Measurement by quantitative PCR of changes in HPRT, PGK-1, PGK-2, APRT, MTase, and Zfy gene transcripts during mouse spermatogenesis

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ABSTRACT

A reverse transcriptase-polymerase chain reaction assay (RT-PCR) was used quantitatively to measure accumulated levels of RNA transcripts in total mouse RNAs derived from male germ cells at various spermatogenic stages. RNA levels for two X-linked enzymes, phosphoglycerate kinase (PGK-1) and hypoxanthine phosphoribosyl transferase (HPRT), both decrease during spermatogenesis, although the transcript levels decrease much more rapidly for PGK-1. RNA for the Y-linked ZFY (zinc finger protein) is elevated in all spermatogenic cell fractions tested, being particularly high in leptotene/zygotene spermatocytes and round spermatids. RNA for adenine phosphoribosyltransferase (APRT) increases 5-fold to a peak during late pachynema. RNA for PGK-2, undetectable in spermatogonial cells, increases at least 50-fold by the round spermatid stage. DNA (cytosine-5-)-methyltransferase (MTase) transcript levels are over an order of magnitude higher throughout spermatogenesis than in non-dividing liver cells.

INTRODUCTION

Many studies of gene expression are limited by the sensitivity of standard hybridization techniques, and, for this reason, several groups have recently made use of the polymerase chain reaction for the detection of specific mRNAs after reverse transcription to DNA (RT-PCR) (1, 2, 3, 4, 5, 6). Because of the increasing use of RT-PCR, we investigated the quantitative aspects of the assay. By limiting the amount of input template and the number of cycles of PCR, we obtained a signal which was linearly related to specific RNA levels over at least a thousand-fold range, with reproducibility adequate for any study where changes in RNA levels are greater than two-fold.

We have used the assay to investigate changes in specific RNA transcripts of X-linked and autosomal genes during mouse spermatogenesis. Our work was initiated as a study of the X chromosome inactivation which occurs during mouse spermatogenesis (7, 8, 9, 10, 11). In addition to the two X-linked

genes, PGK-1 and HPRT, the Y-linked Zfy genes were also included in the study because they may be involved in spermatogenesis (12).

Three other genes were investigated. The autosomal gene for adenine phosphoribosyltransferase (APRT), was included in the study because, like HPRT, APRT is part of the purine salvage pathway and is expressed ubiquitously at low levels. Phosphoglycerate kinase-2 (PGK-2), an autosomal, testis-specific isozyme of PGK-1, was included as a control because it is known to be expressed only in meiotic and post-meiotic male germ cells (13). DNA (cytosine-5-)-methyltransferase (MTase) is part of a gene silencing system probably involved in both X chromosome inactivation and genomic imprinting (14, 15, 16). We find that each of the genes studied shows a unique pattern of transcript level changes during spermatogenesis.

MATERIALS AND METHODS

Isolation of spermatogenic cells and purification of RNA

Fractionated germ cells at various stages of development were prepared as described (7, 8) from Swiss Webster mice (Charles River). In brief, seminiferous cords were isolated from testes of prepuberal mice, the germ cells were dissociated by collagenase and trypsin treatment, then seminiferous cells were separated by sedimentation velocity at unit gravity. Types A and B spermatogonia were isolated from 8-day-old mice, leptotene/zygotene and 'p17' pachytene spermatocytes from 17-day-old mice, and 'p60' pachytene spermatocytes, round spermatids and liver cells from mice 60 or more days old. Sertoli cells were isolated from 6-day-old mice.

Examination by light microscopy showed the fractions to be 80% or more of the designated cell type, with the possible exception of the leptotene/zygotene fraction. For each fraction contamination appeared to be with the developmentally adjacent cell types and Sertoli cells. See RESULTS for additional evidence regarding purity of the fractions.

