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Molecular Analysis of Genomic Imprinting during Mouse Development

by

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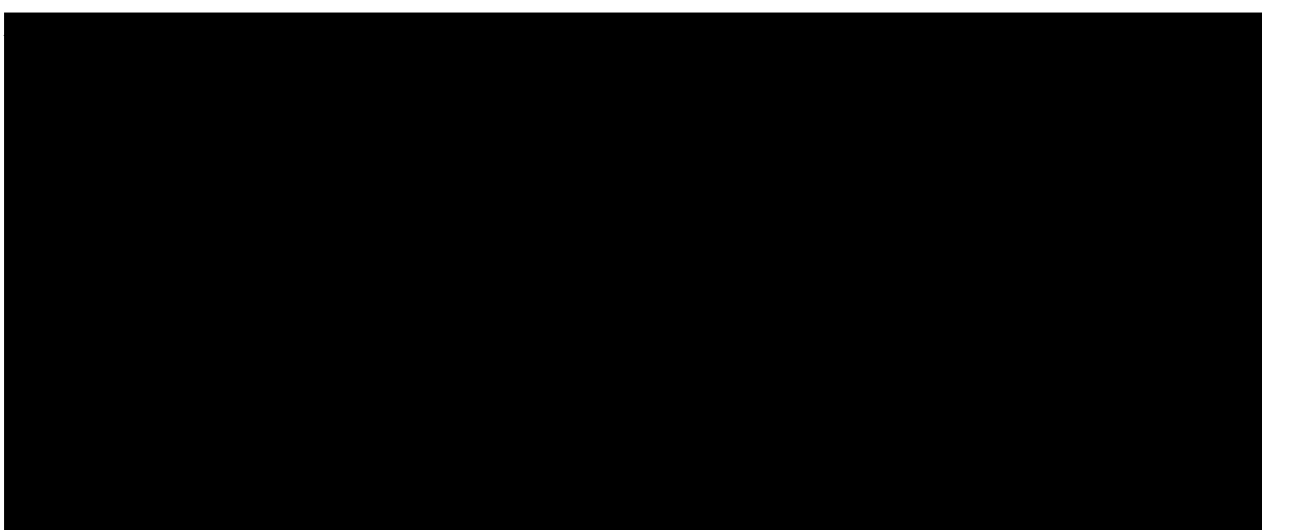

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Molecular Analysis of Genomic Imprinting during Mouse Development

by

Angela Jane Porter Villar

Mammalian development is unique in its requirement for fertilization, owing to genomic imprinting, which results in the functional specialization of the maternal and paternal genomes. Because the study of genomic imprinting requires the ability to distinguish between mRNA transcripts derived from the maternal and paternal alleles, I developed a mRNA phenotyping approach using an interspecies cross between *M. musculus* and *M. spretus* for the detection of allele-specific expression applicable to the developmental and tissue-specific analysis of imprinting.

I analysed a number of candidate imprinted genes mapping to imprinted chromosomal regions in the mouse genome. Using mRNA phenotyping I analysed the allele-specific expression, *Igf-1r*, *Furin*, *H-ras-1*, and *Gabrb3*, mapping to chromosome 7 between known imprinted genes, *Snrpn* and the *Igf-2/H19* domain, and *Myod-1*, mapping proximal to *Snrpn*. Despite their proximity to known imprinted genes, these genes exhibited biallelic expression suggesting that imprinting is not manifested over large chromosome domains but rather is restricted to a small number of discrete genes.

Identifying imprinted genes is important because of the correlation between uniparental chromosomal disomies and/or deficiencies and a number of human diseases, including cancer. I investigated the allele-specific expression pattern of the *Mas* protooncogene based on its proximity to the imprinted gene *Igf-2r* on mouse chromosome 17. mRNA phenotyping analysis demonstrated that *Mas* is parentally imprinted, the maternally inherited allele being transcriptionally repressed in a developmental and tissue-specific manner.

Although the significance of an imprinted protooncogene is not clear, it is intriguing to speculate that allele-specific expression may play a role in controlling oncogenic activity.

With the identification of imprinted genes, I applied mRNA phenotyping to the study of the developmental regulation of genomic imprinting during gametogenesis. Although the mechanism of imprinting is unknown, evidence indicates that imprint switching involves an event that permits expression from both parental alleles presumably as a consequence of imprint erasure. Further analysis of the developmental stage(s) involved in the "erasure" and establishment of the parental imprints may lead to diagnostic or therapeutic interventions for human diseases associated germ cell tumors.

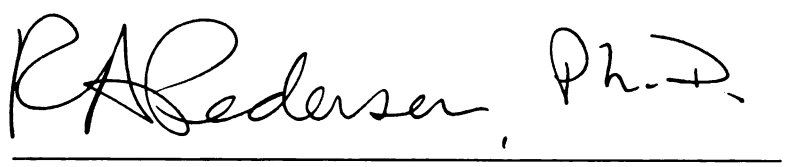

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Table of Contents

List of Tables

Table

List of Figures

Figures

Chapter One

Molecular Analysis of Genomic Imprinting during Mouse Development

By

Angela J. Villar

KEYWORDS: parental imprinting, development, mRNA phenotyping.

The text of this chapter is a reprint of the material as it appears in *Genetics of Gametes and Embryos.* eds. J. Cohen, J. Grifo and A. Handyside, Proceedings of Serono Symposium on *Genetics of Gametes and Embryos,* June 2-5, 1994. The co-author listed in this publication, Dr. Roger A. Pedersen, directed and supervised the research which forms the basis for the dissertation/thesis.

Introduction

Genomic imprinting, defined as the differential expression of alleles inherited from the maternal and paternal genomes (1), adds another dimension to the already complex issue of transcriptional regulation. Unlike most genes which are transcribed by both parental alleles regardless of their dominant or recessive nature, an undefined number of genes are epigenetically modified such that only one copy is actively transcribed. It is unclear, however, whether the imprint that causes differential expression of the parental alleles acts to permit or to suppress gene expression. 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 (4,5); and the non-Mendelian inheritance of genetic traits as a result of the differential epigenetic modification (physical imprinting) of the maternal and paternal autosomes (6,7). Although evidence suggests that the genomic imprint is established in the germline (8,9) 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 (10,11).

The phenomenon of genomic imprinting has been observed in many different phyla from plants to insects (12). However, at least one species of every vertebrate class can reproduce parthenogenetically, with the exception of mammals. The basis for the developmental failure of parthenogenetic embryos has been attributed to the epigenetic modification or imprinting of specific genes

(13-16). As a result offspring only inherit one functional copy of each imprinted gene, i.e., either their maternal or paternal allele is repressed. Despite their absence in nature, however, mouse embryos lacking a male genome or female genome can be produced experimentally. Analysis of these isoparental embryos has provided insight into the biological consequences of genomic imprinting for mammalian development, however, their widespread use in identifying and analyzing the expression of imprinted genes is not feasible because of the limited life span of these embryos.

In this chapter, I will provide a brief overview regarding the phenomenon of genomic imprinting and present an interspecific mouse hybrid approach for the detection of allele-specific expression applicable to 1) the identification of candidate imprinted genes and 2) the developmental analysis of imprinted gene expression. Using this approach I describe several experiments demonstrating the developmental and tissue-specific regulation of parental imprinting.

The Biology of Genomic Imprinting

Embryos lacking a male genome (parthenogenones) may be produced experimentally by activation of the egg by one of a wide variety of stimuli which initiate the program of development *in vivo* and *in vitro* (17,18). At the time of parthenogenetic activation, the mammalian egg is usually arrested in the prophase of the second meiotic division and extrusion of the second polar body leaves the egg haploid. Either suppression of the second polar body extrusion or endoreduplication of the remaining haploid nucleus without cell division will produce a diploid activated egg. Parthenogenetic eggs develop poorly and have abnormal extraembryonic tissues; the yolk sac is small with particularly meagre

vasculature and the ectoplacental cone and trophoblast are sparse (19-21). 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 eleven (approximately 25 somite stage) (22).

Early studies speculated that the possible causes of death of parthenogenones were due to either cytoplasmic or nuclear deficiencies. Nuclear deficiencies would include nonequivalent parental contributions to the zygote nucleus, and/or homozygosity for lethal alleles. Cytoplasmic deficiencies, such as the lack of an extragenetic contribution by the fertilizing sperm or the inability of the parthenogenetic stimulus to mimic appropriately the stimulus provided by the sperm could also result in the death of mammalian parthenogenotes.

Lethal homozygosity does not appear to be responsible for parthenogenetic nonviability. Experiments by Surani et al., (1984) in which parthenogenetic haploid eggs receiving a second pronucleus (either male or female from a fertilized egg of a different strain of mice), indicate that eggs receiving donor male pronuclei develop normally but eggs receiving a female pronucleus only develop to the stage observed in the case of parthenogenotes. Similar studies where the male or female pronucleus was removed from fertilized eggs and replaced with a male or female pronucleus from a second egg indicate that when the added nucleus is of the same parental gender origin as that remaining in the recipient egg, development fails to proceed to term (10). Diploid gynogenetic eggs prepared after fertilization by removing the male pronucleus from digynic triploids develop poorly, but pronuclei from fertilized eggs transferred to enucleated parthenogenetically activated eggs develop normally. This indicates that cytoplasm of activated and fertilized eggs is similar (11). Therefore, the

possibility that an extragenetic physical or physiological sperm contribution is the cause of parthenogenetic lethality can be eliminated. Although Illmensee and Hoppe (1977) reported full-term development of diploid uniparental mouse embryos and of nuclear transplantation embryos that receive a nucleus from an inner mass cell of a parthenogenetic embryo (23,24), similar attempts by other investigators to produce uniparental mice have not resulted in the birth of live progeny (22,25).

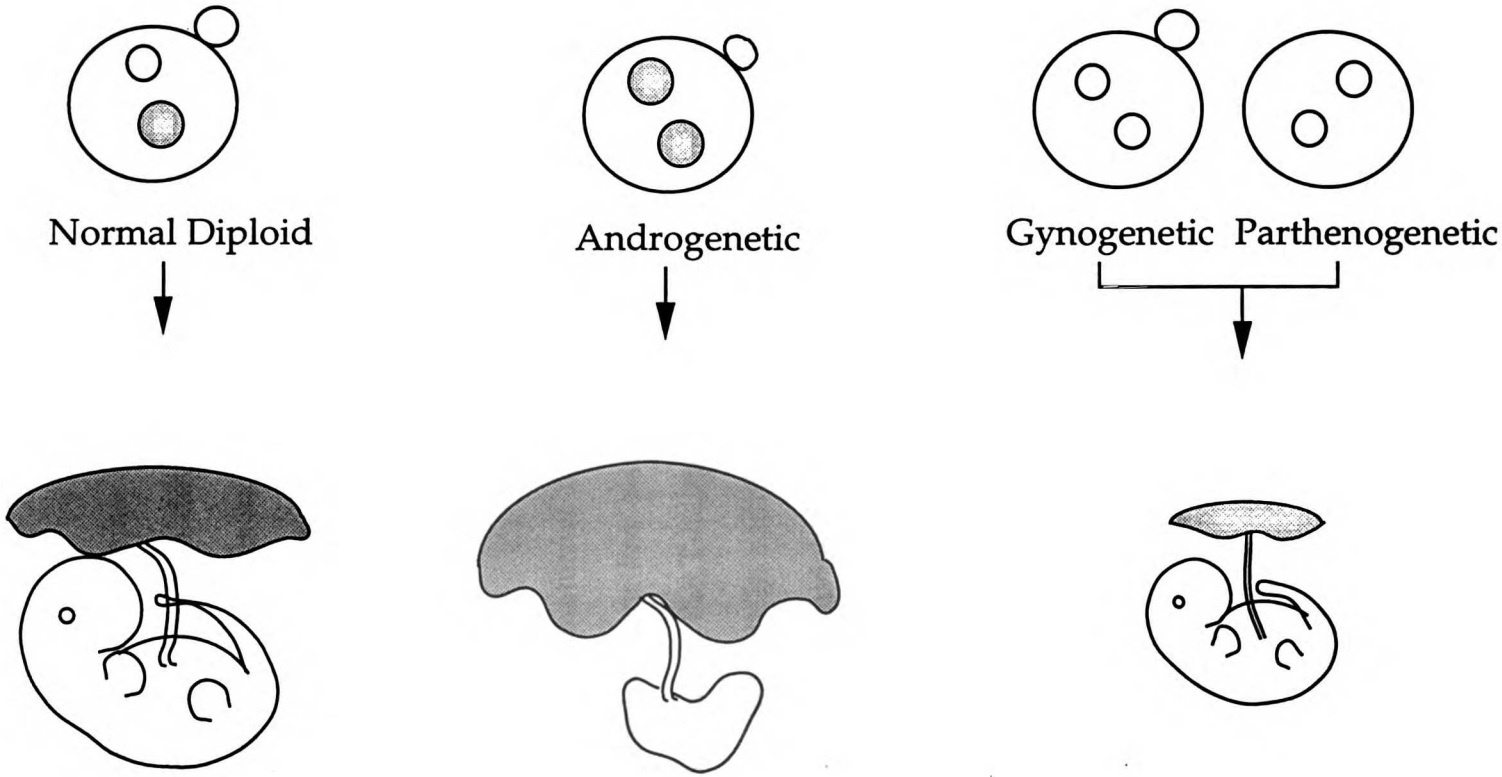
The possibility that the paternal genome would be sufficient for normal development has been addressed through nuclear transfer experiments by constructing zygotes from which the maternal pronucleus was removed and replaced by the paternal pronucleus from another zygote to form androgenetic embryos. Embryos with two male pronuclei have substantial trophoblast but retarded development of the embryo proper (10,13). Approximately 25% of these embryos fail to reach the blastocyst stage because they are YY embryos, and the remaining, XX or XY androgenones rarely reach the 11 somite stage (10,13). Although the contribution of the paternal genome is required for full term development its presence is not a prerequisite until at least the 8-cell stage (26).

In summary, embryos with two female genetic complements are capable of good fetal development but poor development of extraembryonic membranes and placenta. In embryos with two male genetic complements the reverse is true (figure 1). The fact that both parental genomes must be present for normal development suggests that they are functionally different and play complementary roles during embryogenesis (10,20). Despite the fact that parthenogenetic and androgenetic embryos are nonviable and die before or shortly after implantation, embryonic stem cells derived from these embryos are

Figure 1. Experimental evidence for genomic imprinting has been demonstrated by nuclear transplantation experiments. Embryos with two male pronuclei (androgenetic) have substantial trophoblast but retarded development of the embryo proper. Embryos lacking a male genome may be produced experimentally by activation of the egg to initiate the program of development (parthenogenetic) or prepared after fertilization by removing the male pronucleus and replacing it with another female pronucleus (gynogenetic). Embryos with two female genetic complements develop poorly and have abnormal extraembryonic tissues; the yolk sac is small with particularly meagre vasculature and the ectoplacental cone and trophoblast are sparse. The fact that both parental genomes must be present for normal development suggests that they are functionally different and play complementary roles during embryogenesis.

Developmental Consequences of Genomic Imprinting

Maternal
Paternal



viable in culture (27). Although the significance of this is not understood, the results seem to indicate the nonviability of these embryos is not due to a cell autonomous lethality that affects all lineages.

It has been known for some time that chimeras between normal and parthenogenetic embryos can develop to term and that parthenogenetic cells contribute to almost all tissues including germ cells (28-31). Similar results have recently been reported for chimeras between normal and androgenetic embryos, however, there is a high incidence of lethality and the chimeras develop characteristic skeletal abnormalities (32).

Evidence suggesting maternal and paternal genomes play unique roles in mammalian development has also accumulated from several other areas of research. In particular, the T-hairpin tail mutation in mice is lethal only when maternally inherited (33). In addition, DDK females mated with males of another species of mouse are nonviable, however, offspring of the reciprocal cross are normal (34). Recent evidence indicates that the maternal factor responsible for the defective interaction between the DDK cytoplasm and the paternal genome is an RNA stored during oogenesis and active during early cleavage of the embryo (35).

The Mechanism of Genomic Imprinting

Changes in gene activity during development are generally referred to as epigenetic (36). 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 (37). Eukaryotic DNA contains 5-methylcytosine (5MeC) as the sole modified base, which appears exclusively at CpG dinucleotides (38). The transfer of methyl groups from S-adenosyl-L-methionine to certain cytosines in vertebrate DNA is catalysed by a methyltransferase associated with the cell nucleus. The dinucleotide CpG has several interesting features in the DNA of vertebrates. It occurs at about 1/5 of the frequency expected from a random distribution of dinucleotides and between 70-90% of all genomic CpGs in vertebrates are methylated at the 5-position of the cytosine ring (39). There are an estimated 30,000 nonmethylated CpG islands (Hpa II tiny fragments; HTF) per haploid mouse genome. These do not constitute a family of related sequences but represent a distinctive kind of sequence organization. Several studies have suggested a relationship between HTF islands and distinct genes (40-43), although not all genes have HTF islands in their vicinity (e.g., globin genes). 5MeC residues in DNA can be lost by spontaneous deamination to thymidine residues or by programmed conversion of 5MeC to cytosine residues during development (44). Therefore, the maintenance of approximately 0.7-1.0 x 10 5MeC residues per mammalian cell argues for the important function of methylated cytosines in both repeated and nonrepeated DNA sequences (45).

Several lines of evidence have strongly implicated 5MeC in regulating expression of specific genes (46) of chromosome domains (47), and of whole chromosomes (48). Generally, actively transcribed genes can be distinguished from inactive genes by differential susceptibility to DNase I digestion (49). A correlation between DNase I hypersensitivity and DNA hypomethylation would suggest a role of methylation in regulating gene expression. This correlation has been

demonstrated for a number of developmentally regulated genes, such as the globin genes (50), the chicken ovalbumin (51) and the immunoglobulin genes (52), in which gene transcription also coincides with demethylation of some CpG dinucleotides. Therefore, methylatable CpG clusters appear to be capable of altering interactions between the DNA and the chromosomal factors involved in the generation of the DNase I sensitive or insensitive configuration (53). Furthermore, the biological activity of the retroviral genome in transfection assays suggests that in these systems methylation is causally involved in gene inactivation (54,55).

For a number of other cellular genes no clear correlation has been observed between the pattern of methylation and gene expression *in vivo*. For example, during development of chicken or *Xenopus*, the hormone inducible vitellogenin genes (53,56,57) or the α-crystalline gene (58) become expressed despite being highly methylated. Other examples include the α 2(I) collagen gene (41) or X chromosome linked genes in extraembryonic tissues (59). These observations suggest that expression of certain genes may be insensitive to inhibition by methylation. However, it should be kept in mind that all experiments described above used restriction enzymes to determine the *in vivo* methylation status of the respective genes. While the available enzymes recognize 10-15% of the total CpGs present (60), only a small subset of CpGs may be relevant for gene expression. Therefore, it is possible that experiments where no correlation between expression and methylation was found have failed to detect the biologically relevant sites. Ideally, assays should be employed that are independent of sequence-specific restriction enzymes for unequivocally demonstrating that methylation of a given gene is irrelevant for its expression.

DNA sequencing methods which detect methylated bases have been employed for this purpose (61,62).

