Interspecies Approaches for the Analysis of Parental Imprinting During Mouse Development

A. J. Villar and R. A. Pedersen

Genomic imprinting results in the functional specialization of the maternal and paternal genomes during development whereby offspring inherit only one active copy of a gene. Although the reason for its evolutionary genesis remains speculative, the consequences of genomic imprinting are evidenced by the failure of parthenogenetic and androgenetic development in mammals, and parental-specific effects in the etiology of a number of human diseases. While the precise molecular mechanism of imprinting is unknown, evidence suggests that the specialization of the parental genomes is established during gametogenesis when the parental genomes are epigenetically modified to reflect the parent of origin. To examine the epigenetic modification of specific imprinted genes and subsequent differential expression, an assay is required that can distinguish between the maternal and paternal alleles and their respective transcripts. During the past 5 years several strategies have been used to identify imprinted genes, ranging from the fortuitous disruption of specific parental alleles to subtraction hybridization between cDNAs from normal and parthenogenetic embryos. To study the developmental regulation of these imprinted genes during mammalian development, we describe a relatively simple interspecies "mRNA phenotyping" approach applicable to the analysis of allele-specific expression as well as the identification of candidate imprinted genes.

Genomic imprinting, defined as the differential expression of alleles inherited from the maternal and paternal genomes (El-Stratiladis 1994; Pedersen et al. 1993), adds another dimension to the already complex issue of transcriptional regulation. Unlike most genes which are transcribed by both parental alleles, an unknown number of genes are epigenetically modified such that only one copy is actively transcribed, that is, from either the maternal or paternal allele. The term "imprinting" was first used by Crouse (1960) to describe the negative selection against the paternal chromosomes in Sciara and by Lyon and Glenister (1977) to describe uniparental (maternal/paternal) disomy effects. The definition of "imprinting" has since been expanded by developmental geneticists to describe the preferential inactivation of the paternal X-chromosome in extraembryonic tissues of mice and humans (Takagi and Sasaki 1975; West et al. 1977) and the non-Mendelian inheritance of genetic traits as a result of the differential epigenetic modification of the maternal and paternal autosomes (Cattanach and Beechey 1990; Searle et al. 1989). Although evidence suggests that the genomic imprint is established in the germ line (Surani et al. 1988; Swain et al. 1987), the consequences of imprinting are only revealed when manifested in the form of differential transcription (imprinted expression) and differential developmental potential (imprinted phenotype) depending on the parent of origin (Mann and Lovell-Badge 1984; McGrath and Solter 1984).

The effects of differential epigenetic modification of the parental genomes have been observed for more than a dozen genes whose expression is dependent on whether they are transmitted through either the maternal or paternal germ line. Unfortunately there is no clear defining feature common to all imprinted genes, making it difficult to draw conclusions as to the evolutionary role and biological function of genomic imprinting. It has been estimated that there are at least 100 imprinted genes in the mammalian genome (Hayashizaki et al. 1994). As these genes are identified, it is essential to determine the developmental and tissue-specific regulation of their allele-specific expression, not only to understand the cause but also the consequences of genomic imprinting in human disease and
The Biology of Genomic Imprinting

At least one species of every vertebrate class can reproduce parthenogenetically, with the exception of mammals. Despite their absence in nature, however, mouse embryos lacking a paternal genome or maternal genome can be produced experimentally. Analysis of these isoparental embryos has provided insight into the biological consequences of genomic imprinting on mammalian development.

Embryos lacking a paternal genome (parthenogenones) may be produced experimentally by activation of the egg by one of a wide variety of stimuli which initiates the program of development in vivo and in vitro (Cuthbertson 1983; Tarkowski 1975). Parthenogenetic eggs develop poorly and have abnormal extraembryonic tissues; the yolk sac is small with meager vasculature and the ectoplacental cone and trophoblast are sparse (Surani and Barton 1983; Surani et al. 1984; Wakasagi 1974). Development directed by maternal products in the egg and the maternal genome can proceed to advanced stages of development, but fails when rapid growth is needed after day 11 (approximately 25 somite stage) (Graham 1974).