Total RNA from each cell fraction, prepared by a method that removes DNA by differential precipitation (17), was kindly supplied by K. Thomas and P. Tomashefsky (9). The RNA was stored as an ethanol precipitate at $-70°C$. Prior to each

Table 1. Primers Used in the RT-PCR Assay

Gene	Primers Used	Product Size	Bases Spanned	Ref.
PGK-1	TAGTGGCTGAGATGTGGCACAG GCTCACTTCCTTTCTCAGGCAG	166 bp	1364 – 1529	(40)
PGK-2	ATTGTCATAATGTACTTGCTTCC GTTTCCTCCCTAGGAAGGACTGG	225 bp	1254 – 1478	(41)
HPRT	CGAGGAGTCCTGTTGATGTTGC CTGGCCTATAGGCTCATAGTGC	172 bp	685 – 856	(21)
APRT	CCAGCAGCACTAGGAACTGCTT AGGGTGTGTGGGACGTGTACAA	167 bp	2848 – 3014	(42)
MTase*	AGCCAGTTGTGTGACTTGGAAACCA ACCGTTGGCTTTTTGAGTGAGAGTG	141 bp	17 – 157	(43)
ZFY	AAGATAAGCTTACATAATCACATGGA CCTATGAAATCCTTTGCTGCACATGT	618 bp	1336 – 1953 (12)	(24)

*MTase: DNA (cytosine-5-)-methyltransferase

experiment aliquots of RNA were centrifuged with glycogen (40 μg) added as a carrier, rinsed in 70% ethanol, then resuspended either in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM NaCl, or directly in the PCR reaction mix.

The RT-PCR assay

Information about the oligonucleotides used as primers for the PCR reaction is given in Table I. Initially, assays were done as previously described (1). In later experiments, RNA was mixed with the PCR reaction mix (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl$_2$, 50 mM KCl, 0.01% (w/v) gelatin, 200 μM dNTPs, and primers at 1 μM each), and RNasin (Promega, 40 U), in a final volume of 50 μl. After the addition of AMV reverse transcriptase (2 U, Life Sciences), and Taq polymerase (2 U, Cetus Perkin-Elmer), the samples were placed in a thermal cycler at 50°C for 8 min, followed by PCR at 95°C for 1 min, 60°C for 2 min, and 73°C for 2.5 min, except in the case of PGK-2, where the renaturation step was done at 47°C. After PCR, a 20 μl aliquot was subjected to electrophoresis in a 2% agarose gel containing 0.4 μg/ml ethidium bromide in 1× TBE buffer (18).

Quantitation

After gel electrophoresis, the amount of PCR product in each lane was determined in either of two ways: 1) The gel was photographed under UV light onto technical film (Kodak, 4415), with several exposures taken to ensure that the signal from each band was in the linear range of film response. Densitometry was done by use of a video densitometer (Biorad, Model 620), and peak areas were analyzed by means of the 1-D Analyst programs (Biorad). 2) The DNA was transferred onto a nylon membrane (Genetrans 45, Plasco, Inc.) by vacuum blotting (1). Hybridization was done as described (1), at 60°C. Each autoradiogram was analyzed by densitometry as described above; alternatively, the radioactivity in each band was determined directly by means of the AMBIS Radioiosotope Scanning System II (Automated Microbiology System, Inc.).

RESULTS

Quantitation by the RT-PCR assay

Figure 1 shows a dilution series of total RNA from liver or Sertoli cells, assayed for PGK-1 transcripts by RT-PCR. The signal seen for PGK-1 clearly reflects the amount of RNA loaded. To further
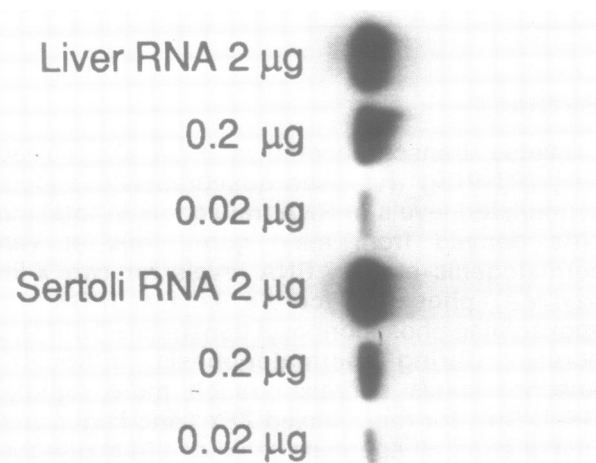
Figure 1. Assay by RT-PCR of PGK-1 transcript levels in mouse liver and Sertoli cells. Samples of total RNA (0.02, 0.2 or 2 μg) were reverse transcribed, then PCR was performed for 20 cycles with the primers for PGK-1 shown in Table 1. The products from the PCR reaction were separated by gel electrophoresis, transferred to a nylon membrane, and hybridized with a ^{32}P-oligonucleotide probe specific for PGK-1 . The probe used was 5' CTCAGCTCGTCTTAC-TGCATCA, beginning at base position 1405 of mouse PGK cDNA (40). The bands seen correspond to the expected size of 166 bp.

