Biallelic expression of imprinted genes in the mouse germ line: implications for erasure, establishment, and mechanisms of genomic imprinting

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Genomic imprinting in mammals determines parental-specific (monoallelic) expression of a relatively small number of genes during development. Imprinting must logically be imparted in the germ line, where inherited maternal and paternal imprinting is erased and new imprinting established according to the individual's sex. We have assessed the allele-specific expression of four imprinted genes, two of which exhibit maternal-specific (*H19* and *Igf2r*) and two of which exhibit paternal-specific (*Igf2* and *Snrpn*) monoallelic somatic expression, in the germ line of F₁ hybrid mice utilizing quantitative RT-PCR single-nucleotide primer extension assays. The expression of each gene was biallelic in the female and male germ line from the time that migratory mitotic PGCs entered the embryonic genital ridge and throughout gametogenesis, except that *H19* RNA was not detected late in gametogenesis. These findings demonstrate that inherited imprinting is erased, or not recognized, in germ cells by the time of genital ridge colonization; also that new imprinting may not be established until late in gametogenesis, or that it is incomplete or not recognized at this stage. Regardless of imprinting status, a generalized neutralization of imprinting is evident in the germ line, associated with the totipotent state of this unique cell lineage.

[Key Words: Imprinting; germ line; embryo; mouse genetics; gene expression; quantitative PCR]

Received May 5, 1995; revised version accepted June 23, 1995.

The epigenetic phenomenon of genomic imprinting determines an inequality of the maternal and paternal genomes in mammals. This is evident from the developmental failure of mouse embryos with two maternal or two paternal genomes, termed parthenogenetic and androgenetic, respectively, and of embryos with uniparental duplication of autosome regions (Lyon and Glenister 1977; Mann and Lovell-Badge 1984; McGrath and Solter 1984; Surani et al. 1984; Cattanach and Kirk 1985). Imprinting is also evident in the preferential inactivation of the paternally derived X chromosome in extraembryonic components of the conceptus (Takagi et al. 1978). As defined by developmental success, there is evidence that two maternally imprinted X chromosomes can adversely affect development, although the observations suggest that the majority of imprints affecting development reside on the autosomes (Mann and Lovell-Badge 1987, 1988; Kaufman et al. 1989; Takagi and Abe 1990). Only a well-defined minor subset of the autosomal component is involved, as abnormal phenotypes and lethality of mice with maternal or paternal duplication is observed for only a small number of autosome regions (Beechey and Cattanach 1995).

It is now generally believed that imprinting influences development through the imposition of parent-of-origin-

dependent gene activity. A growing number of genes that exhibit a monoallelic (+/-) mode of expression are being identified (for list, see Beechey and Cattanach 1995), and at least some of these genes appear to be biallelically expressed (+++) or inactive (---) in embryos and neonates with uniparental inheritance of these genes (Ferguson-Smith et al. 1991, 1993; Cattanach et al. 1992; Sasaki et al. 1992). Some instances have been documented in which the aberrant expression of such genes is known to have a deleterious developmental effect, namely, a --- mode of expression of insulin-like growth factor 2, *Igf2* (small size, DeChiara et al. 1991), insulin-like growth factor 2 receptor, *Igf2r* (perinatal lethality, Lau et al. 1994; Wang et al. 1994), murine achaete-scute homolog 2, *Mash2* (midgestation lethality, Guillemot et al. 1994, 1995), and *H19* (large size, Leighton et al. 1995). These observations demonstrate the necessity for expression of at least some imprinted genes, although it is unclear at this stage if their monoallelic or "imprinted" mode per se is always a necessity for normal development. It does appear important at least for *Igf2*, as a +++ mode of expression of this gene, brought about by deletion of the *H19* gene region, leads to increased size (Leighton et al. 1995).