Although several lines of evidence have strongly implicated 5MeC in the control of gene expression in higher organisms, in most of these studies it is not clear whether methylation is the cause or effect of gene inactivation. Evidence suggests 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 (48,63). Analysis of X chromosome inactivation in marsupials supports the idea that the initiation of gene inactivation can be disassociated from methylation (64). 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 (40,48,49), a prerequisite for the mechanism of imprinting. Evidence that the distribution of 5MeC in DNA is heritable comes from direct and indirect experiments. First, in transfection experiments with globin genes, the DNA remains in either a methylated or an unmethylated state during the growth of individual clones (49). Second, when retrovirus DNA is injected into preimplantation mouse embryos, it can be integrated into the chromosome and is then *de novo* methylated and inactivated (65). This inactivation persists through development into the adult and it can be shown that the methylation of this DNA has been faithfully maintained. Third, the study of X-linked genes in females, such as hypoxanthine-guanine phosphoribosyl transferase (HPRT), phosphoglycerate kinase, and glucose-6-phosphate dehydrogenase has shown that the inactive state of the X chromosome is correlated with methylation in HTF islands that are associated with these genes, whereas these islands are not methylated on the active X chromosome (48). Since

the active and inactive states are stably inherited, the pattern of DNA methylation in these regions of DNA must also be very stably maintained. Finally, it has been shown that in transfection experiments the wild type HPRT+ gene on the inactive X chromosome will not produce HPRT+ colonies in an HPRT- recipient, whereas the same DNA from a cell with HPRT+ on the active X chromosome will produce such colonies (66).

It is generally presumed that the mechanism of imprinting is established sometime during gametogenesis when the male and female genomes are separate and distinct. The pattern of methylation of four transgenes has been investigated in detail (8,67-69). As these transgenes pass from one generation to another their methylation pattern is reversed during successive generations depending on the parental origin. In general, the transgene inherited from the father is less methylated than if it were inherited from the mother. This would seem to contradict the observation that in unique and some repetitive sequences, sperm DNA is more methylated than oocyte DNA (70). However, 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. The differential methylation patterns of known endogenous imprinted genes are discussed in the following section.

Recently, it has been suggested that replication timing may play a role in the activity of genes that display allele-specific expression (71,72). While there is evidence of an association between asynchronous replication and imprinted chromosome regions, it is not clear whether there is a relationship between DNA replication timing and gene activity. Until the mechanisms of imprinting are fully understood, however, it is possible that such effects of asynchronous replication

could constitute a form of imprinting and, in fact, could be associated with the mechanism by which certain genes are differentially expressed. Of interest will be to determine whether differential methylation patterns play a role in the asynchronous replication of imprinted regions.

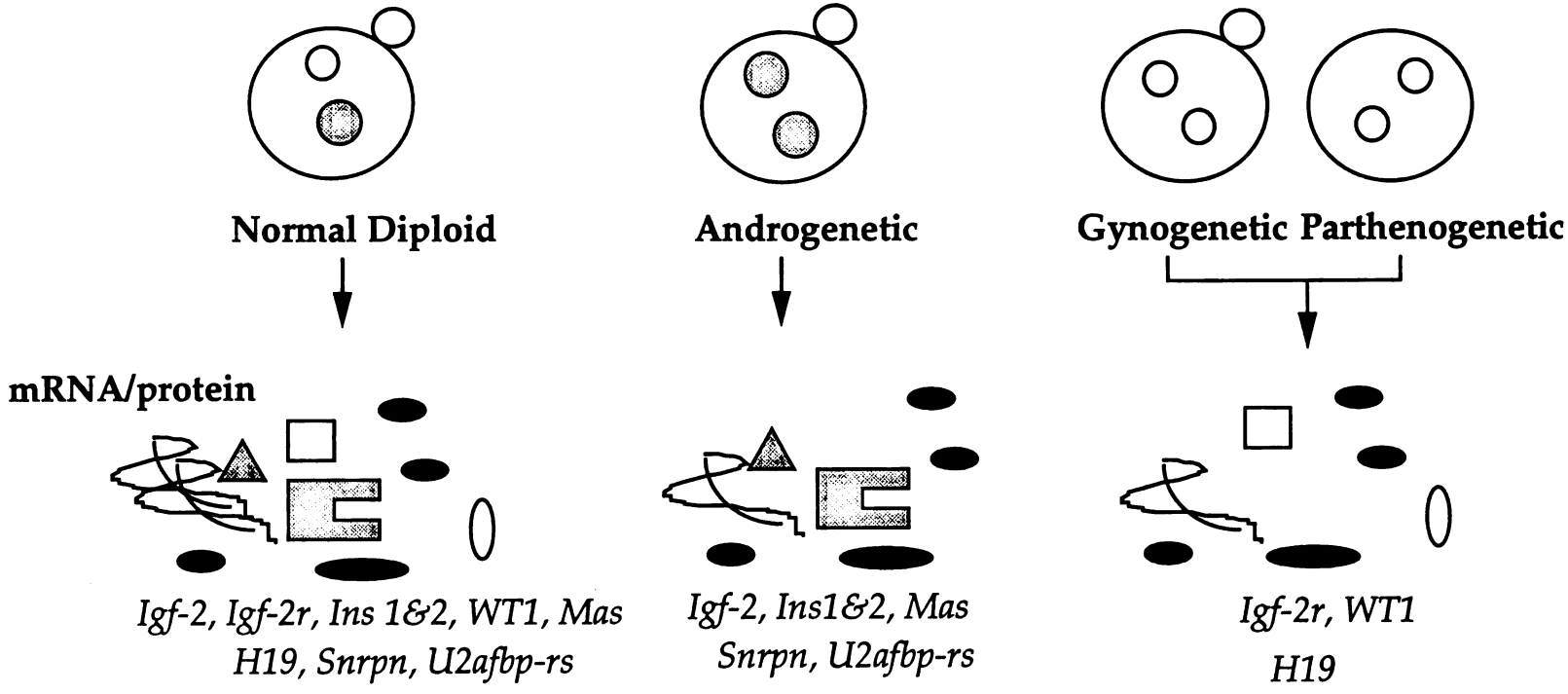
Although the mechanism of imprinting remains unknown, the effects of the differential modification of the parental genomes has been observed for several genes whose expression is dependent on whether they are transmitted through the male or female germ line.

Identities and Expression Patterns of Known Imprinted Genes

Evidence that the maternal and paternal genomes are not functionally equivalent arose from the study of chromosomally balanced mice that inherited maternal duplication/paternal deficiency or paternal duplication/maternal deficiency for certain chromosomal regions (73,74). Despite the fact that these mice inherited a complete set of genetic blueprints, the uniparental inheritance of whole or portions of particular autosomes caused them to develop abnormally. 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 (3) (figure 2). An imprint map has been developed that defines the chromosomal regions shown to have defective complementation depending on the parent of origin. 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 (73-76). Embryos with maternal or paternal duplications and the respective paternal

Figure 2. Although the male and female gamete possess a complete set of genetic blueprints, their developmental potentials are limited due to epigenetic modifications acquired during transmission through the germline. As a result expression of an undefined number of genes is hemizygous and dependent on the parent of origin. Those genes expressed exclusively by the paternal allele include, *Snrpn, U2afbp-rs, Igf-2, Ins1 & 2* and *Mas*. Maternal allele-specific expression has been observed for *H19, Igf-2r* and *WT1*. The role these genes play in the reciprocal phenotype of the developing androgenetic and parthenogenetic/gynogenetic embryo remains a basic question in developmental biology.

Genetic Consequences of Genomic Imprinting



and maternal deficiencies generally display a variety of abnormal phenotypes. The chromosomes affected by paternal deficiency (i.e., in parthenogenones) are 2, 6, 7, and 11. Those affected by maternal deficiency (i.e., in androgenones) are chromosomes 2, 7, 11, and 17. Duplications of chromosomes 1, 5, 9, 14, and 17 are not lethal but cause differential recovery of offspring. The remaining autosomes of the mouse genome either have not been tested (chromosomes 8, 10, 12, and 18) or show completely normal complementation when inherited as duplications or deficiencies. Thus, the correlation between parental chromosome duplication/deficiency and anomolous 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. For example, paternal disomy of distal 7 is associated with early prenatal death, whereas maternal disomy of distal 7 is associated with late prenatal death (77).

Igf-2 was the first gene to be associated with an imprinted phenotype. *Igf-2* is a single copy gene mapping to the distal end of mouse chromosome 7, encoding a 67 amino acid polypeptide with a mitogenic function mediated through the *Igf-1r* gene (78). It is highly expressed in both embryonic and extraembryonic during development, however, postnatally the expression is suppressed in most tissues with the exception of the choroid plexus and the leptomeninges. DeChiara et al., (1990) demonstrated that germline transmission of the inactivated *Igf-2* (Insulin-like growth factor 2) gene from male chimeras yielded heterozygous progeny 40% smaller than their littermates. Progeny homozygous for the inactivated *Igf-2* gene also displayed this phenotype (79) suggesting that the maternally inherited *Igf-2* gene was imprinted in such a way as to resemble the inactive gene. Furthermore, reciprocal crosses in which germline transmission of the

inactivated gene was inherited from female chimeras yielded heterozygous progeny that were of normal size. Further analyses by nuclease protection and *in situ* hybridization revealed that only the paternal allele was actively expressed (80). The monoallelic expression of the *Igf-2* gene in fetal human liver and kidney in addition to the expression of *Igf-2* from the paternally derived allele in complete hydatidiform moles suggests that the human *Igf-2* gene expression is also dependent on the parent-of-origin. Recently, the parental origin of the expressed allele in human placenta and fetal kidney has been assigned to the allele derived from the father (81,82). An upstream site within the promoter region of *Igf-2* is differentially methylated (83,84) in sperm and maintained throughout embryogenesis, a prerequisite for a role in the mechanism of imprinting. However, the regulation of *Igf-2* imprinting is promoter-specific, the recruitment of P1 responsible for the absence of imprinting in specific tissues (85). If this site serves as the imprint, the methylated site on the paternal allele appears to play a role in sustaining, rather than repressing transcription. In addition *Igf-2* expression is barely detectable in embryos deficient in DNA methylase suggesting that normal levels of methylation are required for maintaining expression of the normally active paternal allele (86).

Human *H19* is tightly linked to *Igf-2* (90 kb apart) on 11.p15.5, a region syntenic with the distal portion of mouse chromosome 7 (87). Despite their proximity, however, *H19* is oppositely imprinted with the paternally inherited allele being transcriptionally silent. In mice *H19* is expressed as early as 4.5 d.g. in extraembryonic tissue and in the embryo proper by 8.5 d.g. with repression in cells of neuroectodermal origin and blood cells (88). After birth the gene is repressed in all tissues except in skeletal muscle, where only the maternal allele is transcribed (89). *H19* encodes one of the most abundant RNAs in the developing

embryo with high levels in endodermal and mesodermal tissues (90,91). Detection of *H19* transcripts in cytoplasmic 28S ribonuceoprotein particles has prompted speculation that it may function as a RNA molecule (92,93). *H19* was the first gene to demonstrate monoallelic expression in humans as determined using restriction site polymorphism in fetal tissues (94). The promoter region of the repressed paternal allele of *H19* is both hypermethylated and heterochromatic, thus despite the opposite imprinted expression of *Igf-2* and *H19*, the methylation sites within the *Igf-2/H19* imprint domain are both derived from the paternal genome (84). The methylation pattern observed in the neonate and adult are also observed in the sperm, thus meeting the requirements of an imprinting mechanism. However, it remains to be determined which sites maintain the differential imprint during the genome-wide demethylation that occurs during preimplantion development. In ES cells and embryos with homozygous deletion of the DNA methylase gene a decrease in overall methylation correlates with activation of the normally silent paternal *H19* allele, suggesting that methylation is required for maintaining the transcriptionally inactive state of the paternal allele (86). Therefore, in the case of *H19*, if methylation serves as the imprint, it appears to repress expression of the paternal allele

Deletion of the T-associated maternal effect *(Tme)* locus is a naturally occurring mutation in the mouse spanning 800-1100 kb on chromosome 17 (33,95). Embryos inheriting the deletion from their mother die at 15 d.g., but survive if the deletion is paternally inherited. The fact that the phenotype was dependent on the parent of origin sugggested the the region contained an imprinted gene(s). Of the four contiguous genes that were analysed, *Igf-2r*, which is identical to the cation independent mannose-6-phosphate receptor, was determined to be

expressed exclusively by the maternal allele; therefore when the *Tme* locus deletion is inherited from the female *Igf-2r* protein is absent (96). One of the functions of *Igf-2r* is to bind *Igf-2* thereby resulting in its degradation in lysosomes, suggesting that *Igf-2r* is involved in the regulation of *Igf-2* levels. Unlike the mouse, the *IGF-2R* gene is generally expressed from both parental alleles in human (97). However, in a minority of individuals the human *IGF-2R* gene is monoallelically expressed demonstrating that, like certain transgenes, the imprinting of *IGF-2R* may be a polymorphic trait dependent on genetic background and imprint modifier genes (98). In the case of *Igf-2r*, there is a methylation site in the body of the gene inherited from the female gamete, whereas the promoter region of the inactive paternal allele is methylated (99). Similar to *Igf-2*, the expression of *Igf-2r* in DNA MTase mutant embryos is essentially undetectable compared to the normal and heterozygous embryos suggesting that methylation is required for maintaining the expression of *Igf-2r*, albeit from the maternal allele (86). Interestingly, a change in *Igf-2r* expression is only observed in embryos with the more severe mutation of the MTase allele suggesting that *Igf-2r* gene expression is quantitatively less sensitive to DNA demethylation.

Using a strategy similar to that used to identify *H19* as an imprinted gene, the mouse *Snrpn* gene was shown to be expressed exclusively by the paternal allele, primarily in the brain and heart (100). The *Snrpn* gene encodes a nuclear protein associated with the small nuclear ribonucleoprotein particles (snRNPs) and maps to the central portion of mouse chromosome 7, a region syntenic to human chromosome 15q11-13 (101). It is presumed that the human *SNRPN* gene, which is also imprinted (102), is responsible for the Prader-Willi syndrome (PWS) associated with a maternal deletion or paternal duplication of 15q12 region.

Although more than one gene may be involved in the etiology of this disease, the potential function of *Snrpn* in the post-transcriptional modifications of various genes may be responsible for the pleiotropic effects exhibited by patients with PWS. The gene responsible for the oppositely imprinted disease Angleman syndrome (AS), is also located in the chromosome 15q12 region, indicating that another imprinted gene near *Snrpn* is expressed only by the maternal allele (103-105). Parent-specific methylation patterns observed within intron 5 of the human *SNRPN* gene indicate that, like *Igf-2r*, the expressed gene is methylated, however, the developmental timing of this imprinting event has yet to be detemined (102).

A unique approach in the identification of imprinted genes takes advantage of the possible correlation between allele-specific expression and the differential methylation of key sites within these genes. Restriction landmark genomic scanning (RLGS) uses methylation sensitive restriction enzymes to identify landmark cleavage sites unique to either the maternal or paternal genomes (106). Using this approach, eight out of 3,500 strain-specific loci in the RLGS profile were determined to be differentially methylated, one of which mapped to an imprinted region of mouse chromosome 11. Sequence analysis revealed that this loci showed significant homology to the human *U2af* gene and was subsequently named U2afbp-related sequence *(U2afbp-rs)* (107). Expression of *U2afbp-rs* is allele-specific and correlates with the demethylated paternal allele, suggesting that the methylation of this loci acts to repress transcription from the maternal allele. The *U2afbp-rs* gene encodes a splicing factor with a function similar to that of *Snrpn*, raising the possibility that the imprinting mechanism may have been conserved during the evolution of splicing factors with common ancestry (106). The fact that the *U2afbp-rs* gene is intronless suggests that this gene became

integrated into the genome as a retroviral copy of a similarly imprinted proto gene.

The human insulin gene and its mouse homologue, *Ins2*, are adjacent to the imprint domain of *Igf-2* and *H19* on syntenic regions 11p15 and distal chromosome 7, respectively (108). In addition to the *Ins2* gene, both rats and mice possess an unlinked functional retroposon, *Ins1*, mapping to mouse chromosome 6. While *Ins1* and *Ins2* are expressed from both parental alleles in embryonic and neonatal pancreas, both genes are parentally imprinted in yolk sac with expression restricted to the paternal alleles (109). It is not clear, however, whether the allele-specific expression of these genes is due to a common imprint signal within their DNA sequences or a consequence of the chromsomal domain surrounding these loci. It will be interesting to determine whether the allele-specific expression observed in the mouse is also characteristic of the human *INS* gene, because the increased risk for insulin dependent diabetes correlates with the preferential inheritance of the paternally derived insulin gene (110).

The Wilms' tumor suppressor *(WT1)* gene in humans is implicated in the etiology of a number of diseases (111-113). Although the *WT1* gene exhibits biallelic expression in the human fetal kidney, maternal allele-specific expression is observed in brain and pre-term placenta. Interestingly, like *IGF-2R*, imprinting appears to be polymorphic within the human population because biallelic expression of *WT1* is also observed in other placentae (114). Comparative analysis between imprinted and non-imprinted tissues may prove to be a useful model for determining the DNA sequences recognized by the mechanism of imprinting and for the identification of imprint modifier genes.

The *Xist* locus is a candidate for the X inactivation center that ensures dosage compensation of X-linked gene products in XX females and XY males, with expression of Xist correlating with X chromosome inactivation (115-118). In mouse and human female embryos X inactivation is imprinted, with the paternal X chromosome preferentially inactivated in the extraembryonic tissues. Interestingly, paternal X chromosome inactivation in trophoblast tissues of female embryos is preceded by the exclusive expression of the paternal allele of *Xist* suggesting the *Xist* gene is imprinted (119). In the embryonic lineages during gastrulation, however, random X inactivation correlates with expression of the maternal and paternal alleles, indicating loss of the *Xist* parental imprint. A role for methylation in the control of Xist expression is supported by the developmental regulation of demethylation of *Xist* in the male germ line as well as the preemptive methylation of the maternal allele prior to the onset of differential *Xist* expression in differentiating ES cells (120). Further studies will be required to determine whether the mechanisms involved in X chromosome imprinting also plays a role in autosomal imprinting.

The parental imprinting of the *Mas* protooncogene in mouse is discussed in further detail below (157). The imprinting of *Mas* is not only interesting, because it is developmentally regulated and tissue-specific, it is significant in light of the growing relationship between genomic imprinting and cancer.

The consequences of genomic imprinting during gametogenesis and its wide ranging implications for mammalian embryogenesis and human disease emphasize the importance of identifying 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. If

imprinted genes are randomly distributed throughout the genome, their number may be small; however, if these genes are clustered, their number may be relatively large. Identification of these genes may also be complicated by developmental and tissue specificity. Table I summarizes the known imprinted genes in mouse and human. Candidate imprinted genes found not to be differentially expressed are listed in table II.

The Evolutionary Role of Genomic Imprinting

Several theories have been proposed to explain the possible origin and biological role of imprinting. It has been speculated that the evolutionary origin of imprinting is based on the conflicting reproductive strategies of male and female mammals by virtue of their disparate bioenergetic roles during development (121). This model assumes that monogamy is the exception and that multiple paternity within or between litters is common. Consequently, the females increase their fitness by maximizing the number of offspring. Conversely, the male, who plays no direct physiological role in development, increase their fitness through progeny that compete efficiently for maternal resources at the expense of the progeny of other males. The opposite imprinting of the paternally expressed mitogenic factor, *Igf-2* and the maternally expressed *Igf-2r*, that functions as a sink for *Igf-2*, may be a consequence of such a conflict between the sexes. The biological role of genomic imprinting may also have arisen from evolutionary pressures to maintain heterosis, thereby eliminating the possibility of parthenogenetic reproduction (12).

