of filter paper, and the edges of the coverslip were sealed with nail polish. That same day, the germ cells were examined under ×1000 magnification on a Zeiss Photoscope. Photographs were taken with Panatomic-X film. The number of germ cells per ovary was counted by scanning the entire monolayer ovary squash with an ocular grid.

RESULTS

G6PD Electrophoresis. Because G6PD is a dimeric enzyme, three kinds of dimers will be produced as a result of random monomer association in cells synthesizing two distinguishable monomers. Thus, a heterodimer will be present only in heterozygous cells that have two active X chromosomes. No heterodimer band would be expected in somatic tissues from heterozygous females, according to the Lyon hypothesis (1), and none was observed (Fig. 1). As the specific activities of the two homodimers are approximately equal (unpublished result), cells in which both X chromosomes are active should have twice as much activity in the heterodimer form of the enzyme, which constitutes half of the G6PD molecules, as in either of the homodimer forms. Residual enzyme from a previous one X-active state would decrease the relative intensity of the heterodimer form. A progressive decrease in the relative intensity of the heteropolymer can be seen in germ cell-rich preparations from day 14 back through day 11. On day 14, the pattern is similar to that for mature oocytes, where the intensity of the heterodimer is about double that of the homodimers (lanes c and d). On day 11, the heterodimeric band is substantially less intense than the homodimeric forms (lane a).

The electrophoretic phenotype for germ cell-rich preparations from days 10–13 is given in Table 1. The relative intensity of the bands was estimated by comparison with standards that had been serially diluted. On day 10, no heterodimer band was detected in three separate experiments. The lower limit for detection of a heterodimer band was also estimated by comparison with serially diluted standards. These comparisons suggested that a heterodimer band could have been detected had it been approximately a 10th as intense as either homodimer in two cases and a quarter as intense in the third case. Consideration of the G6PD activities further indicate that, in the two most sensitive cases, the expression of a heterodimer was less than 10% of that which could have been produced by the germ cells of the sample. A heterodimer band was first detected on day 11. However, the relative intensity of the heterodimer and homodimer bands ranged from undetectable to equal. The manner in which the relative intensity of the heterodimer band varied was consistent with other characteristics of the samples. The sample lacking a detectable heterodimer band had the lowest activity per cell in the germ cell-rich preparations and the lowest percentage of germ cells among the day 11 preparations. The ratio of the intensity of the heterodimers to that of the homo-

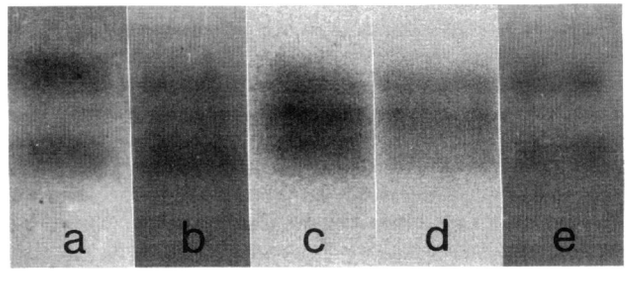

FIG. 1. Electrophoretic phenotypes for germ cell and somatic cell preparations from females heterozygous for G6PD. Lanes: a, b, and c, germ cell-rich preparations from days 11, 12, and 14, respectively; d, sample of ovulated oocytes from mature females: e, homogenate from a fetal female head. Electrophoresis was from bottom to top.