Because at least some imprints appear necessary for

normal somatic and extraembryonic development, it is pertinent to ask whether imprinting is also a feature of, and perhaps also necessary for, germ cell development. Whereas functional oocytes of parthenogenetic origin can be obtained in chimeras (Stevens 1978; Allen et al. 1994), it is possible that deficient developmental cues in parthenogenetic germ cells may be compensated by association with normal or wild-type germ cells. On this point, it has been discovered recently that migratory primordial germ cells (PGCs) become associated by a network of cytoplasmic bridges upon approaching the genital ridge, and this network persists after entry into the gonad (Gomperts et al. 1994). Whether androgenetic germ cells can be formed and rescued in chimeras remains to be determined, although the very low level contribution of the androgenetic component generally precludes this possibility (Mann et al. 1990). Also, it is necessary to ask for how long imprinting may persist in the germ line, as inherited maternal and paternal imprints must be erased at some stage, and new imprints established according to the individual's sex. To summarize germ cell development, the progenitor cells of the germ line reside at the proximal portion of the primitive ectoderm of the 6.5 days postcoitus (dpc) (pregastrulation stage) egg cylinder, and PGCs are first seen as a discrete alkaline phosphatase positive population of ~100 cells in the base of the extraembryonic allantois at 7.5 dpc (late gastrulation stage). Here, as judged by the pattern of intracellular alkaline phosphatase staining, they undergo maturation and move, probably passively, into the embryonic hindgut endoderm by 8.5 dpc (early somite stage). From this location, they migrate actively to the genital ridges, and probably all PGCs have arrived by 11.5 dpc when the genital ridge is still sexually indifferent. Mitosis ceases in the female germ line at 13.5 dpc, when meiosis commences, and in the male germ line at 14.0 dpc. Meiosis in the latter commences at 7.0 days postpartum (dpp) (Eddy et al. 1981; Ginsburg et al. 1990; Hogan et al. 1994; Lawson and Hage 1994).

Although parental-specific monoallelic expression in the soma is indicative of the presence of imprinting, biallelic expression of *Igf2* in the choroid plexus, leptomeninges (DeChiara et al. 1991), and human tumors (Ogawa et al. 1993; Rainier et al. 1993) has been interpreted to represent imprinting loss. On this same basis of allele-specific expression, we have attempted to gain information about the existence of imprinting in the germ line and its times of erasure and establishment: Simplistically, (1) somatic patterns of allele-specific expression in both germ lines would be indicative of the presence of inherited imprints, (2) biallelic expression in both germ lines would be indicative of the loss of inherited imprints, and (3) appropriate sex-specific biallelic expression, for example, for an imprinted gene paternally expressed in the soma, biallelic expression in the male, and lack of expression in the female germ line, would be indicative of the establishment of new imprints. These expectations are based on the assumption that the presence of imprinting will always result in parental-specific expression, which may not necessarily be the case (dis-

cussed later). Thus, for two imprinted genes that are somatically maternally expressed [*H19* (Bartolomei et al. 1991) and *Igf2r* (Barlow et al. 1991)], and a further two that are somatically paternally expressed [*Igf2* (DeChiara et al. 1991) and small nuclear ribonucleoprotein N, *Snrpn* (Leff et al. 1992; Cattanach et al. 1992)], we provide a quantitative assessment of allele-specific expression throughout germ cell development. Implications of the findings for the presence of imprinting in germ cells, mechanisms of imprinting, and germ cell development are discussed.

Results

Quantitative RT-PCR SNUPE assays

The assay (Singer-Sam et al. 1992; Buzin et al. 1994) is based on a single known nucleotide difference between allelic RNAs. Necessary sequence differences were found in two inbred strains, C57BL/6J (B6) (*Mus musculus*) and ferally derived CAST/Ei (CS) (*M. musculus castaneus*), and an assay was developed for each gene. Results of mixing experiments show that all four assays are rigorously quantitative when utilizing small quantities of total RNA (Fig. 1A–C; Table 1). These assays were used to determine the relative proportions of maternal and paternal allele-specific RNA in intersubspecific (B6×CS)_{F1}, B6CS_{F1}, hybrid embryos.

Biallelic expression in postmigratory PGCs of the morphologically sexually indifferent genital ridge

In postmigratory PGCs purified from the 11.5 dpc sexually indifferent genital ridge, all four genes examined were expressed biallelically (Fig. 2A). Analyses were also conducted on the whole 11.5 dpc genital ridge, which is comprised of both PGCs and somatic cells (Fig. 2A). For *H19* and *Igf2*, monoallelic expression was observed, demonstrating that the somatic cell component exhibited monoallelic expression of these two genes. To account for the complete quenching of the PGC-derived paternal *H19* and maternal *Igf2* signals, the total somatic *H19* and *Igf2* RNA must have been considerably higher than that derived from the germ cell component. This relative difference translates approximately into the amount of RNA per cell, as PGCs comprise a significant proportion of the genital ridge [a crude estimate of 50% by volume based on our estimates of 11.5 dpc genital ridge volume ($13 \times 10^6 \mu\text{m}^3$), PGC cell diameter (14–18 μm), and the number of PGCs present in the 11.5 dpc genital ridge (3000; Tam and Snow 1981)]. In contrast to *H19* and *Igf2*, the whole genital ridge exhibited biallelic expression of *Igf2r* and *Snrpn* (Fig. 2A,B). This result is consistent with comparable amounts of total *Igf2r* and *Snrpn* RNA in the PGC and somatic components, assuming that these two genes were expressed monoallelically in the latter: These two genes, as well as *Igf2* and *H19*, were expressed monoallelically in the adjacent mesonephros, the tissue of origin of the somatic genital