investigate quantitation by RT-PCR, filter bound radioactivity was determined; figure 2 shows data accumulated from several experiments such as the one shown in figure 1. A linear correlation between the amount of template RNA and the PCR signal is seen over a thousand-fold range, with an average deviation from the best fit line of 40%. To obtain a linear response to template concentration, the primers, nucleotides, and polymerase must remain in large excess. Therefore, the appropriate number of PCR cycles must be empirically determined for each primer set, since the number of cycles taken to reach saturation depends on primer efficiency and the abundance of the target transcript. For the experiments reported here, total RNA used varied from 0.25 – 2 μg, and the cycle number varied from 20 – 26 cycles. For each set of primers, a dilution series was used to determine that the results obtained were in the linear range of the assay. Since the exposure time for the autoradiogram shown in Figure 1 was only 40 min with no enhancing screens, it is clear that the ultimate sensitivity, even
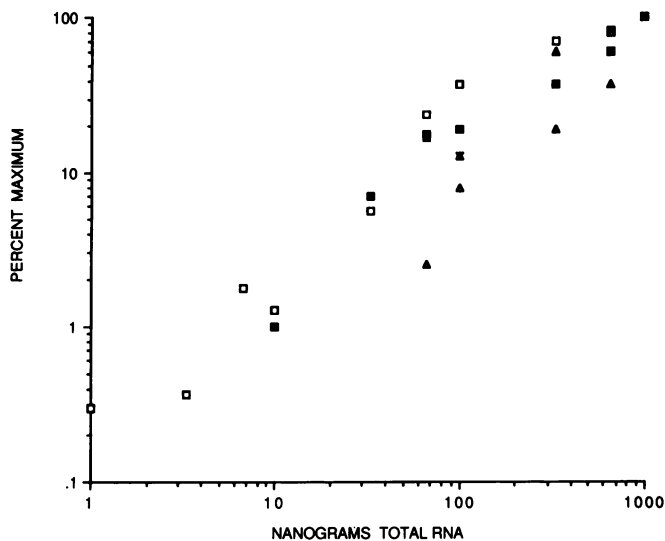
Figure 2. PCR signal as a function of the amount of template RNA. Total mouse liver RNA was diluted in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM NaCl, and added to the PCR reaction mix in the amounts indicated. RT-PCR, gel electrophoresis, hybridization and autoradiography were performed as indicated in Materials and Methods. The cpm in each band were determined by means of a radioiosotope scanning system, and normalized relative to the value obtained for 1 μg of RNA. Each symbol represents an independent experiment. Taq polymerase was added either with reverse transcriptase (open sqares and triangles) or after the reverse transcription step (closed squares and triangles).

without increasing cycle numbers, is much greater than needed for the work reported here.

Measurement of specific RNA transcripts during spermatogenesis

The RT-PCR method was used to assay for specific transcripts in total RNA extracted from cells at various stages of spermatogenesis. Figure 3 shows results of a typical experiment, and Figure 4 summarizes the results obtained for all transcripts assayed. In each case the data were normalized to the stage of maximum expression. For all transcripts studied, the major PCR products were the size expected, and the identity of the product was confirmed by the presence and proper location of restriction enzyme sites and/or hybridization to an internal oligonucleotide probe. As shown in Figs. 1, 3 and 4A, transcripts from the PGK-1 gene decrease to about 5% of somatic cell levels (liver or Sertoli cells) in leptotene/zygotene spermatocytes, and decrease to even lower levels at later stages. In the mixture of type A and B spermatogonia, the level of PGK-1 RNA is already reduced relative to liver. As a control, we measured PGK-2 RNA (Fig 4A). PGK-2 transcripts are first detectable in leptotene/zygotene spermatocytes, earlier than had been previously reported (9), and continued to increase, as expected (13), at least until the round spermatid stage.