The developmental potential of the paternal genome has also been addressed by constructing zygotes from which the maternal pronucleus is removed and replaced by the paternal pronucleus from another zygote to form androgenetic embryos. Embryos with two paternal pronuclei (androgenetic) have substantial trophoblast but retarded development of extraembryonic tissues, it appears that the presence of two active maternal X chromosomes also contributes to this defect (Shoa and Takagi 1990).

Despite the fact that parthenogenetic and androgenetic embryos are nonviable and die before or shortly after implantation, embryonic stem (ES) cells derived from these embryos are viable in vitro and in vivo. Chimeras between normal and parthenogenetic embryos/ES cells can develop to term with parthenogenetic cells contributing to almost all tissues including germ cells (Anderegg and Markert 1986; Stevens 1978; Stevens et al. 1977; Surani et al. 1977). Similar results have been reported for chimeras between normal and androgenetic embryos/ES cells. However, there is a high incidence of lethality and the chimeras develop characteristic skeletal abnormalities (Mann and Stewart 1991).

The Mechanism of Genomic Imprinting

Changes in gene activity during development are generally referred to as epigenetic (Holliday 1987). Epigenetic switches turn particular genes on or off during developmental processes, producing either transient changes in gene activity or a permanent pattern of activities. The significance of epigenetic modification mechanisms is that they provide a molecular basis for the somatic inheritance of a particular pattern of gene activities (Scaroni 1971). Eukaryotic DNA contains 5-methylcytosine (5mC) as the sole modified base, which appears exclusively at CpG dinucleotides (Razin and Riggs 1980).

Although several lines of evidence have strongly implicated 5mC in the control of gene expression in higher organisms, in most of these studies it is not clear whether methylation is the cause or consequence of gene inactivation. Evidence suggests that DNA methylation may be superimposed on prior events that are themselves the primary mechanisms regulating activity of genes or chromatin domains to stabilize inactive regions (Lock et al. 1987; Monk 1986). Analysis of X chromosome inactivation in marsupials supports the idea that the initiation of gene inactivation can be disassociated from methylation (Kaslow and Migeon 1987). However, some of the strongest evidence that methylation is important for the epigenetic control of gene expression comes from experiments that show the pattern of methylation is inherited through DNA synthesis and cell division (Monk 1986; Razin et al. 1984; Stein et al. 1982), a prerequisite for the mechanism of imprinting.