Figure 1. RT-PCR single-nucleotide primer extension (SNUPE) assay for allele-specific RNA quantitation. (A) Outline of assay: RT-PCR is performed on total RNA isolated from F₁ tissue that potentially contains two allelic species of RNAs, B6 and CS, with lower (L) and upper (U) primers. These primers span an exon region that harbors a single defined base difference between the two mouse strains and at least one intron (see Table 1 footnote). The relative proportions of amplified allele-specific DNA products of RT-PCR reflect the original relative proportions of the two allele-specific RNAs. DNA products are isolated, and each of two equal aliquots is subjected to SNUPE with (1) a SNUPE primer that abuts the known base difference, and (2) a radioactive nucleotide that will incorporate into either the B6- or the CS-amplified DNA. The degree of radioactive incorporation is quantified in each of the two reactions (for details, see below), and the relative incorporation represents the relative proportions of allele-specific RNAs. (B) Example of assay: RT-PCR specific for *Snrpn* RNA was performed on 10 ng of total RNA isolated from CSB6F₁ (CS was maternal parent) and the reciprocal hybrid adult (Ad.) brain, and B6CSF₁ hybrid (B6 was maternal parent) 13.5 dpc whole embryo (Em.). Isolated DNA products of RT-PCR were subjected to SNUPE with [³²P]dGTP (top, B6-specific incorporation) or [³²P]dATP (bottom, CS-specific incorporation). This shows that (1) there was no incorporation into the nonspecific DNA template in any of the lanes, and (2) in these tissues, maternal *Snrpn* RNA is not detectable. (C) Assessment of quantitative abilities of assays: For each gene, an RNA reconstruction experiment is shown below the graph. Total RNA isolated from B6 and CS 13.5 dpc embryos was mixed together in different proportions as indicated. (0) 0% B6 and 100% CS RNA; (1) 1% B6 and 99% CS RNA; (5) 5% B6 and 95% CS RNA, etc., and RT-PCR SNUPE performed according to Singer-Sam et al. (1992) and as outlined in A. Total RNA used was 2 ng of *Igf2r*, 0.5 ng of *H19*, 0.5 ng of *Igf2*, 10 ng of *Snrpn*. The values obtained show that for all genes the relationship was linear (R^2 of correlation is >0.99 in all cases). After electrophoresis, each band was quantified with a PhosphorImager, and autoradiographs were made for the separate B6- and CS-specific gels for production of figures. Autoradiographic exposure times were adjusted so that the 100% B6 and 100% CS bands gave approximately the same degree of exposure. Calculation of actual relative proportions of B6 RNA (y axis on graph) was performed using the PhosphorImager data. First, the B6-specific background counts (position of 0% sample) were subtracted from counts obtained for each B6-specific band, and likewise for the CS-specific data. Second, the B6-specific counts were multiplied by counts obtained for 50% CS RNA divided by the values obtained for 50% B6 RNA. This adjustment eliminated bias coming from possible differences in incorporation efficiencies of the two radionucleotides and from differences in the number of nucleotides that incorporate (see Table 1). Data in the remaining figures were adjusted in the same way using the controls included.

Table 1. RT-PCR and SNUPE information

Gene	Primers	Exon	Fragment size (bp) amplified from		Incorporating radionucleotides	
			DNA	mRNA	B6	CS
<i>Igf2r</i>	U, 5'-CTGGAGGTGATGAGTGTAGCTCTGGC-3'	10				
	L, 5'-GAGTGACGAGCCAACACAGACAGGTC-3'	11	~6,000	235	AA	G
	S, 5'-TGC GG GGC CATC-3'					
<i>H19</i>	U, 5'-CCACTACACTACCTGCCTCAGAATCTGC-3'	4				
	L, 5'-GGTGGGTACTGGGCAGCATTG-3'	5	474	413	AAA	C
	S, 5'-ACATTCATACGGAGAGACTC-3'					
<i>Igf2</i>	U, 5'-GACGTGTCTACCTCTCAGGCCGTACTT-3'	5				
	L, 5'-GGGTGTCAATTGGGTTGTTTAGAGCCA-3'	6	735	492	C	TT
	S, 5'-TCAAATTTGGTTTTTAGAA-3'					
<i>Snrpn</i>	U, 5'-TGCTGCTGTTGCTGCTACTG-3'	6				
	L, 5'-GCAGTAAGAGGGGTCAAAAGC-3'	8	~2,000	356	G	A
	S, 5'-CAATTCACAAGAAGCATT-3'					