Purity of RNA samples

DNA contamination can be ruled out for several reasons. First, the PGK-1 signal decreases to less than 5% of somatic levels after the leptotene/zygotene stage (Fig. 3). Since the PGK-1 primers amplify genomic DNA at least as efficiently as RNA (our unpublished data), it follows that the post-leptotene/zygotene spermatocyte RNA samples are free of detectable DNA. Secondly, a PGK-2 signal is not seen in somatic cells or type
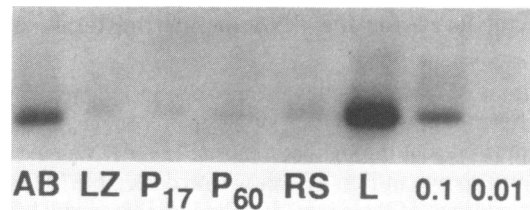
Figure 3. Changes in PGK-1 transcript levels during spermatogenesis. The experimental conditions are the same as those shown in Fig. 1. Liver RNA (L), diluted prior to RT-PCR, is shown in the three right lanes. AB, mixed type A and B spermatogonia; LZ, mixed leptotene and zygotene spermatocytes; P17 and P60, pachytene spermatocytes; R, round spermatids.

A/B spermatogonial preparations; therefore these samples are free of detectable DNA. Since the sensitivity of the assay allows the same samples to be used to measure the other gene targets, it follows that DNA contamination is insignificant. Thirdly, the addition of up to 10% contaminating DNA was found not to affect the results for HPRT.

Even though the cell populations we isolated are known in general to have varying degrees of contamination with other cell types (7, 8), interference by other RNAs can be ruled out, in large part, by the nature of the results we obtained. PGK-1 is a key enzyme of the glycolytic pathway and is ubiquitously expressed in all somatic cell types (19). Since the leptotene/zygotene samples, which might have been the most contaminated with Sertoli cells, have only about 5% of the PGK-1 content of liver and other somatic cells, it follows that contamination with somatic cells expressing PGK-1 is very low. Sertoli cells have about the same level of PGK-1 RNA as liver cells (see Fig. 1), so contamination with Sertoli cells must be 5% or less. Similar reasoning establishes that leptotene/zygotene samples are also not significantly contaminated with stages producing high levels of PGK-2, i.e., pachytene and round spermatids. In addition, the same five samples we used have also been studied by Thomas et al. (9); they assayed by Northern blotting for a Sertoli cell-specific sulfated glycoprotein transcript and concluded that Sertoli cell contamination was not significant.

Transcript levels for the PGK-1, HPRT and APRT genes

The results shown for PGK-1 in Figures 3 and 4A are consistent with cytological data indicating that the single X chromosome in male spermatogenic cells is inactivated early in meiotic prophase. Transcript levels are reduced at least 50-fold by pachynema. The PGK-1 gene probably begins to be shut off during the spermatogonial stage, since our studies (not shown) indicate that primitive A spermatogonia still contain PGK-1 transcripts in amounts comparable to liver or Sertoli cells. Unexpectedly, HPRT decreases during spermatogenesis much more slowly than does PGK-1. As shown in Figure 4B, HPRT RNA in type A/B spermatogonia may be somewhat reduced relative to liver or Sertoli cells, but significant reduction does not occur until later stages and then the level only falls 5 to 10-fold.

APRT and HPRT both function in the purine salvage pathway, are ubiquitously expressed at low levels, and have similar G+C-rich promoters (20, 21). However, as seen in Fig 4B, the expression patterns are quite different between the two genes, with APRT increasing 5-fold to a peak during pachynema as HPRT is decreasing. It is clear that control of transcript levels, and thus the control of the ratio of transcription to degradation, is different between HPRT and APRT.

Transcript levels for the DNA methyltransferase and Zfy genes

The highest MTase RNA levels are seen in leptotene/zygotene and pachytene spermatocytes, but the main conclusion is that the transcript is present throughout meiosis. Liver RNA, the somatic cell control shown in Fig. 4C, shows no detectable MTase RNA under conditions of the assay. It is known that normal liver has very low levels of MTase; lymphocytes and other mitotically active cells express at least 10-fold higher levels of MTase (22). The levels we find in spermatogenic cells are comparable to those in Sertoli cells and hybridoma cells in culture (not shown).