An alternative hypothesis suggests that the functional specialization of the parental genomes was required for the evolution of placentation. The

Table 1. Summary of Imprinted Genes in Mouse and Human

Gene	Expressed allele	Chromosome Mouse	Chromosome Human	Methylated allele	Ref.
Igf-2	paternal	7	11p15	paternal	80,82
Igf-2r	maternal	17	6q25-q27 *	paternal/maternal	96,98
H19	maternal	7	11p15	paternal	89,94,165
Snrpn	paternal	7	15q12	paternal	101,102,164
Ins 1, 2	paternal	6, 7	***		109
U2 afbp-rs	paternal	11	***	maternal	106
*WT1***	maternal	***	11p13		114
Xist	paternal	X	X	maternal	119,120
Mas	paternal	17	***		157

* polymorphic imprinting in human fetuses
** polymorphic imprinting in placentae and brain
*** imprint status not known

Table 2. Summary of Candidate Imprinted Genes Exhibiting Biallelic Expression

	Chromosome		
Gene	**Mouse**	**Human**	**Ref.**
Igf-1r	7	***	156,158,159
Myod-1	7	***	156
H-ras	7	***	156
Gabrb3	7	***	156
Fur	7	***	156
Ins-r	8	***	(unpublished observation)
c-abl	2	***	(unpublished observation)
BRC	***	22	160
Th	7	***	72
PRAD1	***	11	97
C2	***	19	97
WT1[a]	***	11p13	94,166
Pck-1	2	***	161
Gnas	2	***	161
Rb	14	***	(unpublished observation)
Tcp-1	17	***	96
Plg	17	***	96
Sod-2	17	***	96
Ins-1 and *2*[b]	7	***	72,109
IGF2R[c]	***	6q25-q27	97
Igf-2	7*	11p15**	80, 163
DM-kinase	7	19q13	162

[a] in tumors and fetal kidney; polymorphic imprinting in placetae and fetal brains.

[b] in embryonic pancreas.

[c] polymorphic imprinting in human fetuses

* in choroid plexis and leptomeninges

** in liver approximately 6 mons after birth

*** imprint status not known

relationship between invasive trophoblast and metastatic disease has prompted the alternative hypothesis that the evolutionary role of imprinting was to protect the mammalian female germline from ovarian tumors by inactivating growth promoting genes to reduce the risk of potentially lethal consequences of spontaneous egg activation (as occurs in LT/Sv mice and humans) (122). On the other hand, the behavior of ovarian germ cell tumors may simply be a reflection of genomic imprinting, as suggested by Porter and Gilks (1992), rather than a mechanism specifically evolved to protect the female mammal.

Recently, it has been suggested that imprinting may play a role in evolution by promoting speciation events (124). This model proposes that simultaneous switching of the imprint pattern at male and female target genes would result in the reproductive isolation of recipient offspring and precipitate the formation of a new species.

Presently all of these hypotheses are plausible, however, each of these theories yield predictions that are testable. For example, the identification of new imprinted genes will be necessary to determine the extent of genomic imprinting among mammals and other vertebrates (125). The existence of genes that are imprinted regardless of genetic background would suggest the evolutionary conservation of imprinted genes and provide evidence for the possible evolutionary origins and role of genomic imprinting.

Genomic Imprinting and Disease

The profound effects of genomic imprinting on the development of parthenogenetic and androgenetic embryos imply that imprinted genes play a

major role in the differentiation and proliferation of both embryonic and extraembryonic tissues. Although the reason for its evolutionary genesis remains speculative, the functionally hemizygous state of a select number of genes appears not only normal but is probably required for proper mammalian development as evidenced by human ovarian teratomas and gestational trophoblastic neoplasia (126,127). There are 18 mapped human disease loci and chromosome regions in which the manifestation or severity of pathological effects is thought to be the result of genomic imprinting (128-131). For instance, PWS involves maternal uniparental disomy of 15q11-12, for which the *Snrpn* gene is a candidate (132,133). In contrast, AS results from a maternally transmitted deletion of a specific region on 15q11-12 (133); however, the imprinted AS gene, has yet to be determined (103). There are also dominant mutations and recessive diseases in which their manifestation, onset or severity is affected by the parental origin, such as, Albright's hereditary osteodystrophy (135), chronic myeloid leukemia (136), insulin dependent diabetes (137), and hereditary paraganglioma (138).

The association between imprinting and cancer involving either loss of tumor suppressor gene regulation or the activation of growth promoting genes suggests a novel mechanism of tumorogensis (127,139,140). Evidence for a relationship between imprinting and growth has been observed in mice inheriting duplications of particular chromosome regions, specifically, proximal chromosome 11 and distal chromosome 7 (74). In addition, chimeras derived from androgenetic and normal embryos exhibit enhanced growth, whereas growth of their parthenogenetic counterparts is retarded (32,141,142). Therefore it is not surprising that there is strong correlation between imprinting and tumorigenesis.

Igf-2 and *H19* have been implicated in the development of specific growth disorders and tumor formation. Overexpression of *Igf-2*, due either to the loss of the imprint or to paternal duplication of the 11p15 chromosomal region, has been implicated in some patients with Beckwith-Wiedemann syndrome (BWS), which is characterized by multiorgan overgrowth and embryonal tumors (143,144). In addition, a correlation between Wilms' tumor and a bias in the loss of the maternal *H19* allele has prompted speculation that *H19* plays a role in tumor suppression (92,145). Although the *Mas* protooncogene is not associated with a known mouse mutation or human genetic disease, tumorigenicity assays imply that the oncogenic activity of *Mas* is a result of inappropriate expression (146-149). Thus, the loss of imprinting of an otherwise tightly controlled gene, such as *Mas* (157) may represent an a new category of heritable mutation. Finally, the consequences of functional hemizygousity may also be reflected in an increased sensitvity of imprinted genes to environmental agents that disrupt gene expression by damaging genes, altering gene dosage, or affecting gene transcription.

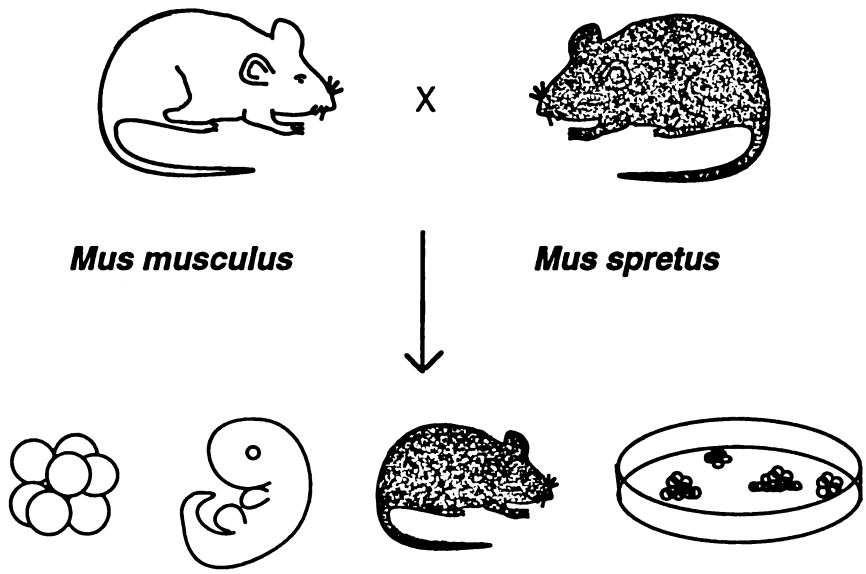
Analysis of Genomic Imprinting using Interspecific Hybrids

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 female (parthenogenone) or male (androgenone) genome would be by subtracted cDNA libraries or comparative two-dimensional gel analysis of proteins, of such uniparental embryos, the feasibility of these approaches is restricted by the limited life span of isoparental embryos, the paucity of material and the abnormal lineage representation, i.e., some tissues are abnormal or missing in parthenogenones and androgenones (10,150). Alternative approaches for the detection and analysis of imprinted genes have evolved from the analysis of isoparental <-> normal chimeras (141,151,152) or of mice inheriting whole or parts of chromosomes from only one parent (153). In addition, isoparental embryonic stem (ES) cell lines have been generated (32,152,154,155) to identify imprinted genes and to study the temporal establishment and maintenance of the imprint. 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, e.g., production of parthenogenetic and androgenetic embryos from which ES cells are derived, or simply impractical for the analysis of imprinting when genetic material is limiting, e.g., early embryonic development. For these reason we developed a powerful alternative 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 *Mus musculus* and *Mus spretus* (156) (figure 3).

Figure 3. 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 *Mus musculus* and *Mus spretus*.

Interspecific Mouse Hybrid Approach



Analyze Hybrids as:

Embryos

Fetuses

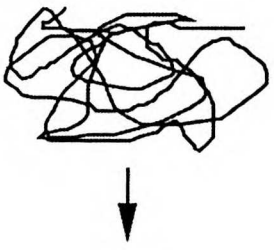
Newborns

Embryonic Stem Cells

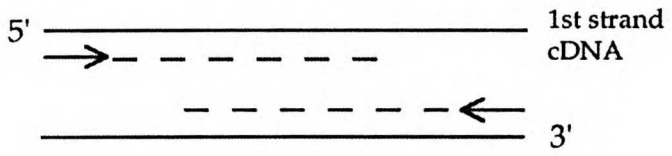
Figure 4. mRNA phenotyping analysis scheme. Total RNA is isolated from *M. musculus, M. spretus* and F1 hybrid progeny produced by crossing *M. musculus* and *M. spretus*. Each RNA preparation is reverse transcribed into cDNA and amplified by PCR with the use of primer pairs designed for specific detection of cDNA target sequences. The *M. musculus* and *M. spretus* PCR product are sequenced to identify base-pair differences that generate species-specific restriction enzyme sites. The PCR products generated from F1 hybrids are then digested with restriction enzymes specific to each species to determine their parental origin. Restriction fragments are resolved electrophoretically on polyacrylamide or agarose gels and visualized with ethidium bromide. The mRNA phenotype of the F1 hybrid is compared to the *M. musculus* and *M. spretus* parental mRNA phenotypes to determine whether expression is biallelic or allele-specific.

mRNA Phenotyping Analysis

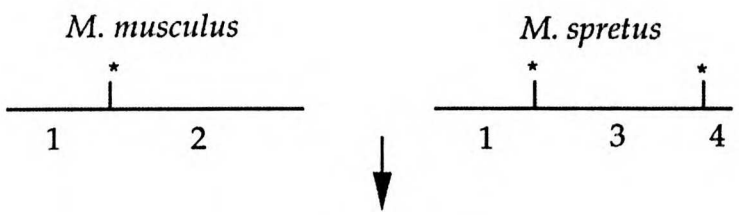
Total Cellular RNA Preparation



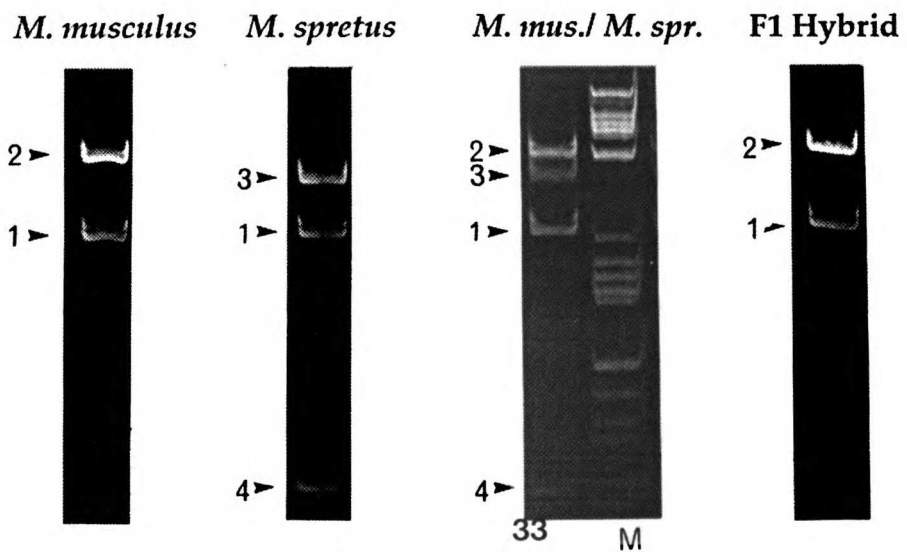
RT-PCR



Sequence PCR Product



RFLP Analysis



By exploiting the genetic diversity of these two divergent mouse species, I analysed the developmental and tissue-specific regulation of genomic imprinting using mRNA phenotyping to distinguish between maternal and paternal allele expression in hybrid progeny and ES cells (figure 4). To detect allele-specific expression I used reverse transcription-polymerase chain reaction (RT-PCR) to amplify the cDNA of interest and restriction fragment length polymorphisms (RFLPs), i.e., mRNA phenotype, 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. spretus* females failed to produce progeny, backcross matings between F1 females (derived from matings of *M. musculus* females and *M. spretus)* males and *M. musculus* males were performed to verify parental imprinting (figure 5). By demonstrating that the mRNA phenotype expressed by heterozygous F2 progeny is dependent on the parent of origin rather than species of origin precludes the possibility that the allele-specific expression is a result of a dominant species effect or selective PCR amplification. In addition, analysis of imprinting using this approach avoids potential experimental artifacts in gene regulation because both parental genomes are present and structural and functional gene relationships are maintained.

In light of our current understanding of the underlying mechanisms involved in the differential expression of the parental genomes, I demonstrate the spatially restricted imprinting of mouse chromosome 7 in chapter two (figure 6). Using mRNA phenotyping I examined the tissue specific expression of the *Igf-1r, H-ras-1, Gabrb3,* and *furin* which map between *Snrpn* and the *Igf-2/H19* domain, and *Myod-1,* which maps proximal to *Snrpn* and found that all of these genes were expressed by both parental alleles. These data suggest that unlike X chromosome

Figure 5. To verify parental imprinting, F1 hybrid females generated from a interspecies cross between a *M. musculus* female and a *M. spretus* male are backcrossed to a *M. musculus* male. Because of meiotic recombination, only 50% of F2 progeny will be heterozygous for the gene of interest, i.e., only individuals that inherit the *M. spretus* allele from the female will be informative (the other 50% inherit the *M. musculus* allele thereby precluding the possiblity of distinguishing between the parental alleles). Heterozygous individuals can be identified by RFLP analysis of the gene's DNA sequence or by mRNA phenotyping analysis of the gene of interest in non-imprinted tissues.

Backcross Mating Strategy to Verify Parental Imprinting

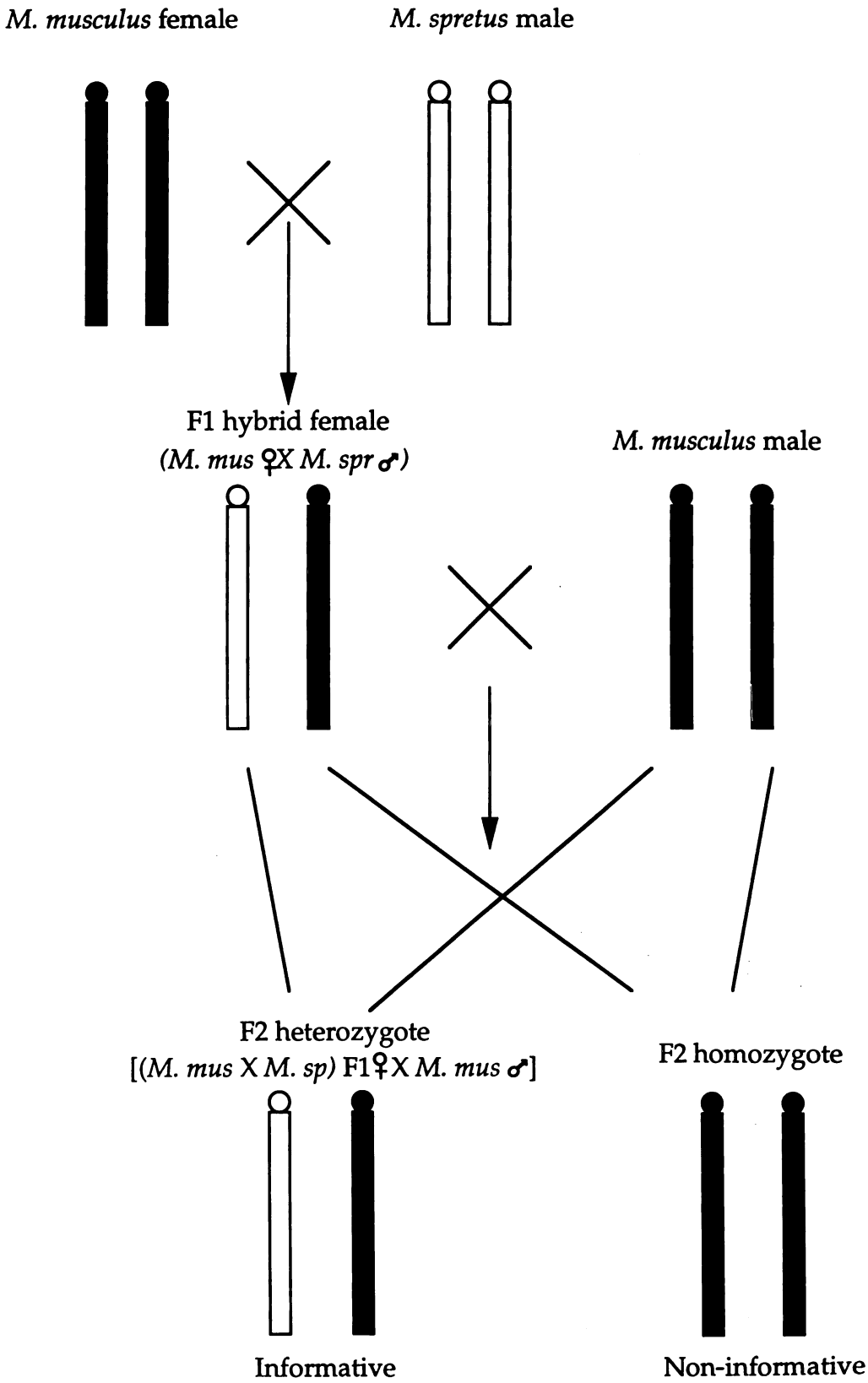
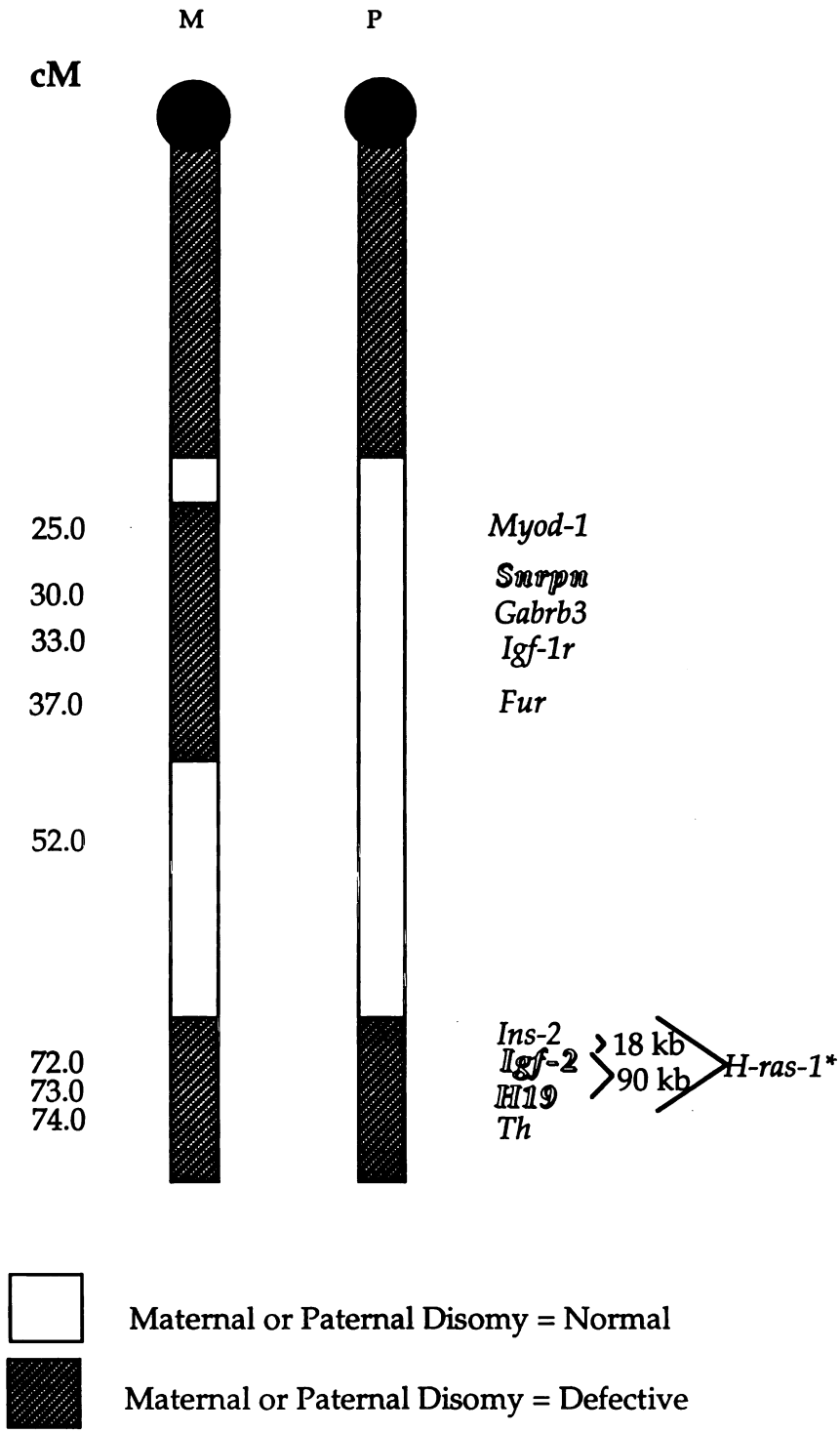