(U, L) Upper and lower RT-PCR primers, (S) SNUPE primers. The U and L primers anneal to different exons; thus, potentially amplified genomic DNA would be separated from the RNA-specific product because of its greater size; note the difference in genomic DNA- and RNA-specific fragment lengths. In practice, genomic DNA amplification was never observed. Incorporating radionucleotides are those that incorporate into B6- or CS-amplified DNA with SNUPE and, in some cases, are >1. To obtain necessary sequence information for the two different mouse strains, exon fragments were amplified from genomic DNA or total RNA and subcloned into the pCR II vector with the TA Cloning Kit (Invitrogen, San Diego, CA) for sequencing. Sources of information for primer design; (*Igf2r*) kindly provided by Peter Rotwein prior to publication (Szebenyi and Rotwein 1994); (*H19*) (Pachnis et al. 1988); (*Igf2*) (Rotwein and Hall 1990); (*Snrpn*) (Gerrelli et al. 1991).

ridge. Also, they were expressed monoallelically in ovarian follicle and testicular Sertoli cells (see below), which are the differentiated derivatives of the 11.5 dpc germ cell supporting cell lineage.

Biallelic and sexually indifferent expression of imprinted genes in the developing ovary and testis, and in oocytes and spermatogenic cells

The analysis of the whole gonad and mesonephros conducted at 11.5 dpc was also conducted on sexually dimorphic gonads at 12.5–15.5 dpc (Fig. 3A,B). Ovary and testis exhibited biallelic expression of *Igf2r* and *Snrpn*, as was observed at 11.5 dpc. Differences in the degree of bias in allele specific expression obtained, for example, in testis, maternal *Snrpn* RNA was 50% of the total at 12.5 dpc, and 9% of the total at 15.5 dpc, may have reflected relative changes in germ cells and somatic cells with respect to total expression level, and/or cell number. Taking into account the fact that the mesonephros and developing metanephros (m'nephros) continued to display monoallelic expression of all four imprinted genes (Fig. 3A,B), these results strongly suggest that biallelic expression in the germ line of at least *Igf2r* and *Snrpn* continued in the female after the onset and arrest of meiosis at 13.5 dpc and in the male after mitotic arrest at 14.0 dpc. Ovary and testis at 12.5–15.5 dpc exhibited

monoallelic expression of *H19* and *Igf2* (Fig. 3A,B), as was observed at 11.5 dpc. Although we did not isolate germ cells at these stages to examine allele-specific expression, it is probable that continuing biallelic expression in this component was again quenched by considerably higher total amounts of somatic RNA.

Biallelic expression of *Igf2r*, *Igf2*, and *Snrpn* in ovulated unfertilized oocytes of superovulated adult females, and monoallelic expression of these same genes in somatic follicle cells that surrounded these oocytes, was observed (Fig. 3A); we could not detect *H19* RNA in either cell type. In the whole postpartum testis, which is comprised of spermatogenic and somatic cells, the allele-specific expression pattern of the four genes at 6.0 dpp was similar to the situation in the embryonic testis, in that monoallelic expression of *Igf2* and *H19*, and biallelic expression of *Igf2r* and *Snrpn*, was observed (Fig. 4A). In comparison, monoallelic expression of all four genes was observed in the kidney (Fig. 4A). *Igf2* and *H19* displayed biallelic expression in the testis from 10.0 to 16.0 dpp (Fig. 4A). This probably reflected a gradual accumulation of biallelically expressing spermatogenic cell types, beginning with the onset of meiosis at 7.0 dpp. Biallelic expression in the germ cell component of the postpartum testis was confirmed in purified spermatogenic cell types: All four genes were biallelically expressed in mitotic spermatogonia, purified from 7.0 dpp testes. *Igf2r*,

Figure 2. Allele-specific expression in sexually indifferent 11.5 dpc embryonic gonads. (A) Unsexed embryos. (GON) Whole gonad; (MN) mesonephros; (PGC) purified PGCs. The two lanes under each heading represent two individual B6CSF₁ embryos, except for PGC, where cells were purified from groups of ~10 gonads. RT-PCR was performed on one-fifth of the total RNA obtained per gonad and mesonephros, and on ~100 PGCs purified to homogeneity by micromanipulation (see Materials and methods). Each imprinted gene is represented by two rows of autoradiograph bands, with the *top* and *bottom* representing the presumptive inactive and active alleles, respectively, with the parental origin given at *right*. The number above each band is the amount of RNA of the presumptive inactive allele expressed as a percentage of the total amount, rounded off as follows: 0%, 0.49% or less, and 1%, 0.5% to 1.49%, etc. The first three lanes (labeled %) are RNA quantitation controls performed along with the experimentals so as to allow for the adjustment of experimental values according to the legend to Fig. 1C: (1) 0% B6 and 100% CS RNA; (2) 50% B6 and 50% CS RNA; (3) 100% B6 and 0% CS RNA. (B) Sexed embryos. (M) Male whole gonad; (F) female whole gonad. Before carrying out RT-PCR SNuPE assays, the sexes of embryos were determined using a PCR assay for the Y chromosome-linked *Zfy* gene (Singer-Sam et al. 1990). Other details are as described in A.