Fig.4C also shows the results obtained for Zfy. All five fractions tested have increased RNA levels over those found in liver cells, with leptotene/zygotene spermatocytes and round spermatids showing maximum levels. In all cases only one PCR product was found, of the size expected from mRNA of both the *zfy-1* and *zfy-2* genes in *Mus musculus* domesticus strains (12, 23, 24); thus, we may be measuring the sum of the two transcripts.

DISCUSSION

We have described here the quantitative use of RT-PCR to measure changes in specific RNA levels. When applied to DNA, the PCR reaction can be used quantitatively (as little as 4% standard deviation (19)), even without especially prepared internal standards. When applied to RNA, a reverse transcription step is necessary, and this probably introduces some additional variation. However, our studies confirm that the PCR product is linearly related to the template RNA concentration, and the reaction is sufficiently reproducible for the reliable detection of greater than two-fold differences (see Figures 2 and 4). The assay is very sensitive, requiring as little as 1/1000th the amount of total RNA used for Northern blots (Fig. 1; ref.(9)).

RT-PCR was used to measure relative levels of accumulation of several specific transcripts at different stages in spermatogenesis. One advantage of the assay is that it requires so little material that we have been able to use the same samples to ascertain RNA purity and to measure RNA transcript levels for all of the genes studied. We have not measured absolute transcript levels or rates of transcription, although, in the case of the PGK-2 gene, we have been able to observe the *initiation* of transcription.

Two of the RNAs we assayed, PGK-1 and HPRT, are X-linked. In 1965 Monesi (25) showed that the X chromosome was present as a heteropycnotic body by lepotonema of male meiotic prophase. He also showed by autoradiography after ^{3}H-uridine labeling, that both the X and Y chromosomes of mouse spermatocytes appear transcriptionally inactive at leptonema, and remain so throughout prophase. However, the direct demonstration of inactivation of specific X-linked genes expressed at low levels was not feasible. Therefore, the exact timing of X chromosome inactivation during spermatogenesis, and the relationship of X-inactivation to normal sperm development were unknown (26, 27).