It is generally presumed that the mechanism of imprinting is established in the gametes when the paternal and maternal development. In addition to providing a brief introduction to the phenomenon of genomic imprinting, we describe an interspecies mRNA phenotyping approach for the detection of allele-specific gene expression applicable to the general analysis of genomic imprinting throughout mammalian development.
Polymorphic imprinting in placenta and brain. asynchronous replication and histone modifications in genomic imprinting, including DNA methylation and other potential mechanisms in 1993). For further discussion of the role of methylation, DNA methylase, suggesting normal levels of expression is barely detectable in embryos deficient in DNA methylase, suggesting normal levels of methylation play a role in sustaining rather than repressing transcription (Li et al. 1993). Further discussion of the role of methylation and other potential mechanisms in genomic imprinting, including asynchronous replication and histone acetylation (see Gold and Pedersen 1994; Ohlsson et al. 1995; Reik and Allen 1994). Genomes are separated. The pattern of methylation of four transgenes has been investigated in detail (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987). As these transgenes pass from one generation to another their methylation pattern is reversed during successive generations depending on the parent of origin. In general, the transgene is less methylated when inherited from the father. This would seem to contradict the observation that in unique and some repetitive sequences, sperm DNA is more methylated than oocyte DNA (Sanford et al. 1987); however, these differences are lost soon after fertilization (Howlett and Reik 1991; Kafri et al. 1992). It should be kept in mind that regardless of global methylation patterns, differential methylation of specific sites and/or chromosome domains may be the key to the phenomenon of imprinting. It is unclear, however, whether the imprint that causes differential expression of the parental alleles acts to permit or to suppress gene expression. For example, in ES cells and embryos with a homozygous deletion of the DNA methylase gene, a decrease in overall methylation correlates with activation of the normally silent paternal H19 allele, suggesting methylation is required for maintaining the transcriptionally inactive state (Li et al. 1993). In contrast, Igf2 expression is rarely detectable in embryos deficient in DNA methylase, suggesting normal levels of methylation play a role in sustaining rather than repressing transcription (Li et al. 1993). For further discussion of the role of methylation and other potential mechanisms in genomic imprinting, including asynchronous replication and histone acetylation (see Gold and Pedersen 1994; Ohlsson et al. 1995; Reik and Allen 1994). Identities and Expression Patterns of Known Imprinted Genes The effects of parental inheritance of specific regions of the genome have been evaluated by means of intercrosses between mice carrying either Robertsonian or reciprocal translocations of nonhomologous chromosomes (Beechey and Searle 1987; Cattanach and Kirk 1985; Searle and Beechey 1979, 1985). Embryos with maternal or paternal duplications and the respective paternal and maternal deficiencies display a variety of abnormal phenotypes. This phenomenon was recognized to result from the differential expression of the maternal and paternal chromosomes, the implication being that there are specific genes preferentially expressed when inherited from one parent but not the other (Lyon and Glenister 1977). An imprint map has been developed that defines the chromosomal regions shown to have defective complementation depending on the parent of origin. The chromosomes affected by paternal deficiency (i.e., in parthenogenotes) are 2, 6, 7, 11, and 12. Those affected by maternal deficiency (i.e., in androgenotes) are chromosomes 2, 7, 11, 12, and 17. Duplications of chromosomes 1, 5, 9, 14, and 17 are not lethal but may be associated with differential recovery of offspring. The remaining autosomes of the mouse genome either have not been tested (chromosomes 8, 10, and 18) or show completely normal complementation when inherited as duplications or deficiencies. Thus the correlations between parental chromosome duplication/deficiency and anomalous phenotypes provide evidence that differential expression of maternally and paternally derived alleles results in the overproduction or absence of stage- and cell-specific expression of developmentally essential genes. The consequences of genomic imprinting in the germ line and its wide-ranging implications for mammalian embryogenesis and human disease emphasize the importance of identifying and characterizing imprinted genes. The actual number of imprinted genes is not known because the only information available is the fact that certain chromosomes contain regions displaying a parental effect. Identification of these genes may also be complicated by developmental and tissue specificity. Table 1 summarizes the known imprinted genes in mouse and human (Barlow 1995; Efstratiadis 1994).