Igf2, and *Snrpn* were biallelically expressed in meiotic pachytene spermatocytes, purified from adult testes, although *H19* RNA was not detected (Fig. 4B). RNA of both allelic forms of *Igf2r*, *Igf2*, and *Snrpn* was also de-

tected in groups of postmeiotic haploid round spermatids, purified from adult testes, whereas *H19* RNA was again not detected (Fig. 4B). Monoallelic expression in the somatic component was evident for *Igf2* and *H19* in the analysis of whole testis at 6.0 dpp (Fig. 4A), and for *Igf2r* and *Snrpn* in the examination of Sertoli cells purified from 7.0 dpp testis (Fig. 4B). Sertoli cells also exhibited monoallelic expression of *Igf2* and strongly biased allele-specific expression of *H19* (Fig. 4B).

The fact that all four genes were biallelically expressed in the whole 10.0 to 16.0 dpp testis (Fig. 4A) demonstrates that the total RNA of each gene in the germ cell component was comparable to that in the somatic cell

Figure 3. Allele-specific expression of imprinted genes in sexually dimorphic 12.5 to 15.5 dpc embryonic gonads. (A) Ovary and adjacent meso/metanephros (m'nephros). Also shown are ovulated unfertilized oocytes and follicle cells isolated from superovulated adult B6CSF₁ females. RT-PCR was performed on one-fifth of the total RNA obtained from each gonad and m'nephros, and from 10 zona-free oocytes. No *H19* RNA was detected in oocytes and follicle cells, and very little *Igf2* RNA was detected in follicle cells. The negative signal for *H19* was also obtained using groups of 50 oocytes (data not shown). (B) Testis and adjacent m'nephros. Other details are as described in the legend to Fig. 2.

component. This would also be true on an RNA per cell basis, as the total number of germ cells is similar to the number of Sertoli cells at these stages (Bellvé 1993). The results of the whole study described above are summarized in Figure 5.

Figure 4. Allele-specific expression in neonatal testis and spermatogenic cells. (A) Neonatal testes: Analyses were conducted on 20 ng of total RNA isolated from one whole testis and kidney of one B6CSF₁ individual for each stage. All six individuals used were littermates. Other details are as described in the legend to Fig. 2, except that the fourth lane is a control, using 100 ng total RNA isolated from the whole 13.5 dpc B6CSF₁ embryo minus the head. (B) Spermatogenic and Sertoli cells: (SG) 7.5 dpp mitotic spermatogonia, (PS) adult meiotic pachytene spermatocytes (ST) adult postmeiotic haploid round spermatids (SER) 7.5 dpp Sertoli cells. (PGC) PGCs isolated from sexually indifferent 11.5 dpc gonads shown for comparison (these lanes are also shown in Fig. 2). RT-PCR SNUPE was performed on groups of 100 spermatogenic or Sertoli cells purified to homogeneity by micromanipulation (see Materials and methods). The negative signal for *H19* in PS and ST was also obtained using groups of 300–500 cells (data not shown).

Figure 5. Summary of allele-specific expression of imprinted genes in the germ line and soma. Total circle area represents 100% expression, contributed by both alleles. The area of the shaded portion represents that proportion coming from the allele normally inactive in the soma. If this is the maternal or paternal allele, the shading is on the left or right, respectively. For spermatids, split circles represent their haploid status.

Expression may be monoallelic in premigratory PGCs at the base of the allantois

It is not possible manually to purify premigratory PGCs because of their small numbers and because they do not reside in a very discrete structure such as the genital ridge. Nevertheless, as determined by alkaline phosphatase staining, ~125 are congregated at the base of the allantois of 7.5 dpc late gastrulation presomite-stage embryos (Ginsburg et al. 1990). We therefore compared the allele-specific expression of the four genes in the base and tip of the allantois, the latter of which is devoid of germ cells. The allantoic base section dissected was approximately spherical and 160 μ m in diameter. Thus, assuming an average cell diameter of 10 μ m, the total number of cells is 4000, placing the proportion of PGCs in this dissected portion crudely at 3%. The SNUPE assays can distinguish a 1% proportion of expression (Fig. 1); thus, if expression of the four genes in PGCs in this region was biallelic, and at similar or greater than somatic levels, this might be detectable. For *Igf2r* and *Igf2*,

oocytes, it is unknown if the “inappropriate” expression of *Snrpn* and *Igf2* (maternally inactive in the soma) represents that occurring during the oocyte growth phase or during the later maturation phases. Many RNA species produced during oocyte growth are stored and dormant (Huarte et al. 1987). Similarly, in groups of haploid round spermatids, existent RNA of *Igf2r* (paternally inactive in the soma) could have represented that expressed at earlier spermatogenic stages, or at this early haploid stage. The fact that mice can be produced by the fusion of a haploid round spermatid with an ovulated oocyte strongly suggests that paternal imprinting is complete by this stage of spermatogenesis (Ogura et al. 1994). Nevertheless, although there is little evidence for regionalized differentiation of the egg cytoplasm, there remains the formal possibility that the maternal and paternal genomes may thereby be differentially modified, with initial or primary imprints being imparted after introduction of the paternal nucleus into the egg cytoplasm.