Table 1. G6PD activity and electrophoretic phenotype of germ cell preparations

Day of development	Germ cell-rich			Germ cell-poor	
	Activity*	% germ cells	Dimeric ratio	Activity	% germ cells
10	32	37	0	33	12
	45	64	0	35	14
	50	64	0	20	11
11	44	48	0.5	19	15
	27	38	0	8	7
	44	58	1	13	10
	48	56	0.5	21	23
12	56	73	1.5	28	47
	86	59	2	45	37
	71	52	2	47	35
	48	57	1.5	19	26
13	147	68	2	88	41
	157	71	2	45	24
	140	69	2	49	11

Germ cell preparations were made from fetal ovaries from single litters at different stages. From these preparations, aliquots were assayed. G6PD activity was measured on $1.7 \times 58 \times 10^3$ cells per sample, depending on the age. At least 145 cells were scored to determine the % germ cells. The electrophoretic phenotype for germ cell-rich preparations was determined as the ratio of the intensity of the heterodimer band to that of either homodimer band.

* Expressed as (fmol of NADPH produced per hr)/cell.

dimers is less than two on day 12 but, by day 13, it does not appear different from the two-fold ratio of mature oocytes. The expression of the X chromosome in germ cells was not ffected by performing the matings reciprocally (*G6PD-A* ♀♀ × *G6PD-B* ♂♂).

G6PD Activities. The G6PD activities for germ cell-rich and germ cell-poor preparations are also given in Table 1. The activities are given in Table 1 as per cell, without distinguishing between germ cells and somatic cells. On day 10, the activitiy per cell of germ cell-rich preparations was slightly higher than that of the germ cell-poor preparations, suggesting that the activity per germ cell is at least as great as the activity per somatic cell. On later days, both the level of activity and the relative difference between germ cell-rich and germ cell-poor preparations increased, indicating that the activity per germ cell is much greater than that per somatic cell.

Cytology. Cytological preparations of ovaries were analyzed to follow the progression of germ cells through oogenesis. Representative germ cell nuclei at different stages are shown in Fig. 2. In leptotene, the first stage of meiotic prophase, scattered clumps of condensing chromosomes can be observed. The homologous chromosomes begin to pair in early zygotene and by late zygotene are completely paired. Throughout leptotene, zygotene, and into pachytene, the chromosomes continue to condense.

The distribution of germ cell stages in single ovaries is given in Table 2. The first germ cells enter meiosis on day 11 and, on day 12, most of the germ cells have entered meiosis. Because fetuses were selected for similar developmental stages by anatomical criteria, most of the variability in the stage reached by germ cells on a given day results from heterogeneity within an ovary. Litters in which the oocytes were slightly advanced cytologically also had electrophoretic patterns and activities that had characteristics of slightly older litters.

Another characteristic of oogenesis is an increase in the number of germ cells, as a result of mitosis, until all the germ cells have entered meiosis. The number of germ cells per ovary is given in Table 3. The number increased ≈2.8-fold between days 10 and 11 and, again, between days 11 and 12. This observed rate of increase in the number of germ cells is similar to that reported for *M. musculus* (32). For the four days after