**Table 1. Summary of imprinted genes in mouse and human**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expressed allele</th>
<th>Mouse</th>
<th>Human</th>
<th>Methylated allele</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Igf2</td>
<td>Paternal</td>
<td>11p15</td>
<td>Paternal</td>
<td></td>
<td>DeChiara et al. 1991; Giauque et al. 1993; Sasaki et al. 1992</td>
</tr>
<tr>
<td>Igf2r</td>
<td>Maternal</td>
<td>6q25-q27*</td>
<td>Maternal</td>
<td></td>
<td>Barlow et al. 1991; Stoger et al. 1993; Xu et al. 1993</td>
</tr>
<tr>
<td>H19</td>
<td>Maternal</td>
<td>7</td>
<td></td>
<td>15q12</td>
<td>Glenn et al. 1993; Lell et al. 1992; Reed and Lelli 1994</td>
</tr>
<tr>
<td>Srrtn</td>
<td>Paternal</td>
<td>6, 7</td>
<td></td>
<td>n.d.</td>
<td>Giddings et al. 1994</td>
</tr>
<tr>
<td>Ins1, Ins2</td>
<td>Paternal</td>
<td>11</td>
<td></td>
<td>11p13</td>
<td>Hayashizaki et al. 1994</td>
</tr>
<tr>
<td>Xist</td>
<td>Paternal</td>
<td>X</td>
<td></td>
<td>X</td>
<td>Kay et al. 1993; Norris et al. 1994</td>
</tr>
<tr>
<td>Mas</td>
<td>Paternal</td>
<td>7</td>
<td></td>
<td>n.d.</td>
<td>Villar and Pedersen, 1994a</td>
</tr>
<tr>
<td>Mash2</td>
<td>Maternal</td>
<td>7</td>
<td></td>
<td>15q</td>
<td>Guillen et al. 1994</td>
</tr>
<tr>
<td>ZNF127</td>
<td>Maternal</td>
<td>7</td>
<td></td>
<td>15p</td>
<td>Dritschel et al. 1992; Efstratiadis 1994</td>
</tr>
<tr>
<td>PAR1/PAR5</td>
<td>Paternal</td>
<td>15q</td>
<td></td>
<td>n.d.</td>
<td>Nicholls 1994</td>
</tr>
<tr>
<td>Ipf1</td>
<td>Paternal</td>
<td>15q</td>
<td></td>
<td>n.d.</td>
<td>Wierick et al. 1994</td>
</tr>
<tr>
<td>Pegl/Mest</td>
<td>Paternal</td>
<td>6</td>
<td></td>
<td>n.d.</td>
<td>Kaneko et al. 1995</td>
</tr>
</tbody>
</table>

* Polymorphic imprinting in placenta and brain.
* Polymorphic imprinting in human fetuses.
* Imprinting status not known
n.d. = not done.

The effects of parental inheritance of specific regions of the genome have been evaluated by means of intercrosses between mice carrying either Robertsonian or reciprocal translocations of nonhomologous chromosomes (Beechey and Searle 1987; Cattanach and Kirk 1985; Searle and Beechey 1979, 1985). Embryos with maternal or paternal duplications and the respective paternal and maternal deficiencies display a variety of abnormal phenotypes. This phenomenon was recognized to result from the differential expression of the maternal and paternal chromosomes, the implication being that there are specific genes preferentially expressed when inherited from one parent but not the other (Lyon and Glenister 1977). An imprint map has been developed that defines the chromosomal regions shown to have defective complementation depending on the parent of origin. The chromosomes affected by paternal deficiency (i.e., in parthenogenotes) are 2, 6, 7, 11, and 12. Those affected by maternal deficiency (i.e., in androgenotes) are chromosomes 2, 7, 11, 12, and 17. Duplications of chromosomes 1, 5, 9, 14, and 17 are not lethal but may be associated with differential recovery of offspring. The remaining autosomes of the mouse genome either have not been tested (chromosomes 8, 10, and 18) or show completely normal complementation when inherited as duplications or deficiencies. Thus the correlations between parental chromosome duplication/deficiency and anomalous phenotypes provide evidence that differential expression of maternally and paternally derived alleles results in the overproduction or absence of stage- and cell-specific expression of developmentally essential genes.

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**Analysis of Genomic Imprinting Using Interspecific Hybrids**

The key to the analysis of imprinting is the ability to distinguish between the parental genomes. Nuclear transfer and classical genetic analysis indicate that there are an undefined number of genes differentially expressed when contributed by the maternal or paternal gamete. Therefore, it is logical to assume the mRNA and protein profiles of androgenotes and parthenogenotes would be unique and differ from a normal embryo of the same developmental stage. Although the simplest approach to the identification of genes uniquely expressed by the maternal or paternal ge-
Analyze Hybrids as:

- Embryos
- Fetuses
- Newborns
- Embryonic Stem Cells

Figure 2. Interspecific mouse hybrid approach for the detection of allele-specific expression applicable to the analysis of imprinted genes at any stage of development using an interspecies cross between *M. musculus* and *M. spretus*.