The chromatin structures of the maternal and paternal pronuclei are not identical, and it has been suggested that consequent differential accessibility of transcription factors and methylation enzymes could lead to the imprinting of genes (Wiekowski et al. 1993). However, in this case, differences in maternal and paternal pronuclear chromatin structure could be thought of as one of a series of imprinting events that could eventually lead to monoallelic expression; that is, rather than being a one-time event late in gametogenesis or in the zygote, imprinting may be a multistep process such that prezygotic, then sequential zygotc and postzygotc epigenetic events occur in *cis* before monoallelic expression is established (Allen and Mooslehner 1992; Chaillet 1992; Bartolomei et al. 1993; Brandeis et al. 1993; Ferguson-Smith et al. 1993; Feil et al. 1994; Latham et al. 1994; Szabó and Mann 1994).

Bypass of imprinting

Whereas absence of imprinting, or incomplete imprinting, may explain the biallelic and sexually indifferent expression of imprinted genes in the germ line, an alternative explanation is that fully established inherited and/or new imprints are not recognized or bypassed. It is of interest that pluripotent cell lines isolated from PGCs of the male 12.5 dpc genital ridge can invoke skeletal defects in chimeras reminiscent of those specific to androgenetic chimeras (Mann et al. 1990; Labosky et al. 1994). Although this might be indicative of an early establishment of paternal imprints, it is not known whether the two phenotypes have the same etiology and whether pluripotent cell lines isolated from female genital ridges might also invoke the abnormalities.

If imprinting is bypassed in the germ line, how might this be achieved? Bypass of imprinting has been suggested as a mechanism in explanation of the biallelic expression of imprinted genes during preimplantation development, embryonic stem (ES) cells, exceptional tissues, and tumors (Barlow 1994; Efstratiadis 1994; Latham et al. 1994; Szabó and Mann 1994). More specif-

ically, *trans*-acting factors, which interact with imprints in a stage- and cell-specific manner to activate or inactivate alleles, have been invoked to explain transitions from biallelic to monoallelic modes of expression, and vice versa (Barlow et al. 1994; Latham et al. 1994). For the germ line, an alternative model based on chromatin structure is also possible: Experiments involving the injection of promoter–enhancer constructs into ova have indicated that enhancers act by relieving repression of promoters. Moreover, alteration of chromatin structure in the zygote can result in bypass of this enhancer-dependent promoter activity (Majumder et al. 1993; Wiekowski et al. 1993). Thus, if imprinting were to act by blocking enhancer function, thereby causing a gene to remain inactive in *cis*, this function could be bypassed in the germ line by some generalized alteration in chromatin structure. This model is depicted in Figure 7. In this model, alterations in chromatin structure, specific to germ cells, allows promoter activity regardless of the status of enhancer functionality. This model accounts for the biallelic expression in both the female and male germ lines of all four imprinted genes examined. More specifically, it readily accounts for the biallelic coexpression of *H19* and *Igf2* in PGCs and spermatogonia, as in

Figure 7. Enhancer imprinting model. Somatic cells: (a) Transcription is allowed by the chromatin structure that results from the interaction between the enhancer and the promoter. (b) Transcription is disallowed by a constrained chromatin structure, e.g., in the 5' region, as the imprint, e.g., CpG methylation, blocks interaction between the enhancer and the promoter. Germ cells: (c,d) a unique chromatin conformation is conducive to promoter activity, irrespective of the status of interaction between the enhancer and the promoter. In these cells, levels of expression would not be expected to be as high as in somatic cells that fully utilize the enhancers.

somatic tissues, mutually exclusive expression of *H19* and *Igf2* in *cis* is enhancer dependent (Bartolomei et al. 1993; Leighton et al. 1995).