Figure 6. Imprint and linkage map of mouse chromosome 7 showing the location of the candidate imprinted genes relative to the known imprinted genes *Igf-2*, *H19* and *Snrpn*. The imprinted genes are shown in outline type. Map distances are expressed in centiMorgans (cM) from the centromere.

Spatially Restricted Imprinting of Mouse Chromosome 7



* maps distal to *Hbb* (52cM) and proximal to *int-2* (74 cM) (Kemp et al., 1993).

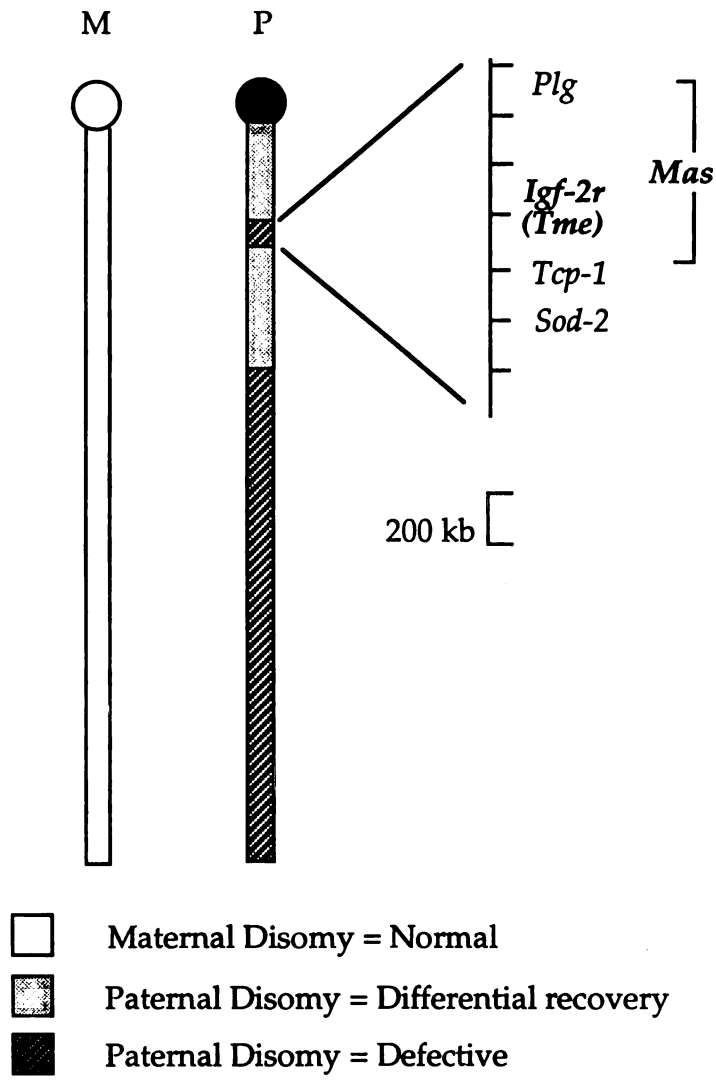
inactivation, autosomal imprinting does not affect blocks of genes, but rather is restricted to either specific genes or "imprint domains" as proposed by Bartolomei et al., (1991) based on the oppositely imprinted genes *Igf-2* and *H19*. Interestingly, the close proximity of oppositely imprintied genes *Igf-2r* and *Mas* on chromosome 17 supports the proposed imprint domain model. However, recent evidence that the mouse *Ins-2* gene, mapping immediately 5' to *Igf-2*, is also imprinted suggests an expanded region of imprinting on the distal region of chromosome 7.

Because there is currently no systematic approach for identifying imprinted genes, it is necessary to take advantage of genetic studies that have identified particular imprinted chromosomal regions and to consider the biological consequences of their uniparental inheritance. 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 my search for new imprinted genes I identified the parental imprinting of the *Mas* protooncogene in the mouse (157). I analyzed the allele-specific expression of the *Mas* protooncogene based on its proximity to the imprinted gene *Igf-2r* on the proximal portion of mouse chromosome 17 (figure 7). 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. This function was predicted by its structure of seven transmembrane domains with homology to the vascular angiotensin II receptor. 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

Figure 7. Imprinting map of mouse chromosome 17 showing the location of the *Mas* protooncogene relative to the known imprinted gene *Igf-2r*.

Parental Imprinting of Chromosome 17



unidentified ligand. In chapter three, I demonstrate that *Mas* is parentally imprinted, the maternally inherited allele being transcriptionally repressed in a developmental and tissue-specific manner (157). The relationship between genomic imprinting and tumorogenesis has been shown to be involve the loss of tumor suppressor gene regulation (139-140). 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.

Finally, in chapter four I analyze the allele-specific expression of known imprinted genes to determine the developmental regulation of genomic imprinting during gametogenesis. 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, such that males transmit only the paternal imprint and females transmit only the maternal imprint to the next generation. In addition, it has been speculated that erasure of genomic imprinting may be a prerequisite for the establishment of parent-specific imprints in the gametes. Because the mechanism of imprinting is unknown, however, it is not possible to follow the presumptive changes in the epigenetic modification of the germ line. Assuming that changes in imprinted expression reflect the alterations in the physical imprint, we analysed the allele-specific expression of *Igf-2*, *Igf-2r* and *H19*, in the developing testis, the neonate and adult ovary, as well as the germinal vesicle and ovulated oocyte of F1 females derived from an interspecies cross between *Mus musculus* females and *Mus spretus* males. Interestingly, the neonate testis and ovary maintained the imprints, however, it is not clear whether we were detecting monoallelic

expression from the somatic cell component, the germ cell component, or both. In the male, relaxation of imprinting was detected by 7 days after birth and continued during testis development (Villar AJ, Eddy EM, Pedersen RA, unpublished observations). In the female, relaxation of the *Igf-2* and *Igf-2r* parental imprints was observed in the germinal vesicle oocyte and adult ovary (Villar AJ, unpublished observations). These preliminary results provide evidence that imprint switching is initiated during gametogenesis with the relaxation of imprinting, presumably as a consequence of imprint erasure. Further studies will be required to determine the temporal regulation in the establishment and manifestation of the parental imprints.

In summary, the analysis of species-specific mRNA phenotypes for the detection of allele-specific expression is a sensitive and efficient approach that provides novel insight into the developmental and tissue-specific regulation of genomic imprinting. Because this approach can be extended to the analysis of candidate imprinted genes in other vertebrates, including humans, its application to other vertebrate systems will provide valuable imformation to address the biological and evolutionary role of genomic imprinting.

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Chapter Two

Spatially restricted imprinting of mouse chromosome 7

by

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The text of this chapter is a reprint of the material as it appears in *Molecular Development and Reproduction.* The co-author listed in this publication, Dr. Roger A. Pedersen, directed and supervised the research which forms the basis for the dissertation/thesis.

ABSTRACT

Three of the four known imprinted genes *(Igf-2, H19* and *Snrpn)* map to mouse chromosome 7. We used mRNA phenotyping to examine the tissue-specific transcription of *Igf-1r, H-ras-1,* and *Gabrb3,* which map to chromosome 7 between *Snrpn* and the *Igf-2/H19* domain, and *Myod-1,* which maps proximal to *Snrpn.* We found that all of these genes were expressed by both parental alleles in tissues from day 1 neonates. The fact that imprinted genes can flank or map closely to genes that escape such epigenetic modification suggests that autosomal imprinting is not manifested globally along imprinted chromosomes but rather is spatially restricted, perhaps even defined by specific DNA consensus sequences or an "imprint box" associated with imprintable genes.

Key Words: mRNA phenotyping, Autosomal imprinting, Chromosome 7, Interspecies cross

INTRODUCTION

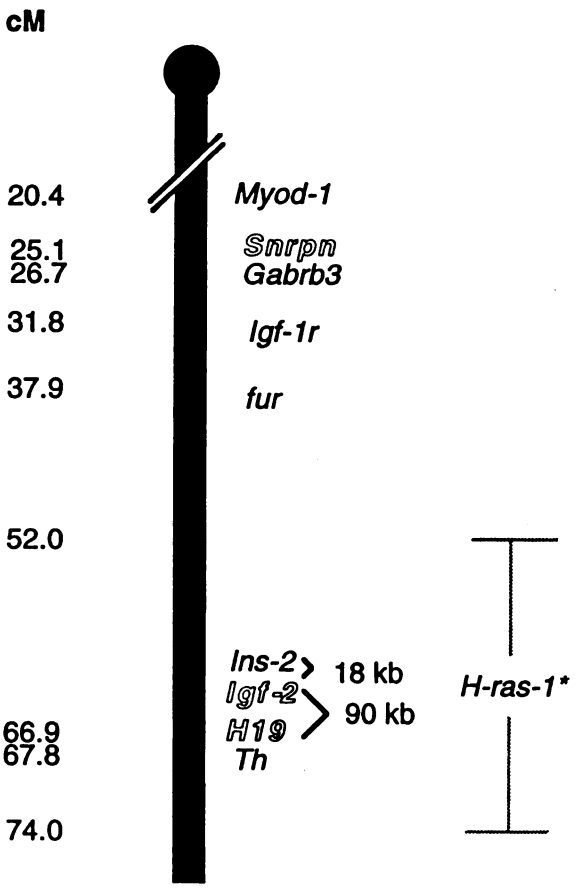
Genomic imprinting is the epigenetic modification of parental genomes during gametogenesis that results in their differential expression during development. Experimental evidence for genomic imprinting has generally been restricted to the mouse, in which contributions from both the maternal and paternal genomes are required for normal development (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1986, 1987). The significance of genomic imprinting has been illustrated not only by its profound effect on mammalian gene expression, but also by its possible role in the establishment and development of the primary cell lineages (unpublished observations; Pedersen et al., 1993). To detect the differential expression of parental alleles, it is necessary to distinguish between the maternal and paternal transcripts of candidate imprinted genes. Four imprinted genes have been identified: insulin-like growth factor-II *(Igf-2)* (DeChiara et al., 1990, 1991), insulin-like growth factor type-II receptor *(Igf-2r)* (Barlow et al., 1991), *H19* (function of transcript unknown) (Bartolomei et al., 1991), and a small nuclear ribonucleoprotein-associated polypeptide SmN *(Snrpn)* (Leff et al., 1992). Although various strategies have been used to demonstrate the differential expression of each of these genes, there currently is no systematic approach for identifying imprinted genes; therefore, it is necessary to take advantage of genetic studies that have identified particular imprinted chromosomal regions and to consider the biological consequences of uniparental inheritance (Searle and Beechey, 1978; Cattanach and Kirk, 1985; Beechey and Searle, 1987; Cattanach and Beechey, 1990; Pedersen et al., 1993). For instance, we chose myoblast differentiation factor 1 *(Myod-1)* as a candidate imprinted gene, not only because of its map position on mouse chromosome 7, but also because of its specific expression during skeletal myogenesis (Sassoon et al., 1989). The fate of isoparental cells in skeletal muscle of aggregation chimeras

differs from that of the normal, fertilized embryo (Stevens et al., 1977; Surani et al., 1977; Nagy et al., 1987; Paldi et al., 1989; Surani et al., 1988): parthenogenetic cells are selected against after 13.5 days of gestation in skeletal muscle (Fundele et al., 1990), suggesting the differential expression of a gene involved in skeletal myogenesis.

Similar considerations led us to study four genes that map to the imprinted region of mouse chromosome 7 (Fig. 1): insulin-like growth factor-1 receptor *(Igf-1r),* Harvey rat sarcoma virus oncogene *(H-ras-1),* gamma-aminobutyric acid receptor β3 *(Gabrb3)* and *Myod-1.* These loci map to mouse chromosome 7 in the following order from the centromere: *Myod-1...(Gabrb3-**Snrpn**)...Igf-1r...Fur...(H-ras-1)...Ins-2...**Igf-2**...**H19**...Th* (imprinted genes are in bold type) (Kemp et al., 1993; Leff et al., 1992; Zemel et al., 1992; Copeland et al., 1992; Saunders and Seldin, 1990). By analyzing the mRNA phenotypes of F1 hybrid neonates from *Mus musculus* females mated with *Mus spretus* males and backcross progeny, we confirmed the imprinted status of *Igf-2* and determined biallelic expression for the selected candidate imprinted genes.

Fig. 2-1. Linkage map of mouse chromosome 7 showing the location of the candidate imprinted genes relative to the known imprinted genes *Igf-2, H19* and *Snrpn* (Leff et al., 1992; Zemel et al., 1992; Copeland et al., 1992; Saunders and Seldin, 1990). A revised location for *H-ras-1* places the gene distal to *Hbb* (52 cM) and proximal to *Int-2* (74 cM) (Kemp et al., 1993). In addition, insulin-2 *(Ins-2),* tyrosine hydroxylase *(Th)* (Kitsberg et al., 1993), and furin *(Fur)* (unpublished observation) are not imprinted. The imprinted genes are shown in outline type. Map distances are expressed in centiMorgans (cM) from the centromere.

Mouse Chromosome 7



*maps distal to *Hbb* (52 cM) and proximal to *int-2* (74 cM) (Kemp et al., 1993).

MATERIALS AND METHODS

Isolation of RNA from Day 1 Neonates

M. musculus (C57BL/6J, Jackson Laboratories, Bar Harbor, ME) and *M. spretus* (Jackson Laboratories) females were caged with males of the same species for natural mating. Natural interspecies matings were also performed between *M. musculus* females and *M. spretus* males to produce F1 hybrids. These F1 hybrid females were then mated with *M. musculus* males to derive backcross progeny. Day 1 neonates were killed by decapitation and dissected for tissue collection. Total RNA was prepared from pooled tissues of littermates by the GuSCN-CsCl gradient ultracentrifuge technique (Chirgwin et al., 1979). Dissected tissues were solubilized in 2.4 ml of GuSCN and then layered over 1.6 ml of 5.7 M CsCl and centrifuged for 12-16 h at 35,000 rpm in a SW60 rotor in a Beckman L5-75 ultracentrifuge. RNA concentrations were determined by $A_{260,}$ and integrity was determined by electrophoresis on formaldehyde gels and by ethidium bromide staining to visualize ribosomal RNAs.

RT-PCR

Total RNA from liver, tongue, skin, lung, heart, and brain was isolated from day 1 neonate progeny of *M. musculus*, *M. spretus*, F1 hybrids produced by crossing *M. musculus* females and *M. spretus* males, and backcross progeny produced by mating F1 hybrid females and *M. musculus* males. Although the tissues we selected to study do not represent all of the tissues in which these genes are expressed,

they do represent the mesoderm, ectoderm, and endoderm cell lineages. Each preparation was reverse transcribed into cDNA as described by Rappolee et al. (1988 a,b), and one tenth of the mixture was amplified by PCR with the use of

primer pairs designed for specific detection of *Igf-2*, *Igf-1r*, *H-ras-1*, *Gabrb3*, and *Myod-1* cDNA target sequences. Briefly, the RNAs were incubated at 42°C for 1 h with 0.2 μg of random hexamer (Pharmacia, Gaithersburg, MD) and 40 units of reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a 10-μl mixture. The total reaction mixture was diluted with 20 μl of 1X RT reaction buffer. Three microliters of the RT reaction mixture was amplified with 2.5 units of AmpliTaq polymerase (Perkin Elmer-Cetus, Norwalk, CT) in a final volume of 50 μl containing 10 mM Tris-HCl buffer (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 1 μg acetylated bovine serum albumin, 0.7 μg of each sequence-specific primer, and 2 mM each dNTP (Pharmacia). The mixture was overlaid with mineral oil to prevent evaporation, and cDNA sequences were amplified by PCR for 35-45 cycles in a DNA thermal cycler (Perkin Elmer-Cetus) programmed for a 94°C denaturation, 55°C annealing, and 72°C primer extension step for each cycle.

Primer pairs were generated by using PCR Mate (Applied Biosystems, Foster City, CA). The *Igf-2* primer pair was designed to detect the presence of *Igf-2* mRNA (cDNA) via a predicted 255-bp PCR product (Dull et al., 1984). The cDNAs for *Igf-1r*, *H-ras-1*, *Gabrb3*, and *Myod-1* were amplified by PCR to yield expected products of 354 bp, 309 bp, 258 bp, and 482 bp, respectively. The following primers were used for PCR amplification: *Igf-2:* 5' GGCCCCGGAGAGACTCTGTGC 3' and 5'GCCCACGGGGTATCTGGGGAA 3'; *Igf-1r:* 5' ATGCTGTTTGAACTGATGCGCATGTGCTGG 3' and 5' CCGCTCGTTCTTGCGGCCCCCGTTCATGTG 3'; *H-ras-1:* GCAGCCGCTGTAGAAGCTATG 3' and 5' CCTGTACTGATGGATGTCCTC 3'; *Gabrb3:* 5' GTTGGTGACACCAGGAATTCAGC 3' and 5' GTACAGCCAGTAAACTAAGTTG 3'; *Myod-1:* 5' GCATGCCTGGGAGATAAATATAGCC 3' and 5' AGAGATCGACTGCACAGCAGAGGG 3'. The absence of PCR product from total mRNA that had not been reverse transcribed indicated the absence of

contaminating genomic sequences. The identity of PCR products was verified by restriction enzyme analysis (see below).