The genome would be by subtracted cDNA libraries (Kaneko-Ishino et al. 1995) or comparative two-dimensional gel analysis of proteins (Bowden et al. 1996) of such uniparental embryos, the feasibility of these approaches is restricted by the limited lifespan of isoparental embryos, the paucity of material, and the abnormal lineage representation, that is, some tissues are abnormal or missing in parthenogenones and androgenones (McGrath and Solter 1984; Sturm et al. 1994). Alternative approaches for the detection and analysis of imprinted genes have evolved from the analysis of isoparental-normal chimeras (Mann et al. 1990; Nagy et al. 1987; Paldi et al. 1989) or of mice inheriting whole or parts of chromosomes from only one parent (Ferguson-Smith et al. 1991). In addition, isoparental embryonic stem cell lines have been generated (Allen et al. 1994; ...
Mann et al. 1990; Mann and Stewart 1991; Szabo and Mann 1994). Although these strategies have been successfully used to distinguish between maternal and paternal allele expression, they have the disadvantage of being labor intensive and time consuming; an example is the production of parthenogenetic and androgenetic embryos from which ES cells are derived. For these reasons several laboratories have developed alternative approaches for the detection of allele-specific expression applicable to the analysis of imprinted genes at any stage of development using interspecies crosses (Figure 2). For instance, RNase protection (Bartolomei et al. 1991) and reverse transcription-polymerase chain reaction (RT-PCR) single nucleotide primer extension (Szabo and Mann 1995a) assays, based on defined nucleotide differences between allelic RNAs of interspecies hybrids, permit sensitive and quantitative analysis of allele-specific expression.

By exploiting the genetic diversity between *M. musculus* and *M. spreitus*, we analyzed the developmental and tissue-specific regulation of genomic imprinting using mRNA phenotyping to distinguish between maternal and paternal allele expression in hybrid progeny (Villar and Pedersen 1994b; Figure 3). To detect allele-specific expression we used RT-PCR to amplify the cDNA of interest and restriction fragment length polymorphisms (RFLPs), that is, mRNA phenotypes, unique to the two species. Thus the mRNA phenotype exhibited by the progeny from an interspecies cross reflects the parent of origin. Because reciprocal cross-matings between *M. musculus* males and *M. spreitus* females failed to produce progeny, backcross matings between F₁ females (derived from matings of *M. musculus* females and *M. spreitus* males) and *M. musculus* males were performed to verify parental imprinting. By demonstrating that the mRNA phenotype expressed by heterozygous F₂ progeny is dependent on the parent of origin rather than the species of origin precludes the possibility that the allele-specific expression is a result of a dominant species effect or selective PCR amplification.

Evidence suggests that imprinted DNA sequences are associated with CpG islands, monoparental methylation, and direct repeats (Neumann et al., 1995). Although these features have been proven useful in strategies designed to identify imprinted regions within the genome e.g., restriction landmark genomic scanning (RLGS) with methylation sensitive enzymes (Hayashizaki et al. 1994), the search for imprinted genes continues. The advantage of the mRNA phenotyping approach for the detection of allele-specific expression is that it is efficient enough to be used as a simple screening assay for candidate imprinted genes. In our search for new imprinted genes we identified the parental imprinting of the *Mas* protooncogene in the mouse (Villar and Pedersen 1994a) and analyzed its allele-specific expression based on its proximity to the imprinted gene *Igf-2r* on the proximal portion of mouse chromosome 17. The *Mas* oncogene, encoding a mitogenic peptide receptor, was identified by Young et al. (1986) because of its tumorigenic potential in transfected NIH/3T3 cells. Although *Mas* was initially believed to function as a transducer of certain angiotensin effects, recent evidence suggests that *Mas* is not a classical angiotensin receptor but rather a receptor for an unidentified ligand. Using mRNA phenotyping we demonstrated that *Mas* is parentally imprinted, the paternally inherited allele being transcriptionally repressed in a developmental and tissue-specific manner (Villar and Pedersen 1994a). The relationship between genomic imprinting and tumorogenesis has been shown to involve the loss of tumor suppressor gene regulation (Hochberg et al. 1994; Rainier et al. 1993). The identification of *Mas* as an imprinted gene provides the first evidence that loss of imprinting may also play a role in the activation of a protooncogene.