How might the alteration in chromatin structure suggested in the model be achieved? Hyperacetylation of core histones is known to alter chromatin structure, and transcriptionally active chromatin is hyperacetylated (Turner 1991; Jeppesen and Turner 1993). Cytosine methylation can influence chromatin structure (Zacharias 1993). In addition, the presence of methylation at specific sites, such as promoters and CpG islands on the X chromosome, can be associated with gene repression (for review, see Jost and Saluz 1993). Also, *Xenopus* germ cells possess at least one unique histone variant (Chao and Wolffe 1994; Dworkin-Rastl et al. 1994). Although the histone acetylation status of germ cells is unknown, PGCs of the 12.5 dpc gonad are known to be globally undermethylated (Monk et al. 1987; Kafri et al. 1992), which could contribute to a relaxation of constraints in chromatin structure. These considerations of imprinting bypass could be relevant to the biallelic expression of imprinted genes in methylation-deficient mouse embryos (Li et al. 1993). In this instance, rather than the loss of imprints being the primary event, generalized alterations in chromatin structure, caused by methylation deficiency, may lead to the bypass of fully imprinted regulatory elements.

Germ cell development

Although the status of imprinting in the germ line will be facilitated by the unequivocal identification of an imprinting mechanism, our results demonstrate that from the time PGCs enter the genital ridge, and possibly earlier, the germ line has no requirement for genomic imprinting in terms of monoallelic expression, that is, if the function of imprinting is to invoke monoallelic expression, then this function is neutralized in the germ line. One possible reason for this neutralization may be to retain the potential for the activity of those imprinted genes that would otherwise exhibit a $-/-$ mode of expression. However, on this point, both sexes of mice with no *Igf2* or *H19* activity are fully fertile (DeChiara et al. 1991; Leighton et al. 1995). The findings also imply that there is no functional difference between the inherited maternal and paternal genomes in germ cell development, at least by the time of genital ridge colonization. A logical extension of this implication is that PGCs of parthenogenetic and androgenetic origin may have the same potential as wild-type PGCs for normal development and differentiation in either sexual pathway.

Imprinting neutralization would appear to be a salient feature of the germ line and is coincident with the unique developmental potentiality of this cell lineage. It has been suggested that a restoration of totipotency in germ cells, subsequent to prior restrictive events in their primitive ectoderm cell progenitors, is linked with the onset of meiosis, X chromosome reactivation, and erasure of imprinting (Monk 1981, 1988). However, if there is a linkage between imprinting neutralization and nu-

clear totipotency in the germ line, then the restoration of totipotency must occur sometime during the mitotic phase of germ cell development, and before sexual differentiation of the gonad.

Materials and methods

Quantitative RT-PCR SNUPE assays

These assays were performed essentially as described (Singer-Sam et al. 1992).

Preparation of RNA Tissue samples were collected in 0.1 ml of RNazol (Tel-Test Inc., Friendswood, TX). To facilitate precipitation of RNA, 2 μ l of 0.25% linear polyacrylamide was added as carrier (Gaillard and Strauss 1990). The pellet was dissolved in water containing 1 U/ μ l of RNasin (Promega) and 10 mM DTT, which were carried over in the RT-PCR reaction.

RT-PCR All reactions were carried out in a Programmable Thermal Controller (MJ Research Inc., Watertown, MA). RNA was preassociated with 2.5 μ M of the lower primer, in 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 5 mM MgCl₂, and 1 mM dNTPs by incubating at 64°C for 10 min and then slowly cooling to 42°C for 15 min. At this point, 2.5 U/ μ l of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) and 1 U/ μ l of RNasin was added, and the incubation continued at 42°C for 15 min. The RT reaction was stopped by heating to 99°C for 5 min. Samples were then cooled to 65°C and kept at this temperature while being pipetted into tubes containing PCR master mix, and then the PCR reaction was started. The final concentrations of ingredients in 0.1-ml PCR reactions were 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1 mM MgCl₂, 200 μ M dNTPs, 0.5 μ M of the lower and upper primers and 2.5 units per reaction of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). PCR run conditions were 95°C for 30 sec, 42°C for 30 sec, and 72°C for 2 min for 40 cycles. PCR primers had a T_m of 52–64°C at 2 mM MgCl₂, and were designed with the aid of a computer program (Oligo 4.0, National Bioscience Inc., Plymouth MN). Isolation of amplified DNA products: These were subjected to agarose gel electrophoresis in 1 $\times$ TAE buffer, and the single bands obtained were isolated with the Prep-A-Gene DNA Purification Kit (Bio-Rad). DNA was suspended in water at $\sim$ 2 ng/ μ l.