Cloning and Sequencing of PCR Products

The PCR products from both *M. musculus* and *M. spretus* were resolved electrophoretically on 2% agarose gels and extracted by using Geneclean (Bio101, La Jolla, CA). The purified PCR products were cloned by means of the TA cloning system (Invitrogen, San Diego, CA), designed for direct insertion of PCR products. Briefly, ligations were set up as a 1:1 molar ratio of pCR vector (Invitrogen) to PCR product and incubated at 12°C for 4 h. The ligation mixtures were used to transform competent *Escherichia coli* cells, which were then plated onto Luria-Bertani agar plates containing kanamycin (50 μg/ml) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (40 mg/ml stock solution). Preparations of single-stranded plasmid DNA containing the appropriate PCR product were prepared and sequenced by the dideoxy-chain termination method (Sanger et al., 1977). The sequencing reaction was resolved on a 7 M urea, 6% acrylamide gel. After fixation in 5% methanol and 5% acetic acid, the gel was dried under vacuum and autoradiographed. The identity of the PCR products was verified by comparison with published sequences.

Species-Specific Restriction Enzyme Digestion of PCR Products

The PCR products generated from day 1 neonates were digested with restriction enzymes specific to each species to determine their parental origin. All restriction fragments were resolved electrophoretically on 8% polyacrylamide gels except for those of *Myod-1*, which were visualized on a 2% agarose gel. The amplification of nonspecific DNA bands for *Igf-2* and *H-ras -1* was consistent and did not interfere with our analysis.

RESULTS

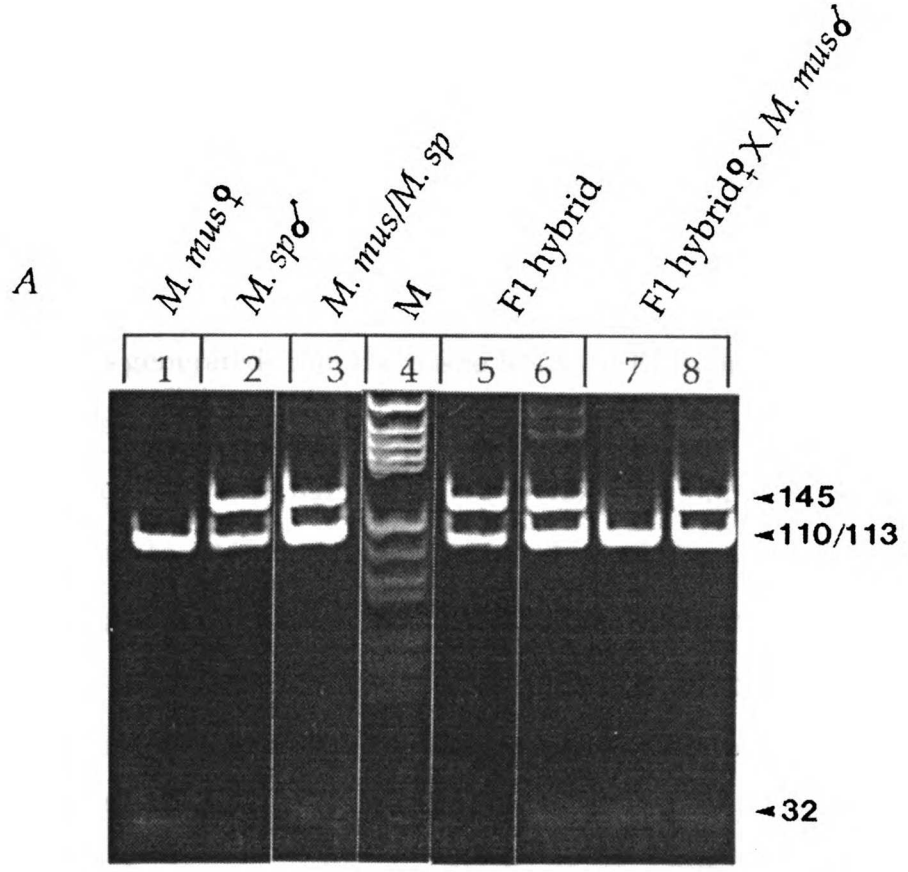
Allele-Specific Expression of *Igf-2*

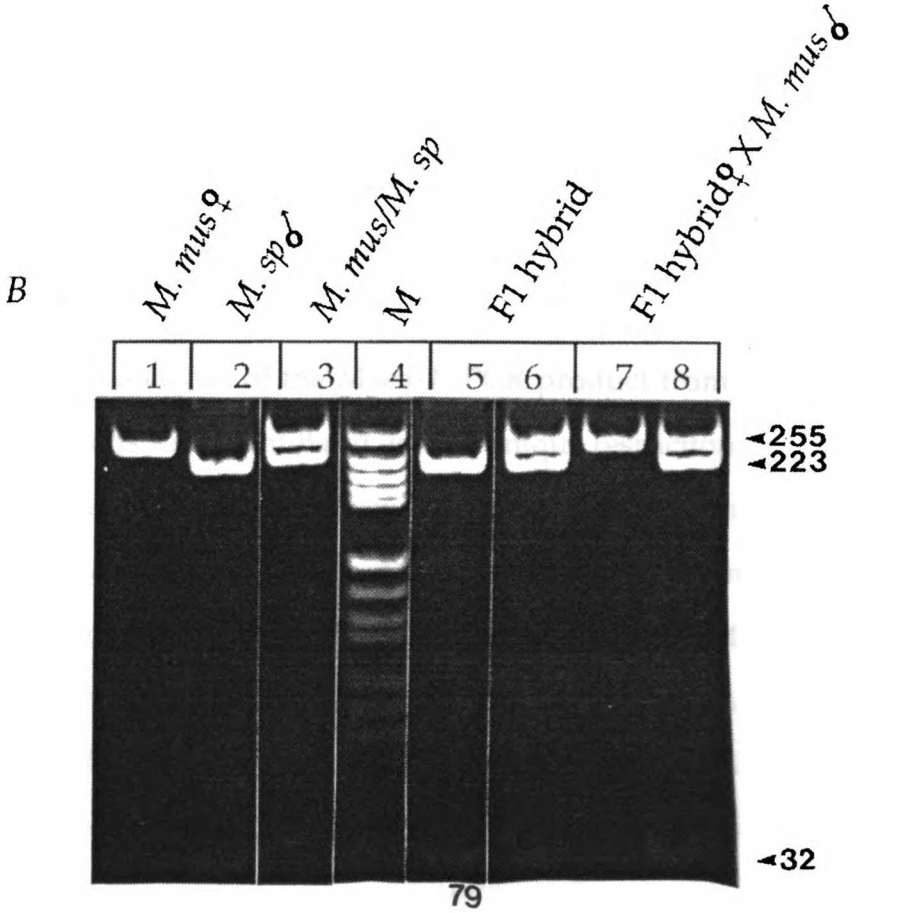
To detect allele-specific expression, we analyzed the mRNA phenotypes of day 1 neonates using a combination of reverse transcription-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphisms (RFLPs) unique to each of the two species. The gene for *Igf-2*, previously shown to be imprinted (DeChiara et al., 1991), was used to validate our approach.

DNA sequencing of the cloned *Igf-2* PCR products revealed a single base-pair (bp) difference between *M. musculus* and *M. spretus* in the 255-bp amplified target sequence. This sequence difference produced diagnostic RFLPs that were used as parental allele-specific markers to study the parental origin of mRNAs transcribed by the F1 progeny of *M. musculus* females mated with *M. spretus* males and of a backcross between F1 females and *M. musculus* males. We analyzed mRNA (cDNA) from the interspecies cross as well as the backcross to demonstrate that allele-specific expression is not species dependent and can be erased and reestablished in the germline of the next generation. PCR products were digested with AluI to yield the three expected fragments for *M. musculus* and the two fragments for *M. spretus* (Fig. 2A). Similarly, *Mbo* I produced an undigested product for *M. musculus* and the cleavage fragments expected for *M. spretus* (Fig. 2B). As predicted from previous studies showing the imprinted status of *Igf-2*, the *Igf-2* PCR product from the F1 hybrids was digested with AluI to yield *M. spretus*-specific fragments (Fig. 2A, lane 3) and with MboI to yield *M. spretus*-specific fragments (Fig. 2B, lane 3) in RNA from liver, tongue, skin, lung, and heart. (Only tongue is shown.) These enzymes yielded the male *M.*

musculus-specific fragments in the same tissues from backcross progeny heterozygous for the *Igf-2* allele (Fig. 2A,B, lane 5). Conversely, AluI digestion of *Igf-2* products from brain (Fig. 2A, lanes 4 and 6), and MboI digestion of *Igf-2* products from brain (Fig. 2B, lanes 4 and 6) produced fragments that were not allele specific. The biallelic expression of *Igf-2* in the brain and the species-specific RFLPs permitted the identification of the heterozygous individuals, i.e., those that had inherited the *M. spretus* allele from the mother. The sizes of fragments digested by the various enzymes are shown in Table 1. These enzyme restriction patterns confirm that *Igf-2* is expressed only from the paternally inherited allele in liver, tongue, skin, lung and heart, whereas *Igf-2* is expressed by both parental alleles in the brain.

Fig. 2-2. mRNA phenotyping analysis of *Igf-2* PCR products from tissue RNA of *M. musculus*, *M. spretus*, and their hybrid progeny. Allele-specific *Igf-2* mRNA from day 1 neonates was detected by means of restriction enzyme digestion to generate species-specific RFLPs. *A*, *Igf-2* PCR products digested with *Alu* I: tongue from *M. musculus* (lane 1) and *M. spretus* (lane 2); tongue (lane 3) and brain (lane 4) from F1 hybrids (*M. musculus* females X *M. spretus* males); tongue (lane 5) and brain (lane 6) from backcross progeny (F1 hybrid females X *M. musculus* males). Lane 7 is an assay control in which equal amounts of tongue RNA from *M. musculus* and *M. spretus* were used in the PCR. *B*, *Igf-2* PCR products digested with *Mbo* I. Lanes are as described in *A*. The molecular size marker (M) is DNA molecular weight marker V pBR322 DNA-*Hae* III (Boehringer Mannheim, Indianapolis, IN). Arrows indicate enzyme restriction fragments (bp).


A
M. mus♀
M. sp♂
M. mus/M. sp
M
F1 hybrid
F1 hybrid♀ X M. mus♂
1 2 3 4 5 6 7 8
145
110/113
32


B
M. mus♀
M. sp♂
M. mus/M. sp
M
F1 hybrid
F1 hybrid♀ X M. mus♂
1 2 3 4 5 6 7 8
255
223
32

mRNA Phenotyping Analysis of Candidate Imprinted Genes

The *Igf-1r, H-ras-1, Gabrb3,* and *Myod-1* PCR products from day 1 neonate F1 hybrids were cloned and sequenced to identify restriction enzyme sites that would produce fragments unique to either *M. musculus* or *M. spretus*. The *Igf-1r* PCR products generated from the tissue RNAs of F1 hybrids were digested with MspI to produce both the *M. musculus* and *M. spretus* fragments (Fig. 3A). Similarly, digestion with BanII resulted in 187-bp, 93-bp, and 85-bp fragments corresponding to *M. musculus* and 190-bp, 93-bp, and 85-bp fragments corresponding to *M. spretus* (Table 1). Although this restriction enzyme site was not species specific, the 190-bp and 187-bp digestion products were species specific because of a 3-bp repeat in the *M. spretus* DNA sequence. As predicted from the DNA sequence, digestion of the *H-ras* PCR products from the F1 hybrid with DdeI (data not shown) and with AvaII (Fig. 3B) resulted in fragments corresponding to the undigested *M. spretus* PCR product and the two *M. musculus* cleavage fragments in brain, liver, tongue, skin, lung, and heart. The *Gabrb3* PCR product generated from brain RNA of the F1 hybrids was digested with MspI to produce restriction fragments corresponding to the undigested *M. musculus* PCR product and the two *M. spretus* cleavage products (Fig. 3C). Finally, RFLP analysis of the *Myod-1* PCR product from tongue RNA of the F1 hybrids demonstrated that BanII, with a unique restriction site in the *M. musculus* sequence (Fig. 3D), and HinfI, with a unique restriction site in the *M. spretus* sequence (data not shown), both recognized a portion of the *Myod-1* PCR product. Therefore, F1 progeny derived from mating of *M. musculus* females and *M. spretus* males transcribe both maternal and paternal alleles of *Igf-1r, H-ras-1, Gabrb3,* and *Myod-1,* despite the fact that they map to an imprinted chromosome region. A diagram of mouse chromosome 7 (Fig. 1) shows the position of the

Fig. 2-3. mRNA phenotyping analysis of *Igf-1r, H-ras -1, Gabrb3,* and *Myod-1* PCR products from tissue RNA of *M. musculus, M. spretus,* and their hybrid progeny. Allele-specific transcription of candidate imprinted genes from day 1 neonates was detected by means of restriction enzyme digestion to generate species-specific RFLPs. *A, Igf-1r* PCR products from tongue RNA digested with *Msp* I: *M. musculus* (lane 1), *M. spretus* (lane 2), F1 hybrids (*M. musculus* females X *M. spretus* males) (lane 3). *B, H-ras-1* PCR products from tongue RNA digested with *Ava* II: *M. musculus* (lane 1), *M. spretus* (lane 2), F1 hybrids (lane 3). *C, Gabrb3* PCR products from brain RNA digested with *Msp* I: *M. musculus* (lane 1), *M. spretus* (lane 2), F1 hybrids (lane 3). *D, Myod-1* PCR products from tongue RNA digested with *Ban* II: *M. musculus* (lane 1), *M. spretus* (lane 2), F1 hybrids (lane 3). Lane 4, in all panels, is an assay control in which equal amounts of tissue RNA from *M. musculus* and *M. spretus* RNA were used in the PCR. The molecular size marker (M) is the 1-kb ladder (Gibco BRL, Gaithersburg, MD) for panels *A, C* , and *D.* The size marker in panel *B* is DNA molecular weight marker V pBR322 DNA-*Hae* III (Boehringer Mannheim). Arrows indicate enzyme restriction fragments (bp).

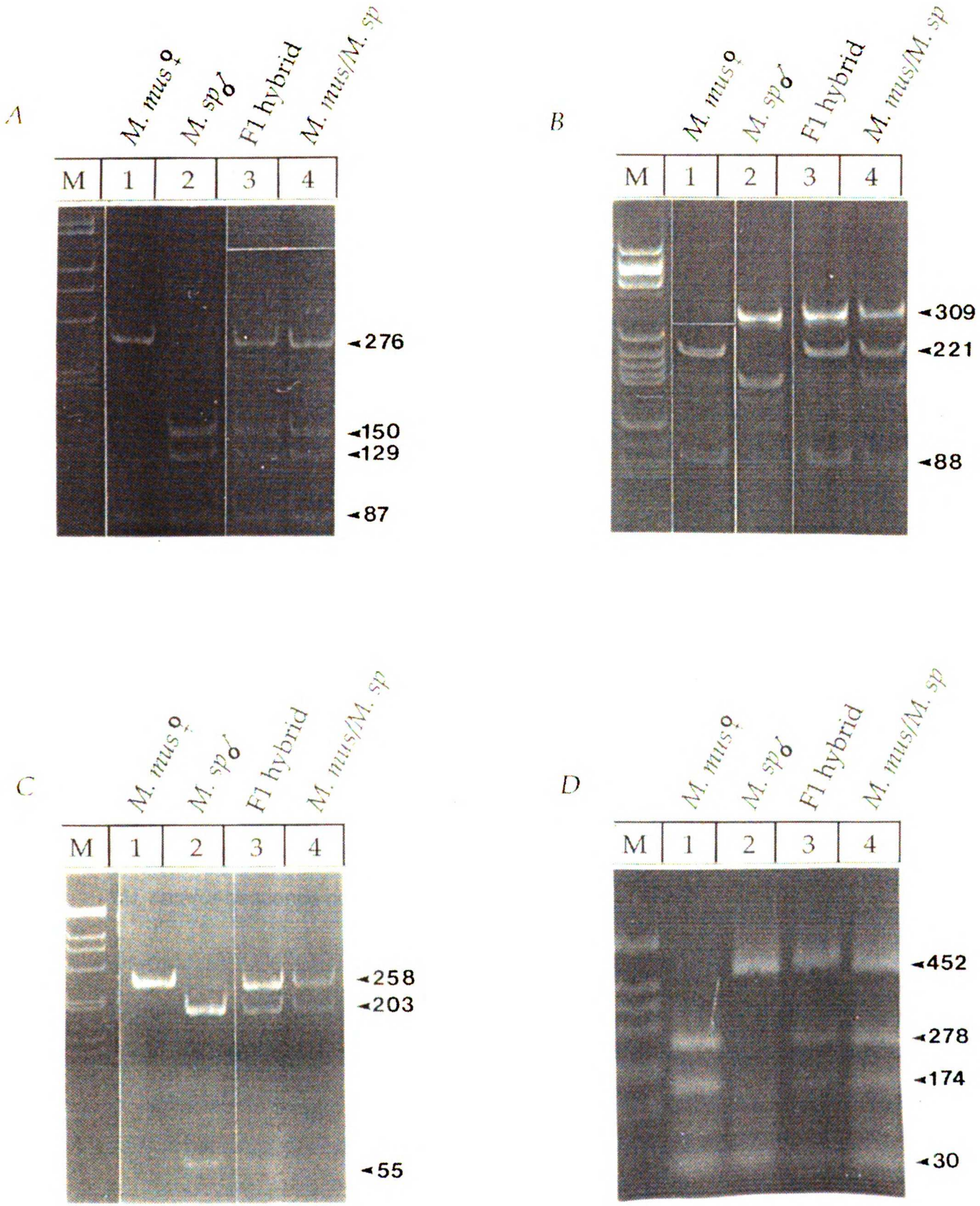

A
M. mus ♀
M. sp ♂
F1 hybrid
M. mus/M. sp
M
1
2
3
4
276
150
129
87
B
M. mus ♀
M. sp ♂
F1 hybrid
M. mus/M. sp
M
1
2
3
4
309
221
88
C
M. mus ♀
M. sp ♂
F1 hybrid
M. mus/M. sp
M
1
2
3
4
258
203
55
D
M. mus ♀
M. sp ♂
F1 hybrid
M. mus/M. sp
M
1
2
3
4
452
278
174
30

TABLE 1 Fragment Sizes of PCR Products Digested with Species-Specific Restriction Enzymes[a]

Gene	Enzyme	*M. musculus* (fragment size in bp)	*M. spretus* (fragment size in bp)	F1 hybrid[b] (fragment size in bp)
Igf-2	*Alu* I	110,113,32	110,145	110,145
	Mbo I	255	223,32	223,32
H-ras	*Dde* I	183, 126	309	309,183,126
	Ava II	221,88	309	309,221,88
	Msp I	172,137	172,137	172,137
Igf-1r	*Msp* I	276,87	150,129,87[c]	276,150,129,87
	Ban II	187,93,85	190,93,85[c]	190,187,93,85
Gabrb3	*Msp* I	258	203,55	258,203,55
	Alu I	148,110	148,110	148,110
Myod-1	*Ban* II	278,174,30	450,30	452,278,174,30
	Hin fI	482	270, 212	482,270,212

[a]All restriction enzymes used to identify the PCR products and confirm transcription from one or both parental alleles are presented.