In light of our current understanding of the underlying mechanisms involved in the differential expression of the parental genomes, we demonstrated spatially restricted imprinting of mouse chromosome 7. Using mRNA phenotyping we examined the tissue-specific expression of *Igf-1r*, *Hras-1*, *Gabbr3*, and *larm*, which map between *Sirrn* and the *Igf2/H19* domain, and *Mycd-1*, which maps proximal to *Sirrn*, and found that all of these genes were expressed by both parental alleles (Villar and Pedersen 1994b). These data suggest that, unlike X chromosome inactivation, autosomal imprinting does not affect large blocks of genes, but rather is restricted to either specific genes or "imprint domains" as proposed by Bartolomei et al. (1993). Interestingly, the close proximity of oppositely imprinted genes *Igf-2r* and *Mas* on chromosome 17 supports the proposed imprint domain model. Recently a functional relationship between *Igf-2* and *H19* has been demonstrated based on the disruption of the allele-specific expression of *Igf-2* in mice inheriting a targeted deletion of maternal *H19* or its paternal enhancers (Leighton 1995a,b). However, evidence that the mouse *Mash2*, *Ins-2*, and *p57* genes are also imprinted suggests an expanded region of imprinting on the distal region of chromosome 7. Whether the imprinting of these genes is mediated by local imprinting signals or by a regional-acting "imprinting center" is not clear.

Finally, several studies have demonstrated tissue-specific and developmental regulation of imprinted expression (Deltour et al. 1995; Latham et al. 1995; Szabo and Mann 1995a) with implications for possible mechanisms in the establishment and maintenance of genomic imprinting (Szabo and Mann 1995a; Villar et al. 1995). Evidence based on nuclear transplantation studies and transgene methylation patterns suggest that the specialization of the parental genomes is established during gametogenesis when the parent-of-origin imprints inherited from the previous generation are switched. Assuming that changes in imprinted expression reflect the alterations in the physical imprint, we analyzed the allele-specific expression of *Igf-2*, *Igf-2r*, and *H19* in the developing testis, the neonate, and the adult ovary, as well as the germinal vesicle and ovulated oocyte of F₁ females derived from an interspecies cross between *M. musculus* females and *M. spreitus* males. Biallelic expression of *Igf-2*, *Igf-2r*, and *H19* was detected during tests development, suggesting erasure of the parental imprint is a function of the germ line. Similarly, relaxation of the *Igf-2* and *Igf-2r* parental imprints was observed in the germinal vesicle oocyte and adult ovary. In fact, biallelic expression of imprinted genes has been demonstrated as early as 11.5 days postcoitus in mitotic postmitragory primordial germ cells. Whether imprint switching is initiated during migration to the genital ridge or upon colonization (Szabo and Mann 1995a), however, has yet to be determined.

In summary, the use of interspecies hybrids in the analysis of species-specific mRNA phenotypes for the detection of allele-specific expression is a sensitive and efficient approach applicable to (1) developmental and tissue-specific regulation and (2) the screening of candidate imprinted genes. Just as interspecies hybrids have proven to be valuable in gene mapping and the analysis of X-linked gene expression, their systematic use in the study of parental-specific expression and DNA methylation offers the potential for ex-
tended analysis in the mouse and other vertebrate systems to address the biological and evolutionary role of genomic imprinting.

References