SNUPE The reaction mix contained, in a total volume of 10 μ l, $\sim$ 10 ng of the isolated DNA fragment, 1 μ M of the SNUPE primer, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.75 units of AmpliTaq DNA polymerase, and 2 μ Ci of [³²P]dNTP according to Table 1. The radionucleotides (3000 Ci/mmole, 10 μ Ci/ml), were diluted 10-fold in water, and 2- μ l aliquots were added to each tube just prior to incubation. All other ingredients were added to the template as a master mix. Primers were kept in small aliquots to avoid repeated freeze-thawing. The SNUPE reaction consisted of one cycle of 95°C for 30 sec, 42°C for 30 sec, and 72°C for 1 min. After electrophoresis of the samples on a 15% denaturing polyacrylamide gel, bands were visualized by autoradiography and quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Production of B6CSF₁ embryos and postnatal mice

Prepubertal B6 female mice (Jackson Laboratory, Bar Harbor, ME) were superovulated by injection of pregnant mare's serum gonadotrophin and human chorionic gonadotrophin (HCG) (Hogan et al. 1994) and caged with CS males (Jackson Labora-

tory) for mating. The resulting B6CSF₁ zygotes were released from oviducts in medium M2 (Wood et al. 1987) and transferred immediately to oviducts of 0.5 dpc pseudopregnant recipients; or B6CSF₁ morulae were flushed from oviducts at 2.5 dpc, cultured overnight in medium CZB plus glucose (Chatot et al. 1989) to the blastocyst stage, and transferred to the uteri of 2.5 dpc pseudopregnant recipients according to Mann (1993), except that the following anesthetic regime was used: Mice were injected with 19 µg of fentanyl citrate (Elkins-Sinn Inc., Cherry Hill, NJ) and 113 µg of midazolam HCl (Versed; Roche Pharma. Inc., Minati, PR) together in 0.5 ml into the body cavity and, after 3 min, were fully anesthetized with a nose cone [3-ml disposable hypodermic syringe cover] containing gauze soaked with methoxyflurane (Metofane, Pitman-Moore Inc., Mundelein, IL), which was removed after commencement of surgery. Following surgery, and before administering a wound-clip, 2 µg of naloxone HCl (Astra Pharma. Prod. Inc., Westborough, MA) in 50 µl was pipetted into the body cavity to reverse the effects of fentanyl; after Flecknell (1993).

Purification of germ and somatic cells

Oocytes and follicle cells Adult B6CSF₁ females were injected with superovulatory hormones as described above, and 14 hr after HCG injection, the oviducts were removed and the masses of unfertilized oocytes and adhering follicle cells were released into medium M2 containing hyaluronidase. Following release of the oocytes from follicle cells, they were rinsed repeatedly in successive changes of medium M2 until all follicle cells were absent; the zonae pellucidae were then removed by acid Tyrode's treatment (Hogan et al. 1994). Small groups of oocytes or much larger numbers of follicle cells were then pipetted into 0.1-ml aliquots of RNazol in microcentrifuge tubes and kept frozen over liquid nitrogen until SNUPE analysis.

Spermatogenic and Sertoli cells Seminiferous tubules were dispersed into a single cell suspension according to Bellvé (1993), except that the base medium used throughout was PB1 (Wood et al. 1987) minus pyruvate, plus nonessential amino acids and glutamine at standard concentrations. For purification, two shallow drops of medium were placed onto the under-surface of a 6-cm tissue culture dish lid and overlaid with paraffin oil, and some of the suspension of spermatogenic cells pipetted into one of the drops. Then, under phase-contrast optics, cells of a particular identity were recognized according to size (Bellvé 1993), aspirated into a glass collection pipette controlled by a micrometer syringe [similar to that used in ES cell blastocyst injection, Hogan et al. (1994)], and expelled into the second drop of medium as discrete groups of 100 cells. This procedure for purification was used instead of the gravity sedimentation method (Bellvé 1993) so as to obtain samples as close to absolute purity as possible for RT-PCR. Each group of cells was placed into RNazol and stored as described above until SNUPE analysis. Pachytene spermatocytes and round spermatids were collected from adult B6CSF₁ testes, and spermatogonia and Sertoli cells were collected from 7.0 dpp B6CSF₁ testes.

PGCs These cells from 11.5 dpc genital ridges were dispersed according to Buehr and McLaren (1993). Then, groups of 100 PGCs were collected by micromanipulation, placed into RNazol, and stored as described above for spermatogenic cells.

Acknowledgments

We thank Gerd Pfeifer for inspiration and advice, Jeanne LeBon and Judy Singer-Sam for technical advice, Hector Rivera for DNA sequencing of subclones, Gerd Pfeifer, Ibolya Kiss, Art Riggs, and Judy Singer-Sam for their comments on the manuscript, and Peter Rotwein for providing *Igf2r* sequence information. This work was supported by a National Institutes of Health award (MGN 1R01 GM48103-01) and a National Science Foundation award (MCB 9405373) to J.R.M.

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Genes Dev. 1995, **9**:

Access the most recent version at doi:[10.1101/gad.9.15.1857](https://doi.org/10.1101/gad.9.15.1857)

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