[b]Result of *M. musculus* female X *M. spretus* male interspecies cross.

[c]*M. spretus* sequence contains a 3-bp duplication.

relevant loci. The sizes of fragments digested by the various enzymes are shown in Table 1.

DISCUSSION

Interspecies hybrids have been used successfully to map genes and to analyze X-linked gene expression (Guenet, 1986; Avner et al., 1988; Grant and Chapman, 1988; Brockdorff et al., 1991). Recently, their use has been extended to the analysis of differential gene expression by the RNase protection assay: Bartolomei et al. (1991) demonstrated that *H19* is only maternally expressed, and Leff et al. (1992) showed that *Snrpn* is only paternally expressed. Using an interspecies cross between *M. musculus* females and *M. spretus* males and a backcross between the F1 hybrid females and *M. musculus* males, we were able to distinguish between maternally and paternally derived transcripts owing to sequence differences that resulted in diagnostic RFLPs specific to each species. This approach is sensitive enough to study allele-specific expression at very early stages of development, when the amount of tissue may be quite limited, and efficient enough to be used as a simple screening assay for candidate imprinted genes. We have validated this experimental approach by using *Igf-2* as a model. The restriction enzyme pattern of *Igf-2* corresponded to the mRNA phenotype of the paternal species in tongue, liver, lung, skin, and heart from day 1 neonates, indicating that *Igf-2* is expressed only by the paternal allele in these tissues; however, after digestion of *Igf-2* PCR products from total brain RNA at this same stage of development, both species-specific enzyme restriction patterns were generated. These observations support the finding of DeChiara et al. (1991) that *Igf-2* is expressed only by the paternal allele, except in the choroid plexus and leptomenige tissues of the brain, where both alleles are expressed.

To determine whether the mechanism of imprinting is manifested in a global or spatially restricted manner, we selected *Igf-1r, Hras, Gabrb3* and *Myod-1* as candidate imprinted genes because of their map positions on mouse chromosome 7, and because of indirect evidence of possible differential expression. Specifically, the *Igf1-r* gene was studied because of the observation by Rappolee et al. (1992) that preimplantation parthenogenones did not express *Igf1-r; H-ras-1* was selected because of the effect of DNA methylation on *c-Ha-ras-1* promoter activity in vivo (Rachal et al., 1989) and evidence for allele-specific methylation of the human *c-Ha-ras-1* gene (Chandler et al., 1987); *Gabrb3* was selected because its map position on mouse chromosome 7 is homologous to a critical region on human chromosome 15 implicated in the Prader-Willi and Angelman syndromes, which are associated with the preferential loss of paternal or maternal genes, respectively, in 15q11-13 (Wagstaff et al., 1991); and *Myod-1* was selected because of its exclusive role in skeletal myogenesis (Sassoon et al., 1989) and the preferential restriction of parthenogenetic cells from this cell lineage between days 13.5 and 15 of gestation (Fundele et al., 1990). By restriction enzyme analysis of PCR products, we found that the F1 progeny of the interspecies cross transcribed both parental alleles of *Igf-1r, H-ras, Gabrb3,* and *Myod-1* in tissues from day 1 neonates. Although PCR is a very sensitive technique capable of amplifying trace amounts of DNA, we did not detect any maternal transcription of *Igf-2* in tissues other than brain, even after reamplification of the PCR product; therefore, we believe that the non-imprinted status of *Igf-1r, H-ras,* and *Gabrb3* in all tissues studied, *Myod-1* in tongue, and *Igf-2* in brain is an accurate interpretation of their biallelic expression. In contrast, Sasaki et al. (1993) were able to detect leaky expression of *Igf-2* from the maternal allele, in mice inheriting a maternal duplication of the distal region of chromosome 7, by increasing the sensitivity for detecting *Igf-2* expression using

Southern blotting of the PCR product. It is also possible that the transcriptional regulation of *Igf-2* may be affected by the uniparental inheritance of other imprinted genes in this region or that the relaxation of the imprint is due to limited imprinting factor(s).

While we cannot exclude the possibility that these genes may be imprinted in other stages of development, other tissues, or different genotypic backgrounds, we consider this unlikely because among the four known imprinted genes, only *Igf-2* shows biallelic expression, and this exception is limited to specific tissues of the 16.5-day embryo and three-week-old brain (DeChiara et al., 1991). Allele-specific expression of *Igf-2* (Ohlsson et al., 1993; Giannoukakis et al., 1993) and *H19* (Rachmilewitz et al., 1992; Zhang and Tycko, 1993) has also been shown to be conserved through evolution.

Although there is as yet no evidence for developmental regulation of imprinting in somatic tissues, transgene expression and imprinting have been shown to be influenced by strain-specific modifiers and/or imprinting genes (Allen et al., 1990; Sapienza, 1989; Reik et al., 1990). The imprinted *Tme* locus also appears to be under such epigenetic control via an unlinked imprintor-1 gene (Forejt and Gregorova, 1992). Our observation that *Igf-1r, Hras, Gabrb3* and *Myod-1* are not qualitatively imprinted even though they map to a region containing three imprinted genes is significant in light of our current limited understanding of genomic imprinting. These results, along with the fact that *Igf-2* and *Snrpn* are expressed only by the paternal allele (DeChiara et al., 1991; Leff et al., 1992), whereas *H19* is expressed only by the maternal allele (Bartolomei et al., 1991), raise the question of how these linked genes can be epigenetically modified to display parental imprinting and how others can escape such modification.

The actual number of imprinted genes is not known, because the only information available is that certain chromosomes contain regions with one or more imprinted genes. Eight mouse autosomes have been shown to have defective complementation that depends on the parental origin of the imprinted chromosomal region (Cattanach and Beechey, 1990). The four identified imprinted genes map to two of these chromosomes; there may be additional imprinted genes on chromosome 7, 17 or other autosomes. Their frequency will depend on whether the mechanism of imprinting affects the transcription of entire chromosome regions or gene clusters, or whether it affects single genes. Our data suggest that autosomal imprinting does not affect blocks of genes, as does X-chromosome inactivation (Gartler et al., 1992), but is restricted to either specific genes or "imprint domains," as suggested by Bartolomei et al. (1991). This conclusion supports the observation of Barlow et al. (1991) that, of the four genes assigned to an 800- to 1,100-kb region of the imprinted *Tme* locus on chromosome 17, only *Igf-2r* is differentially expressed. It has also recently been shown that the insulin-2 *(Ins-2)* and tyrosine hydroxylase *(Th)* genes, which flank *Igf-2* and *H19* on chromosome 7, are not imprinted (Kitsberg et al., 1993). If this conclusion applies to the other imprinted autosomal regions, the number of imprinted genes in the mammalian genome may be quite limited. It also suggests that there may be an imprint recognition sequence or "imprint box," as suggested by DeChiara et al., 1991, that identifies the gene to be imprinted and is recognized by the mechanism of imprinting under the appropriate conditions. Analysis of other genes mapping to the imprinted chromosome regions will allow us to develop a more detailed map of the DNA sequences that are subject to parental imprinting. The possibility that genomic imprinting may affect a relatively small number of genes mapping to regions that display defective complementation depending on parental origin emphasizes the need for an

approach that can specifically identify imprinted endogenous genes. The identification of these genes is essential to understanding the mechanism of genomic imprinting and the consequences of differential gene expression on cellular and genetic processes involved in early mammalian embryogenesis and human disease.

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Chapter Three

Parental imprinting of the *Mas* protooncogene in mouse

by

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The text of this chapter is a reprint of the material as it appears in *Nature Genetics.* The co-author listed in this publication, Dr. Roger A. Pedersen, directed and supervised the research which forms the basis for the dissertation/thesis.

The *Mas* protooncogene on mouse chromosome 17 encodes a mitogenic G-protein-coupled cell surface receptor. We investigated the allele-specific expression pattern of the *Mas* gene on the basis of its localization between *Plg* and *Tcp-1* and its proximity to the known imprinted gene *Igf-2r*. mRNA phenotyping demonstrated exclusive expression from the paternal allele in all embryonic tissues, including visceral yolk sac, between 11 and 12.5 days of gestation. By 13.5 days of gestation the paternal allele-specific expression of *Mas* was restricted to heart, tongue, and visceral yolk sac, whereas all other tissues exhibited relaxation of the parental imprint. These results demonstrate parental imprinting of *Mas* and suggest that the maternally inherited allele is transcriptionally repressed in a developmental and tissue-specific manner.

Although viable parthenogenesis is observed in other classes of vertebrates, mammalian development is unique in its requirement for a paternal genetic contribution [1-4]. The basis for the developmental failure of isoparental embryos (parthenogenones, gynogenones, and androgenones) has been attributed to the epigenetic modification, or imprinting, of specific genes [3-8]. The identification of these genes is essential not only to determine the mechanism of genomic imprinting but to understand the wide-ranging implications of imprinting for mammalian embryogenesis, human disease, and the evolution of sexual reproduction.

We analyzed the allele-specific expression of the *Mas* protooncogene during mouse development on the basis of its proximity to the imprinted gene *Igf-2r* on the proximal portion of mouse chromosome 17 (ref 9). The *Mas* oncogene, identified through its tumorigenic potential in transfected NIH/3T3 cells, encodes a mitogenic peptide receptor thought to transduce extracellular signals to G-proteins [10,11]. This function is predicted by its structure of seven transmembrane domains with homology to the vascular angiotensin II receptor [12]. Although there is no direct evidence for the physiological role of *Mas* as an angiotensin receptor [13], *Mas* has been shown experimentally to bind angiotensin and to function as a transducer of certain angiotensin effects in *Mas*-expressing *Xenopus* oocytes and *Mas*-transfected mammalian cell lines [12].

We used mRNA phenotyping in interspecies mouse hybrids [14] to determine whether the mouse *Mas* gene expression was parentally imprinted. Analysis of restriction fragment length polymorphisms (RFLPs) unique to *Mus musculus* and *M. spretus* allowed us to distinguish between maternal and paternal transcripts. During early stages of fetal development, expression was detected only from the paternal allele in all embryonic tissues, including visceral yolk sac. By 13.5 days of gestation (d.g.), however, repression of the maternal allele was

restricted to specific tissues. A similar pattern of imprinted expression was observed after birth. The identification of *Mas* as an imprinted gene provides the first evidence that the mechanism of imprinting plays a direct role in the transcriptional regulation of an oncogene and suggests a novel mechanism in growth control.

Mas sequence analysis

We identified *Mas* allelic differences between *M. musculus* and *M. spretus* by RFLP analysis of products generated by polymerase chain reaction of cDNA reverse transcribed from total RNA (RT-PCR). Primers specific for *Mas* were designed to amplify a 583-bp PCR product that included 133 bp of the 5' untranslated region (nucleotides -133 to +450 in cDNA) (Fig. 1*a*). The identity of the PCR product was verified by sequencing and was found to be well conserved between the two species. Coincidentally, two independent single-basepair differences generated a *M. spretus*-specific *Tsp*509I restriction site at position -53 and a *M. musculus*-specific *Tsp*509I restriction site at position +311 in the *Mas* cDNA sequence (Fig. 1*b*). The characteristic species-specific RFLPs were visualized on 6% polyacrylamide gels after ethidium bromide staining. We controlled for the possibility of genomic contamination by running concurrent PCR assays of RNA samples that had not been reversed transcribed. These controls invariably gave negative results.

Inactive maternal allele of _Mas_

To detect differential expression of the *Mas* parental alleles, we analyzed the mRNA phenotypes of tissues from fetuses at 11 to 13.5 d.g. and day 1 neonates derived from an interspecies cross between C57BL/6J *(M. musculus)* females and *M. spretus* males. The restriction enzyme pattern of *Mas* PCR products in 11- and

Fig. 3-1 *Mas* protooncogene sequence analysis. *a, M. musculus* sequence of cDNA amplified by mouse *Mas* primers. Sites of basepair differences between *M. musculus* and *M. spretus* are indicated. Primer sequences are in bold type. *b,* Partial nucleotide sequencing of the C57BL/6J *(M. musculus)* and *M. spretus Mas* PCR product. The nucleotide sequence recognized by *Tsp*509I is in outlined type. The restriction maps indicate the positions of the species-specific restriction sites and the sizes of the corresponding restriction enzyme fragments.

a

5' **ACACTT TCCTAACTGA GCCAC**CCTCA CCCCTCAGCT GTCTCTTTCA TTTCAA[T/C]TCT

TGAAACTTTC ATACATCTTG TTTGTTTGAT TTATCTACAG AAAATGACCT GAAGTGACCC

AGGGCAAGAA CTCCTCATGG ACCAGTCAAA TATGACATCC CTTGCTGAAG AGAAAGCCAT

GAATACCTCC AGCAGAAATG CCTCCCTGGG GAGCTCACAC CCACCCATTC TCCTCTGGTT

CCTCTGCTTC CGGATGAGGA GAAATCCTTT CACGGTCTAC ATTACCCACT TGTCCATGGC

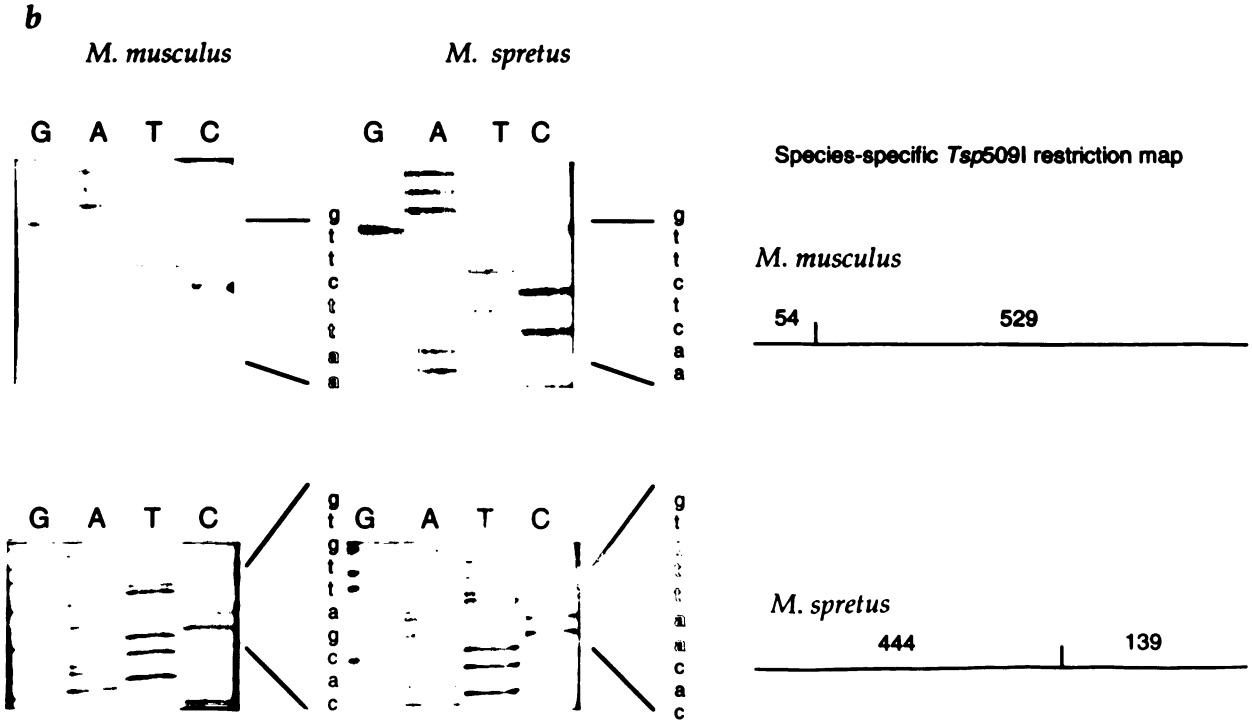
TGATATCTCT CTTCTGTTCT GTATTTTTAT CCTGTCCATT GACTATGCTT TAGACTATGA

ACTCTCTTCT GGCCATCACT ACACAA[T/C]GT GACATTATCG GTGACTTTTC TATTTGGCTA

CAACACGGGC CTCTATCTGC TGACAGCCAT CAGTGTGGAG AGGTGCCTAT CGGTCCTCTA

CCCCATATGG TACAGATGCC ACCGCC**CCAA GCACCAGTCA GCATTCG** 3'

b

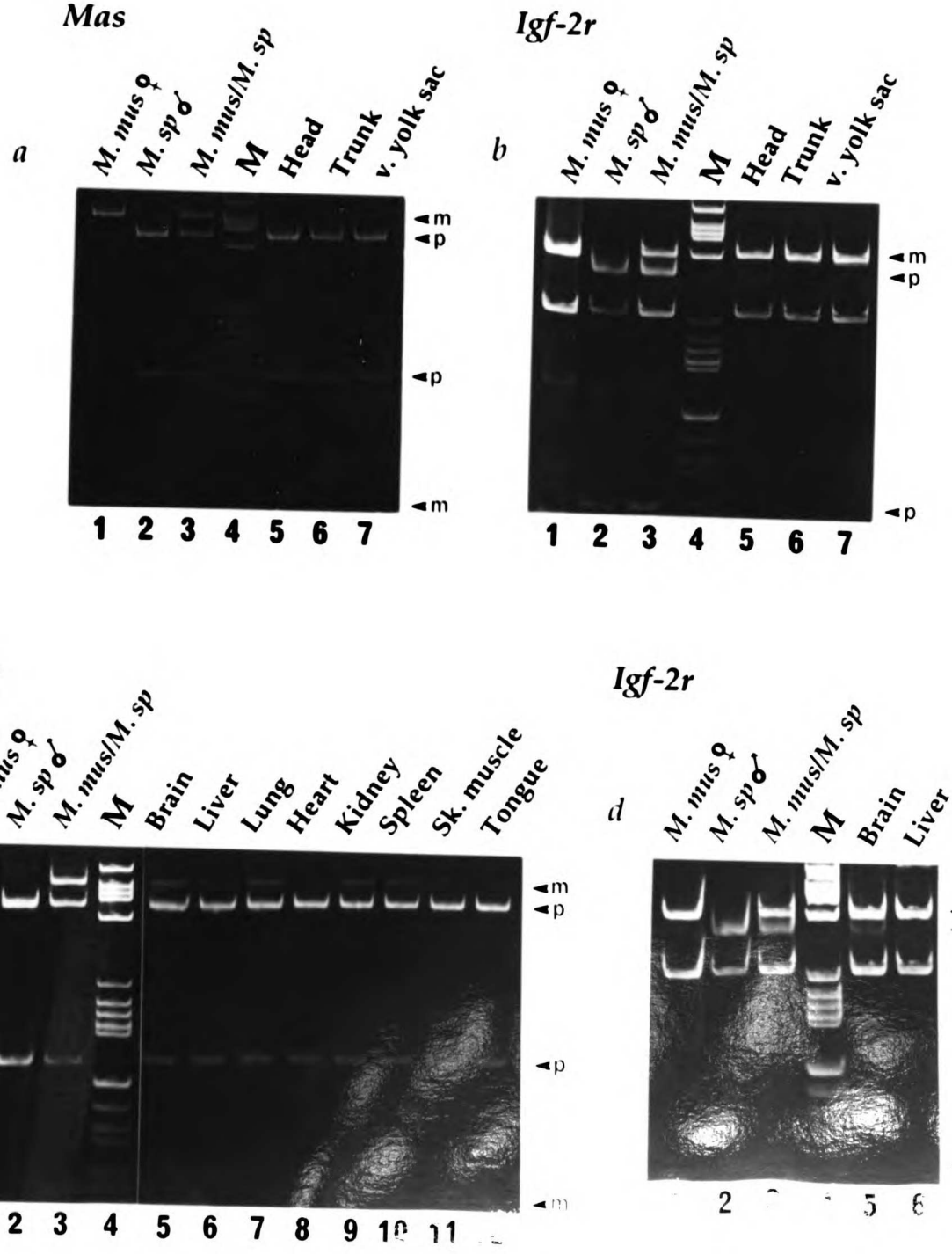


12.5-d.g. fetal F1 hybrids corresponded only to the paternal mRNA phenotype in head, trunk, and visceral yolk sac, indicating that the maternal allele was transcriptionally inactive (Fig. 2*a*). This global pattern of imprinted expression became restricted to specific tissues after 13.5 d.g. At this stage of development the restriction enzyme pattern of the *Mas* PCR products indicated that both the paternal and maternal alleles were expressed in brain, liver, and trunk; however, monoallelic paternal expression was maintained in heart, tongue, and visceral yolk sac. A similar pattern of tissue-specific imprinting was observed in day 1 neonates: brain, liver, lung, kidney, and spleen expressed both parental alleles, whereas heart, skeletal muscle, and tongue expressed only the paternal allele (Fig. 2*c*). The applicability of the RT-PCR approach for assessing allele-specific expression was confirmed by analyzing the maternally expressed imprinted gene *Igf-2r* in 12.5-d.g. fetuses and neonates obtained from the same interspecies crosses. *Igf-2r* primers amplified a 668-bp PCR product, and sequence differences between species produced diagnostic RFLPs that could be used as parental allele-specific markers. A single-basepair difference in the *Igf-2r* cDNA generated a *M. spretus*-specific *ScrF*I site in addition to another *ScrF*I site common to both species. mRNA phenotyping analysis revealed maternal allele-specific expression in all tissues at 12.5 d.g. (Fig. 2*b*) and at birth (Fig. 2*d*), with the exception of head and brain, where expression from both parental alleles was observed. It is interesting that this tissue-specific pattern of relaxation of imprinting observed for *Igf-2r* is identical to that observed for *Igf-2* [5,14], albeit of the opposite parental allele.