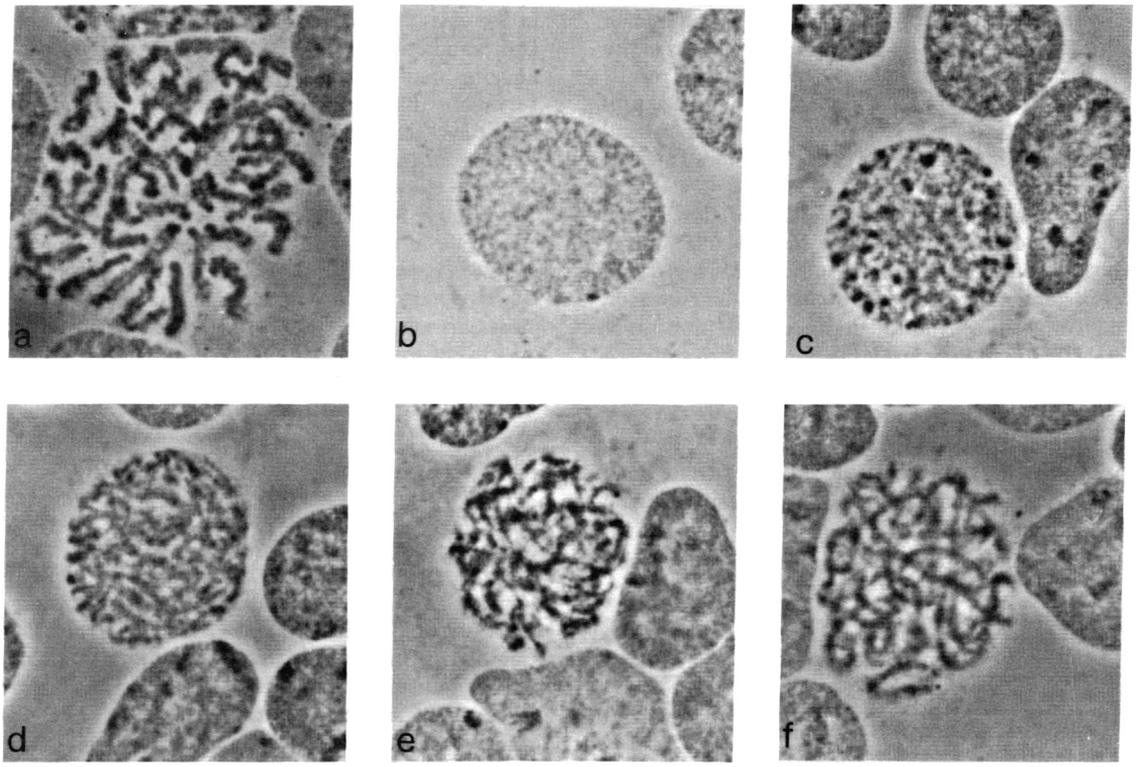

FIG. 2. Representative germ cell stages from fetal ovaries: (*a*) oogonial pro-metaphase, (*b*) oogonial interphase, (*c*) leptotene, (*d*) early zygotene, (*e*) late zygotene, and (*f*) pachytene. The squashes were made in aceto-orcein. (×1000.)

Table 2. Distribution of germ cell stages in single ovaries

Day of development	Germ cells counted, no.	% germ cells at different stages			
		Oogonia	Leptotene	Zygotene	Pachytene
10	300	100			
	350	100			
11	300	94	6		
	300	82	18		
12	400	12	75	13	
13	400	1	7	75	17
	400		6	55	39

Germ cell stages were recorded for random areas of ovary squashes. Each row represents the results for a separate ovary.

day 12, the number remained roughly constant at 11 or 12 thousand per ovary, which is similar to the maximum number reported in *M. musculus* (33, 34). Although the maximum number of germ cells per ovary is not reached until day 12 in *M. caroli*, the cytologically observed asynchrony of germ cell progression makes it likely that some germ cells enter meiosis by day 11.

DISCUSSION

The absence of a detectable heterodimer in germ cell-rich preparations from day 10 is consistent with a single active X chromosome in oogonia. Earlier evidence for an inactive X chromosome in mitotic germ cells had been obtained cytologically by Ohno (17) and, more recently, by Gartler *et al.* (20). The use of G6PD as an indicator of X chromosome expression is directly comparable with the studies in fetal human germ cells by Gartler *et al.* (21) and Migeon and Jelalian (22). The absence of a heterodimer in our study is similar to the absence reported by Gartler *et al.* (21) in 12-week human germ cells and apparently at variance with the detection of a heterodimer by Migeon and Jelalian (22) in 8-week human germ cells. The apparent discrepancy may be due to the uncertainty in estimating human gestational age and the lack of a cytological examination of the germ cells. It is possible, therefore, that the discrepant results reflect a difference in the presence of germ cells in meiotic prophase. In our studies, we observed a faint heterodimer band when as few as 10 percent of the germ cells had entered meiotic prophase.