In this study we have assayed for transcript levels of three genes on the sex chromosomes. We find that PGK-1 transcript levels decrease rapidly early in meiotic prophase, starting with type A/B spermatogonia, and drop rapidly to very low levels, consistent with previous cytological data. HPRT transcripts also decrease, but at a much slower rate. If transcription ceases, the message for HPRT is very stable, since transcript levels decrease only
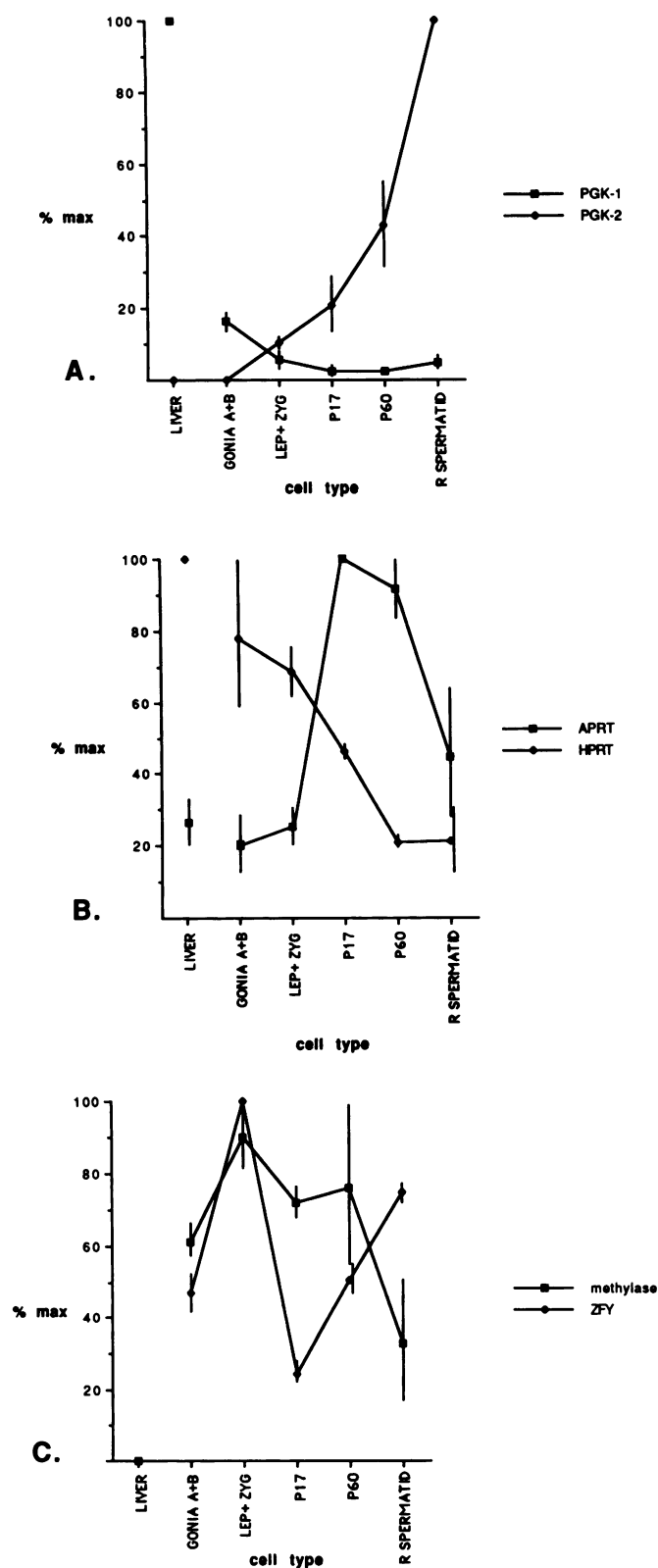
Figure 4. RNA levels during spermatogenesis. Primers and conditions used for the RT-PCR assay are given in Table I and Materials and Methods. Each experiment was done at least in duplicate and the range of values is shown by the error bars. 4A) PGK-1 and PGK-2. 4B) APRT and HPRT. 4C) DNA methylase and Zfy.

5 to 10-fold over a period of 13 days. There are many long-lived mRNAs in meiotic cells (28, 29), and HPRT mRNA could be among them.

We cannot rule out some continued transcription of the HPRT gene. Allsop, et al.(30) showed HPRT protein levels to increase during rat spermatogenesis, and suggested that HPRT may be necessary for spermatogenesis to occur. HPRT$^-$ mice however, undergo normal spermatogenesis (31, 32), so there is no obvious need for stabilization and preservation of HPRT message.

We have found Zfy transcripts to be present in all spermatogenic cells, and particularly high in leptotene/zygotene spermatocytes and round spermatids (Fig. 4C). Two Y-linked Zfy transcripts are known to be expressed in adult mouse testis, *Zfy-1* and *Zfy-2*, the former giving a relatively faint signal (12, 24). With *Mus musculus* musculus strains, the two transcripts are distinguishable by RT-PCR, giving different sized products with the Zfy primers shown in Table I (12, 23). In contrast, with the *Mus musculus* domesticus strains we used for this study, only one PCR product was observed. Two peaks of Zfy expression are seen, and it is tempting to speculate that *Zfy-1* and *Zfy-2* may be transcribed at different times during spermatogenesis. In any event, our finding that Zfy transcripts are present in relative abundance in meiotic spermatocytes and round spermatids is consistent with a role for one or both Zfy genes during sperm development (12, 24, 33, 34).

Recent studies have shown that many genes, perhaps representing 10% of the genome, function differently depending on parental origin (16, 35). This phenomenon of genomic imprinting probably involves DNA methylation. Differences in methylation between male and female gametes have been detected for repetitive DNA sequences (36, 37, 38), and oocyte DNA is globally less methylated than is sperm DNA (36). Therefore, changes in DNA methylase activity during gametogenesis might be important, but only one relevant study has been reported. Reddy and Reddy (39) measured MTase specific activity during maturation of the rat testis, finding activity for the whole testis to be highest at 20–30 days, coinciding with the appearance of spermatids. We find that MTase RNA levels are high in meiotic germ cells, but decreases postmeiotically, so the potential is present for methylation changes relevant to genomic imprinting.

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