Verification of *Mas* imprinting in backcrosses

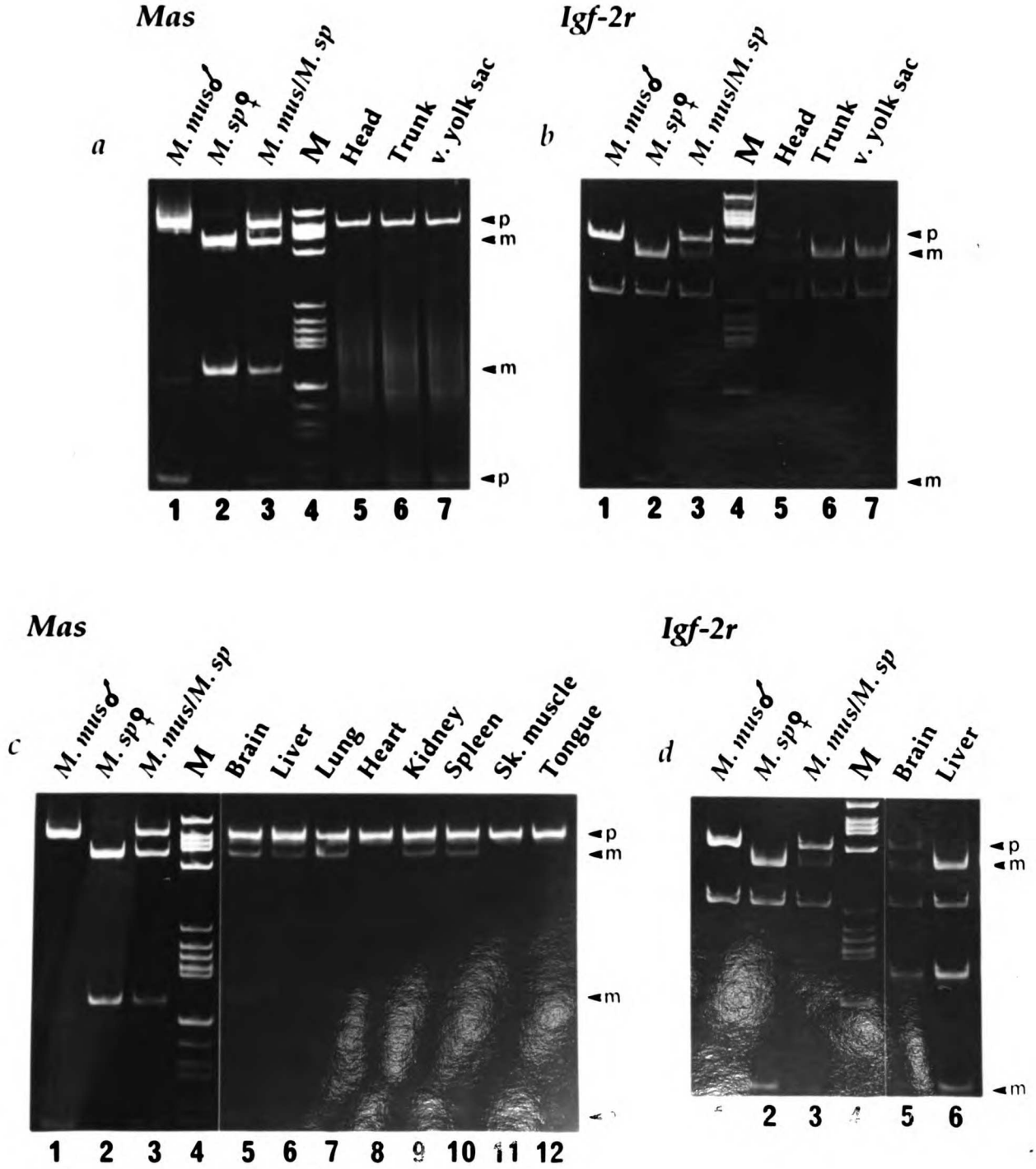
We also analyzed mRNA phenotypes from the backcross progeny between F1 hybrid females and C57BL/6J *(M. musculus)* males to determine whether the

Fig. 3-2 mRNA phenotyping analysis of PCR products from tissue RNA of C57BL/6J *(M. musculus)* females, *M. spretus* males, and their F1 hybrid progeny. Allele-specific *Mas* and *Igf-2r* mRNA from 12.5-d.g. fetuses and day 1 neonates was detected by means of restriction enzyme digestion to generate species-specific RFLPs. *a, Mas* PCR products (from 12.5-d.g. fetuses) digested with *Tsp*509I: lane 1, *M. musculus* brain; lane 2, *M. spretus* brain; lane 3, equal amounts of brain RNA from C57BL/J6 and *M. spretus* ; lane 5, head; lane 6, trunk; lane 7, visceral yolk sac tissues from F1 hybrids. *b, Igf-2r* PCR products (from 12.5-d.g. fetuses) digested with *ScrF*I. Lanes are the same as those described in panel *a. c, Mas* PCR products (from day 1 neonates) digested with *Tsp*509I; lane 1, C57BL/6J brain; lane 2, *M. spretus* brain; lane 3, equal amounts of brain RNA from C57BL/6J and *M. spretus;* lanes 5-12, brain, liver, lung, heart, kidney, spleen, skeletal muscle, and tongue, respectively, from F1 hybrids. *d, Igf-2r* PCR products (from day 1 neonates) digested with *ScrF*I. Lanes are the same as those described in panel *c.* Lanes marked (M) are DNA molecular weight marker V pBR322 DNA-*Hae*III (Boehringer Mannheim, Indianapolis, IN). Arrows indicate paternal- (p) or maternal- (m) specific restriction fragments (in bp).


Mas
Igf-2r
a
b
c
d
M. mus ♀
M. sp ♂
M. mus/M. sp
M
Head
Trunk
v. yolk sac
Brain
Liver
Lung
Heart
Kidney
Spleen
Sk. muscle
Tongue
m
p
1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 9 10 11

paternal allele-specific expression of *Mas* was unique to the F1 hybrids and whether the active *M. spretus* allele (inherited paternally) could be repressed after transmission through the female germline. To determine whether backcross progeny at 11 and 12.5 d.g. had inherited the *M. spretus* allele of *Mas* from the F1 hybrid females, we first analyzed the maternal allele-specific expression of *Igf-2r*. Individual mice exhibiting the *M. spretus*-specific restriction fragments of *Igf-2r* were assumed to have also inherited the *M. spretus* allele of *Mas*, based on the low frequency of recombination observed between these genes during high-resolution mapping [9]. At 13.5 d.g. and at birth, the expression of *Mas* from both parental alleles in several tissues together with the species-specific RFLPs enabled us to identify the heterozygous mice, i.e., those that had inherited the *M. spretus* allele of *Mas* from their mother. Parallel analysis of the allele-specific expression of *Igf-2r* revealed no evidence of recombination in the 16 backcrosses studied. Analysis of the allele-specific expression of *Mas* in backcross progeny verified the globally repressed state of the maternally inherited *(M. spretus)* allele in the head, trunk and visceral yolk sac tissues of 11- and 12.5-d.g. fetuses (Fig. 3*a*). At later stages of development, while the heart, skeletal muscle, and tongue maintained the imprint, all other tissues expressed *Mas* from both parental alleles, as evidenced by the detection of both parental enzyme restriction patterns (Fig. 3*c*). As predicted, digestion of the *Igf-2r* PCR product yielded maternal-specific *(M. spretus)* fragments in all tissues at 12.5 d.g. (Fig. 3*b*) and in day 1 neonates (Fig. 3*d*), indicating exclusive expression from the maternal allele. Again, both maternal and paternal restriction patterns were found in head at 12.5 d.g. and in brain at birth.

Fig. 3-3 mRNA phenotyping analysis of PCR products from tissue RNA of 12.5-d.g. fetus or neonatal backcross progeny derived from the mating of F1 hybrid *(M. musculus* X *M. spretus)* females and C57BL/6J males. Allele-specific *Mas* and *Igf-2r* mRNA from 12.5-d.g. fetuses and day 1 neonates was detected by means of restriction enzyme digestion to generate species-specific RFLPs. *a, Mas* PCR products (from 12.5-d.g. fetuses) digested with *Tsp*509I. *b, Igf-2r* PCR products (from 12.5-d.g. fetuses) digested with *ScrF*I. *c, Mas* PCR products (from day 1 neonates) digested with *Tsp*509I. *d, Igf-2r* PCR products (from day 1 neonates) digested with *ScrF*I. Lanes are the same as those described in Fig. 2. The molecular size markers are identified in the legend to Fig. 2. Arrows indicate paternal- (p) or maternal- (m) specific restriction fragments (in bp).

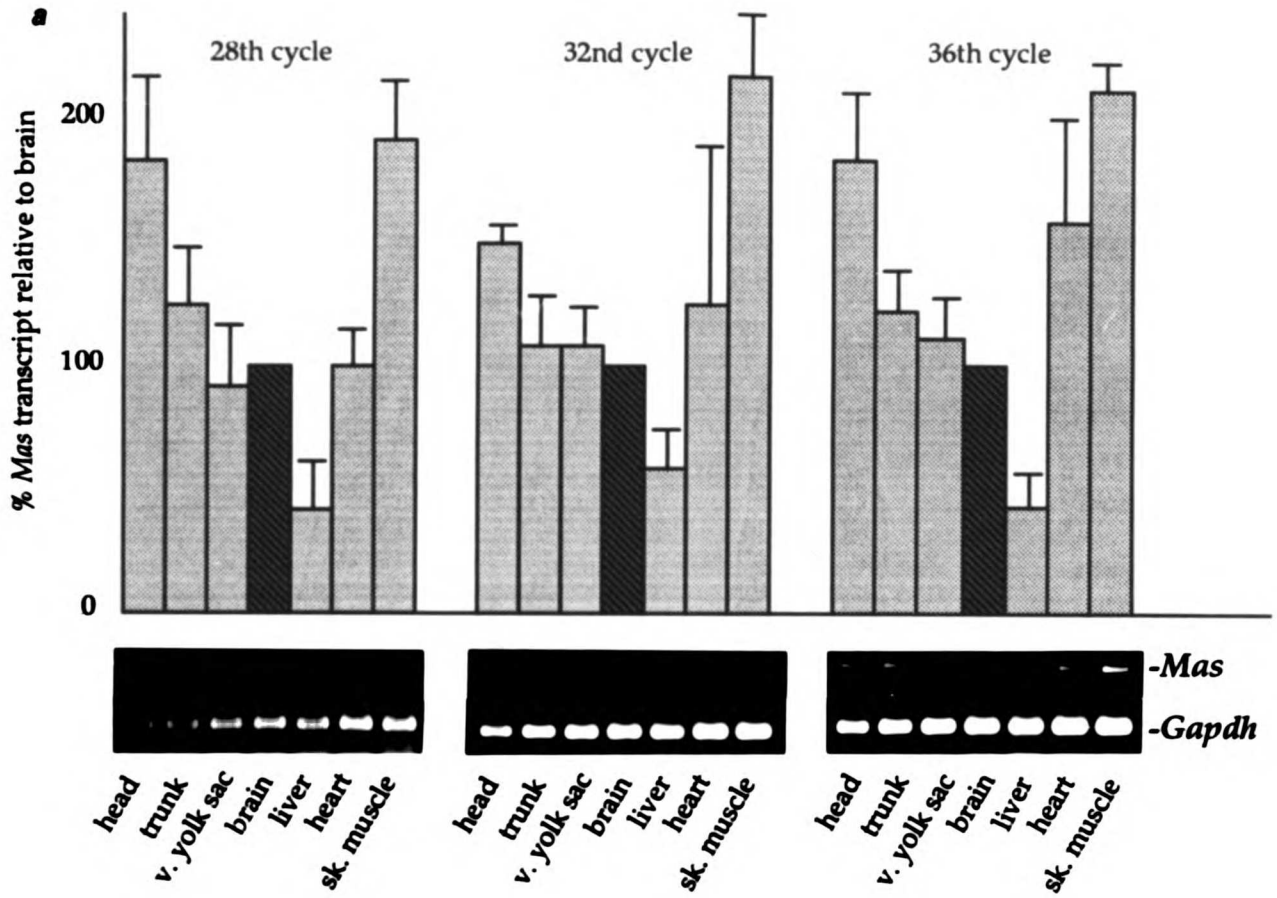

Mas
Igf-2r
a
b
c
d
M. mus♂
M. sp♀
M. mus/M. sp
M
Head
Trunk
v. yolk sac
Brain
Liver
Lung
Heart
Kidney
Spleen
Sk. muscle
Tongue
p
m
1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7 8 9 10 11 12
2 3 4 5 6

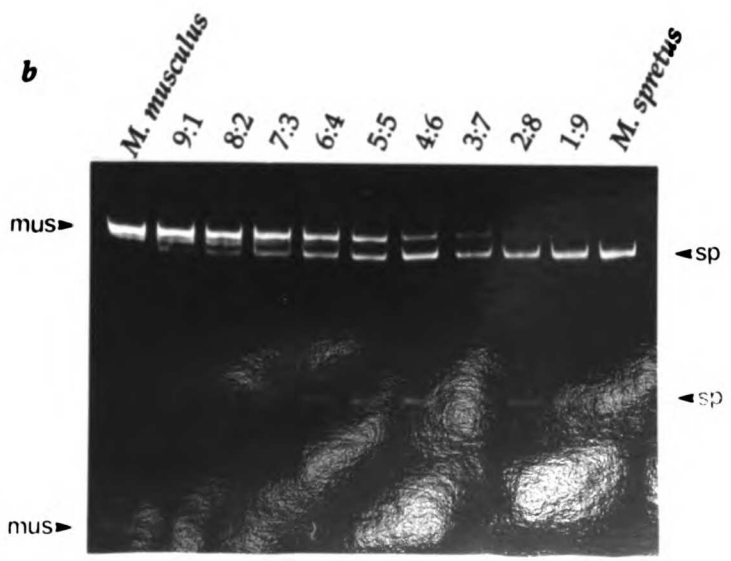
Relative levels of *Mas* transcription

Mas mRNA expression has been observed during development of the rat central nervous system, primarily in the hippocampus, with lower levels of expression in the cortex and thalamus [15]. Because the expression of *Mas* has not been reported in other tissues, we compared the levels of *Mas* expression in several tissues relative to the brain of day 1 neonates by means of a quantitative PCR assay (Fig. 4*a*). We estimated the relative expression of *Mas* in 13.5-d.g. fetus head, trunk, and visceral yolk sac as 170%, 132%, and 111%, respectively, of that in the day 1 neonate brain; and in day 1 neonate liver, heart, and skeletal muscle as 35%, 128%, and 220%, respectively, of that in the day 1 neonate brain (after 36 cycles of amplification).

We observed tissue-specific relaxation of *Mas* imprinting after 13.5 d.g. To determine the relative level of *Mas* expression from the maternal allele, we performed a competitive PCR assay with known amounts of *M. musculus* and *M. spretus* RNAs. The relative percentage of *Mas* transcript from each species was estimated by dividing the densitometric value of the species-specific fragments by the value of the total PCR product (Fig. 4*b*). These values were then compared to the percentage of PCR product represented by the maternal-specific fragments generated from tissue. The relative expression from the *Mas* maternal allele corresponded to 20% *M. musculus* RNA (for the F1 hybrids) and 20% *M. spretus* RNA (for the backcrosses) used in the RT-PCR approach as applied here, suggesting expression from the maternal allele was one fourth that of the paternal allele. Therefore, although mRNA phenotyping detected expression from both parental alleles, the different levels of expression suggest that at least some elements of *Mas* imprinting were retained.