It is conceivable that the lack of a heterodimer in day 10 samples is not due to the presence of only a single active X chromosome in oogonia but is instead the result of a very low G6PD activity level in germ cells relative to somatic cells. However, the percentage of germ cells in the germ cell-rich preparation and the relative G6PD activity levels in germ cell-rich and germ cell-poor samples (see Table 1) argue against this possibility. These data suggest that most of the G6PD activity in the germ cell-rich preparation is germ cell derived. For two of the three day 10 samples, the intensity of the heterodimer band would be expected to be about equal to that of the corresponding homodimer bands. The absence of any detectable heterodimer band on day 10, therefore, is evidence that the germ line is subject to X chromosome inactivation.

Table 3. Number of germ cells per ovary

Day of development				
10	11	12	15	16
1231	4061	11,695	10,232	12,643
1800	4545			

Each number represents the total number of germ cells counted in an ovary squash.

Following X chromosome inactivation, the inactive X chromosome is later reactivated as demonstrated by the presence of a heterodimer band in germ cell-rich preparations from later fetal development and in mature oocytes. Other situations in which chromosome reactivation has been reported include the mammalian X chromosome of sperm after fertilization (35), the marsupial paternal X chromosome during oogenesis (36), and several instances in insects (37–39). In the present study, the first evidence of reactivation of the previously inactive X chromosome occurs on day 11. Day 11 is also when the earliest germ cells enter meiotic prophase as observed cytologically and as estimated from the number of germ cells per ovary. As more germ cells enter meiotic prophase, the relative intensity of the heterodimer band increases. This relationship suggests that reactivation of a previously inactive X chromosome occurs on entry into meiotic prophase or some earlier event and is independent of chromosome pairing.

Collectively, our data and the data of Gartler *et al.* (20) which show a heterochromatic chromosome in the last oogonial mitosis, suggest that X chromosome inactivation occurs in germ cells and that germ cells leave the mitotic cell cycle with an inactive X chromosome. Reactivation could occur anytime between leptotene of meiotic prophase, when a heterodimer band is first detected, and the preceding G1 phase. However, it is expected that reactivation in the germ line would be simpler either before or during the premeiotic replication phase, where only one copy of the inactive chromosome would be involved, than in the following short G2 phase, where two copies of the inactive chromosome would need to be reactivated.

Several properties of X chromosome inactivation are shared by somatic cells and germ cells. The inactive X chromosome in oogonia is transmitted mitotically, just as in somatic cells, as suggested by Johnston's result that oogonia from mice heterozygous for the T(X; 16) translocation express only the maternal chromosome (25). In addition, inactivation of an X chromosome in the germ line must be random, as it is in somatic cells; the two homodimer bands in day 10 samples are approximately equal in staining intensity. Apparently at variance are the observations that, although the inactive X chromosome of germ cells can reactivate, the inactive X chromosome of somatic cells appears incapable of transformation (40), and rarely reactivates in tissue culture, even under selection (9–12). The inability of the inactive X chromosome of somatic cells to transform and reactivate in tissue culture may be a reflection of a difference between germ cells and somatic cells in the maintenance of the inactive state. However, the difference more likely is due to a necessary condition for reactivation that is present only during meiosis or the immediately preceding premeiotic stage.

Some of the reported differences between the mitotic and meiotic cell cycles occurring before prophase include a lengthened DNA synthesis phase in both male and female meiocytes (41–44) and a delayed replication of part of the nuclear genome until meiotic prophase (45). An extremely short G2 phase follows the lengthened premeiotic synthesis phase in the mouse (41). There are likely to be many other differences between premeiotic and premitotic events, some of which may be important to X chromosome reactivation.

We conclude that, as in somatic cells, an inactive X chromosome is present in oogonia as a result of random inactivation and is transmitted mitotically. The inactive X chromosome is reactivated sometime during the period between the premeiotic G_1 phase and prophase, but before chromosome pairing. The ability to reactivate an inactive X chromosome is a property solely of the germ cells and may depend on conditions that are present immediately preceding or during meiosis.

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