Fig. 3-4 Relative levels of *Mas* expression. *a*, Histogram summarizing pooled data from multiple experiments in which the expression of *Mas* in the head, trunk, and yolk sac at 13.5 d.g. and in the liver, heart, and skeletal muscle of day 1 neonates was compared with that of day 1 neonate brain. The percentage of *Mas* expression in each tissue relative to the expression of *Mas* in brain was determined by dividing the ratio of *Mas* PCR product to *Gapdh* PCR product (internal control) from each tissue by the ratio of *Mas* PCR product to *Gapdh* PCR product from the brain. The data are expressed as the mean ± S.E.M. Total RNA (1, 0.5, and 0.25 μg) from each tissue was reversed transcribed into cDNA. One tenth (for *Mas)* and one thirtieth (for *Gapdh)* of the cDNA was amplified in duplicate reactions (thus, the relative intensity of *Mas* transcript to that of *Gapdh* does not necessarily reflect the true mean ratio of *Mas* to *Gapdh* expression). Samples were removed at the 28th, 32nd, and 36th cycles. *b*, mRNA phenotyping analysis of *Mas* PCR products from brain RNA with different ratios of *M. musculus* to *M. spretus*. Allele-specific expression was detected by means of *Tsp*509I restriction enzyme digestion to generate species-specific RFLPs. Arrows indicate *M. spretus*-specific (sp) and *M. musculus*-specific (mus) restriction enzyme fragments.


a
28th cycle
32nd cycle
36th cycle
% Mas transcript relative to brain
200
100
0
-Mas
-Gapdh
head
trunk
v. yolk sac
brain
liver
heart
sk. muscle

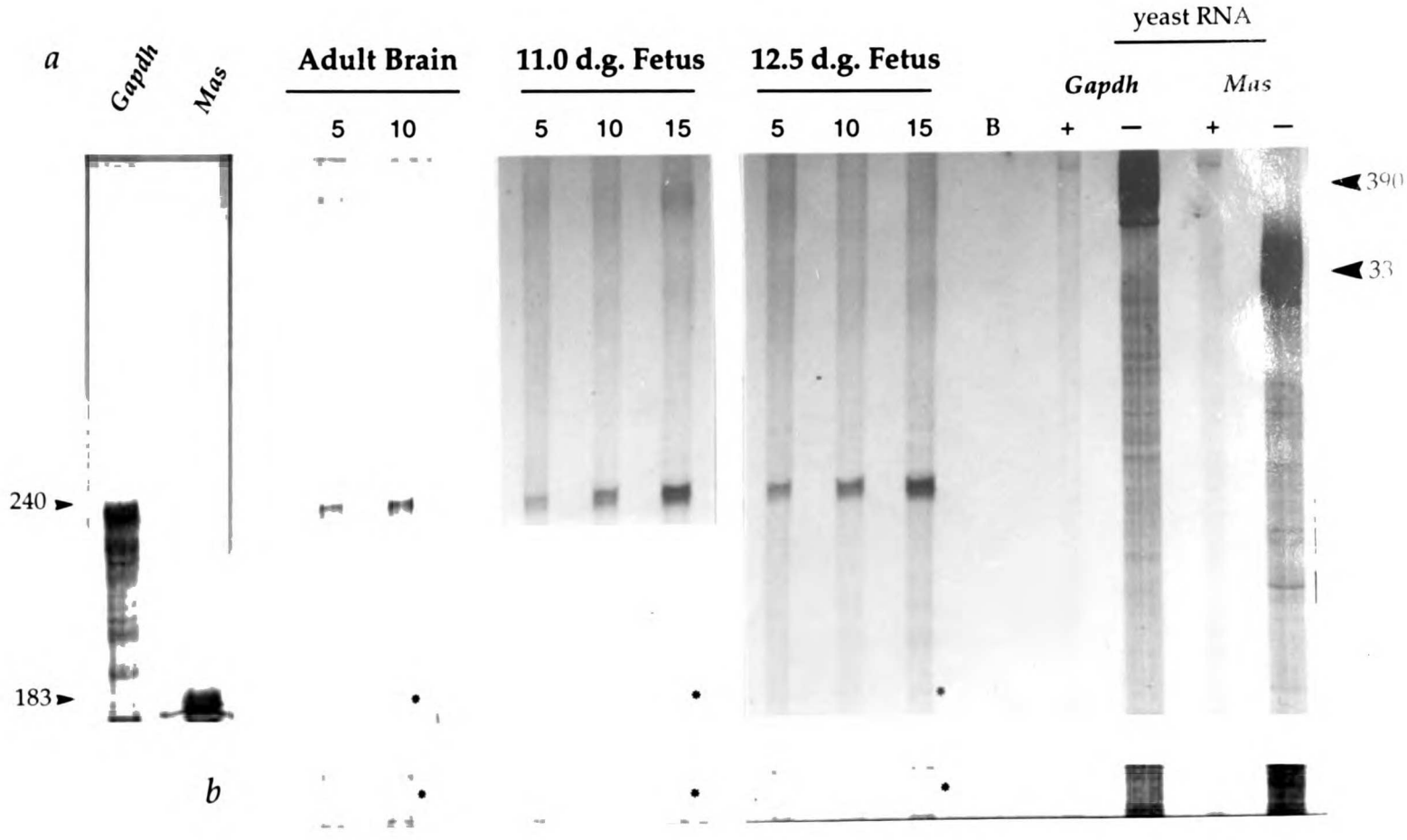

b
M. musculus
9:1
8:2
7:3
6:4
5:5
4:6
3:7
2:8
1:9
M. spretus
mus
sp
sp
mus

To determine the relative levels of *Mas* transcription in the 11.0 and 12.5 d.g. fetuses, we performed RNase protection analyses. Ten micrograms of brain mRNA and 15 μg of fetal mRNA were required in the hybridization reactions to visualize the probe fragment protected by the *Mas* mRNA (Fig. 5 *a* & *b*). We found that the 11.0 and 12.5 d.g. fetal tissues expressed *Mas* as 57 ± 7% and 76 ± 2%, respectively, of that in the adult brain (the data are expressed as the mean ± S.E.M.). The RNase-protected *Gapdh* band served as an internal control and verified the integrity of the mRNA. Thus, the fetus expresses Mas at approximately 2/3 the level observed in the adult brain indicating that transcription is above basal levels. Whether these relatively low levels of *Mas* transcription are functionally relevant in either tissue, however, will be better addressed once antibodies to the Mas protein are available.

Discussion

The importance of identifying imprinted genes has been illustrated not only by their profound effect on mammalian development but by their possible role in the etiology of a number of human diseases, including cancer [16-19]. Our results show that expression of the *Mas* protooncogene in the mouse depends on the parent of origin. mRNA phenotyping analysis revealed global repression of the maternally inherited allele during early fetal development. Tissue-specific relaxation of the imprinted expression was observed at later stages; only in heart, skeletal muscle, tongue, and visceral yolk sac was exclusive paternal allele-specific expression maintained. Although the imprint itself is established sometime during gametogenesis, our data suggest that the differential expression of *Mas* is manifested in a developmental and tissue-specific manner. The *Igf-2* gene and the two mouse insulin genes also exhibit developmental and tissue-specific patterns of imprinting [5,20].

Fig. 3-5 RNase protection assay. *a,* Adult brain mRNA and mRNAs from 11.0 and 12.5 d.g. fetuses were hybridized with ^{35}S-labeled *Mas* and *Gapdh* antisense riboprobes. We used the cloned *Mas* and *Gapdh* (internal control) PCR products as a template for *in vitro* transcription, after linearization with *Hin*dIII. The fragments of the *Mas* probe protected by the mRNA are indicated by asterisks. Yeast RNA was used as a control with (+) and without (-) RNase treatment. *Mas* (183 bp) and *Gapdh* (240 bp) PCR products were also hybridized with their respective antisense probes to verify the size of protected fragments (indicated by small arrows). Large arrows indicate probe size in bp. 5, 10, and 15 indicate micrograms of mRNA. B, blank lane. Denatured samples were separated on a 6% polyacrylamide sequencing gel and subjected to autoradiography for 72 h. *b,* Fragments of the *Mas* probe protected by the mRNA and visualized by autoradiography after 7 days.


a
Gapdh
Mas
240
183
b
Adult Brain
5
10
11.0 d.g. Fetus
5
10
15
12.5 d.g. Fetus
5
10
15
B
yeast RNA
Gapdh
+
—
Mas
+
—
390
33

The protein encoded by *Mas* is structurally similar to a class of receptor peptides that interact with a G-protein to stimulate both DNA synthesis and increase intracellular calcium, a potent second messenger [10,11]. 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 [12,21]. Both *Mas* (this study) and the vascular-type angiotensin II receptor [22] are seen in a wide variety of tissues.

Preliminary studies of known imprinted genes have provided insight into the developmental and tissue-specific regulation of imprinting, as well as into the possible role of methylation [23-26]. Parent-specific methylation patterns have been identified for a number of genes; however, there appears to be little consensus between imprinted expression and the methylation of specific sites. Comparative analysis of the methylation status of *Mas* in different tissues during development may be useful in elucidating the role of methylation in imprinting.

The imprint domain model, based on the imprinted genes *Igf-2* and *H19*, predicts that the differential expression of linked genes is a consequence of a mechanism that biases the competition for common transcription factors according to their maternal or paternal inheritance [27]. The opposite imprinting of *Igf-2r* and *Mas*, located less than 300 kb apart on the proximal portion of mouse chromosome 17, fulfills this requirement of an imprint domain during early fetal development. At later stages, however, the imprinted expression of *Mas* appears to be relaxed in several tissues, whereas the exclusive maternal expression of *Igf-2r* is maintained. These differences in their pattern of imprinting suggest either that *Mas* and *Igf-2r* are not part of the same imprint domain or that the regulation of their differential expression is more complex than that of *H19* and *Igf-2*.

The significance of the exclusive paternal expression of *Mas* in heart and skeletal muscle after 13.5 d.g. is not clear; however, the analogy between

angiotensin-induced cardiac or vascular smooth muscle hypertrophy and oncogene deregulation of proliferation implies a possible correlation between alteration of the *Mas* imprint and cardiovascular disease [28]. It is of interest that the human *MAS* homolog maps to 6q24-q27, a region of the chromosome associated with susceptibility to coronary artery disease [29]. Additional studies will be required to determine whether the allele-specific expression observed in the mouse is also characteristic of the human *MAS* gene.

The relationship between invasive trophoblast and metastatic disease has prompted the speculation that the evolutionary role of imprinting is to protect the mammalian female germline from ovarian tumors [30]. Our demonstration of maternal repression of the *Mas* protooncogene is consistent with this hypothesis. An alternative hypothesis suggests that the evolutionary origin of imprinting was in the conflicting reproductive strategies of male and female mammals by virtue of their disparate bioenergetic roles during development [31]. This model assumes that monogamy is the exception and that multiple paternity within or between litters is common. Consequently, paternal fitness is maximized if the male's progeny can compete for maternal resources at the expense of the progeny of other males. A mitogenic role of *Mas* expressed only by the paternal allele, thus leading to rapid embryonic growth, would be consistent with this hypothesis. Other studies on the extent of imprinting among vertebrate species will be required to distinguish between these hypotheses [32].

Although the *Mas* gene is not associated with a known mouse mutation or human genetic disease, the consequences of genomic imprinting on the development of parthenogenetic and androgenetic embryos imply that imprinted genes play a major role in the differentiation and proliferation of both embryonic and extraembryonic tissues [1,33]. Analysis of *Mas*-transfected cells exhibiting a malignant phenotype reveals that conversion of the normal protooncogene to the

activated oncogene involves the alteration of 5' noncoding sequences associated with its transcriptional regulation [11,34,35]. In fact, tumorigenicity assays demonstrate that overproduction of the normal Mas protein is sufficient to cause a malignant phenotype [11,36]. These observations imply that the oncogenic activity of *Mas* is a result of inappropriate expression of an otherwise tightly controlled gene. We suggest that imprinting may function in the suppression of its transforming potential by restricting expression of *Mas* to one allele. Clearly, the possibility that loss of imprinting plays a role in the mechanism of *Mas* oncogenic activation warrants further investigation.

Methodology

RT-PCR and sequencing. Total RNA was prepared by the GuSCN-CsCl gradient ultracentrifuge technique as described previously [37]. 1 μg of each RNA preparation was reverse transcribed into cDNA using random hexamers as described [38,39], and one tenth of the mixture was amplified by PCR with the use of primer pairs designed for specific detection of *Mas* cDNA target sequences. The primer pair was generated by using PCR Mate (Applied Biosystems, Foster City, CA) and designed to detect the presence of *Mas* mRNA (cDNA) via the predicted 583-bp PCR product. cDNA sequences were amplified by PCR for 40 cycles in a DNA thermal cycler (Perkin Elmer) programmed for a 94°C denaturation, 60°C annealing, and 72°C primer extension step for each cycle. The absence of PCR product from total mRNA that had not been reverse transcribed indicated the absence of contaminating genomic sequences. The identity of PCR products was verified by restriction enzyme analysis (see below). PCR products were prepared and sequenced by the dideoxy-chain termination method [40]. The identity of the PCR products was verified by comparison with GenBank sequences.

Species-specific restriction enzyme digestion of PCR products. To determine their parental origin, we digested the PCR products with the *Tsp*509I restriction enzyme, which has a unique site specific to each species. Restriction fragments were resolved electrophoretically on 6% polyacrylamide gels. The amplification of nonspecific DNA bands for *Mas* and *Igf-2r* was consistent and did not interfere with our analysis.

Mice. Female C57BL/6J *(M. musculus)* mice (Jackson Laboratories, Bar Harbor, ME) were bred to male *M. spretus* mice (Jackson Laboratories). Pregnancy was detected by palpating the females after they were paired for two weeks with a fertile male. Pregnant dams were killed to obtain fetuses at 11 to 13.5 d.g. Head tissue was dissected rostral to the first branchial arch. Neonates were killed by decapitation on day 1. Tissues were pooled from each litter and analyzed together. Total RNA was prepared from head, trunk, and visceral yolk sac (for fetuses) and from brain, liver, lung, heart, kidney, spleen, skeletal muscle, and tongue (for day 1 neonates).

Backcross progeny were obtained by mating female F1 hybrid progeny of C57BL/6J females and *M. spretus* males to C57BL/6J males. Fetuses heterozygous for *Mas* were identified by screening for the presence of the *M. spretus* allele of *Igf-2r*. Neonates heterozygous for *Mas* were identified by expression of the *M. spretus* allele in tissues exhibiting relaxation of the imprint. Tissue RNAs from each fetus and each neonate were isolated and prepared separately.

PCR quantification of *Mas* expression. Relative levels of *Mas* RNA in the head, trunk, and visceral yolk sac at 13.5 d.g. and in the liver, heart, and skeletal muscle of day 1 neonates were estimated by comparing PCR products of *Mas* transcripts in these tissues with that in the day 1 neonate brain. Total RNA (1, 0.5, and 0.25 μg) was copied into cDNA by using reverse transcription as described above. For *Mas*, one tenth of the cDNA was amplified to yield the expected 583-bp product. For *Gapdh*, which served as an internal control, one thirtieth of the cDNA was amplified in a separate tube. Duplicate PCR assays were performed as described above. Samples were removed at the 28th, 32nd, and 36th cycles. Ten microliters of each PCR product was electrophoresed in 1.5% agarose gels

and stained with ethidium bromide. The gels were photographed with Polaroid® type 55 positive-negative film. The negative was scanned by a laser densitometer (Molecular Dynamics, Mountain View, CA), and the band intensities were quantified by Molecular Dynamics ImageQuant™ software. Primers specific for *Gapdh* were designed to amplify a 240-bp PCR product. The *Gapdh* primer sequences are: 5' TGATGACATCAAGAAGGTGGTGAAG 3'and 5' ATGGCCTACATGGCCTCCAAGGA 3' (ref 41). The absence of PCR product from total mRNA that had not been reverse transcribed indicated the absence of contaminating genomic sequences. The identity of PCR products was verified by restriction enzyme analysis.

To determine the relative level of *Mas* transcript from the maternal allele observed in a subset of tissues after 13.5 d.g., we performed RT-PCR by using known ratios of *M. musculus* and *M. spretus* RNA in a total of 1 μg RNA. Densitometric analysis was performed by using ImageQuant™ software to determine the percentage of species-specific fragments relative to the total PCR product. The percentage of PCR product generated by the *M. musculus*-specific fragments (for F1 hybrids) and *M. spretus*-specific fragments (for backcrosses) after mRNA phenotyping was then compared with the values obtained from the control RNA ratios to estimate the relative level of maternal and paternal *Mas* transcripts in each tissue.

RNase protection assay. To determine the relative levels of *Mas* transcription in 11.0 and 12.5 d.g. fetuses we performed RNase protection analyses as follows: Total RNA from C57BL/6J *(M. musculus)* mice was prepared as described above. Poly(A)$^+$ RNA was selected by using the mRNA purification kit from United States Biochemical. The first 183 bp of the *Mas* (583 bp) and the full-length Gapdh (240 bp) PCR products were cloned by using the TA™ Cloning pCR™

vector kit from Invitrogen. The *Mas* primers used to generate the 183-bp product were: 5' ACACTTTCCTAACTGAGCCAC 3' and 5' GAAGAGAAAGCCATGAATACC 3'. The vector was linearized with *Hin*dIII and the RNA probe was synthesized by using [α–^{35}S]ATP (6 μM, 1254 Ci mmol^{-1}) and T7 RNA polymerase (Stratagene). The radiolabeled probes (3 X 10^6 cpm) were annealed to adult brain and fetus mRNA at 45°C for 16 h by using the hybridization solution of the RNase protection kit (Ambion). After RNase degradation, the denatured samples were analyzed on 6% polyacrylamide-8M urea sequencing gels, dried, and subjected to autoradiography for up to 7 days on Hyperfilm-MP (Amersham). To determine the percentage of *Mas*-protected fragments in the fetuses relative to that in the adult brain, we scanned the autoradiograph on a laser densitometer (Molecular Dynamics), and the band intensities were quantified by using ImageQuant™ software.

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Chapter Four

Developmental Regulation of Genomic Imprinting during Gametogenesis

Successful mammalian development requires both the male and female genome, due in part to genomic imprinting, which results in offspring inheriting only one functional copy of a gene either from the mother or father. Evidence suggests that this specialization of the parental genomes is established during gametogenesis when the imprint pattern inherited from the parent is switched to reflect the sex of the progeny. We analysed the allele-specific expression of *Igf-2*, *Igf-2r* and *H19*, in the testes and ovaries of mice derived from an interspecies cross between *Mus musculus* and *Mus spretus* at stages selected to identify the erasure of any pre-existing imprint during meiosis and gametogenesis. While both the neonatal testis and ovary maintained the imprint, in the male, relaxation of imprinting was detected by 7 days after birth and continued during testis development. In the female, relaxation of the *Igf-2* and *Igf-2r* parental imprints was observed in the adult ovary and oocyte. These results 1) indicate that imprinted expression is relaxed during gametogenesis presumably as a consequence of imprint erasure; and 2) predict a subsequent imprinting event whereby the allele-specific expression of *Igf-2*, *Igf-2r*, and *H19* reflects the parent of origin. However, the biallelic expression of *Igf-2* and *Igf-2r* in the hybrid blastocyst indicate that the relaxation of genomic imprinting initiated in the germ line persists during pre-implanation development indicating that the establishment of the imprint and the manifestation of imprinted expression may be temporally distinct events.

Introduction

Although viable spontaneous parthenogenesis is observed in other classes of vertebrates, successful mammalian development requires both the male and female genomes (reviewed by Gold and Pedersen, 1994; Villar and Pedersen, in press). The basis for the developmental failure of isoparental embryos has been attributed to the epigenetic modification or imprinting of specific genes. Despite the accumulation of experimental evidence for genomic imprinting, the primary mechanism of imprinting remains elusive. Although identification of the mechanism of genomic imprinting is important, analysis of the developmental regulation of imprinting is also essential in order to understand the consequences of differential gene expression on cellular and genetic processes involved in early mammalian embryogenesis. 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 (Swain et al., 1987; Surani et al., 1988; Chaillet et al., 1993). Thus, the effect of gametogenesis is to switch the parent-of-origin imprints inherited from the previous generation such that males transmit only the paternal imprint and females transmit only the maternal imprint to the next generation.

Imprint switching has been observed for a number of endogenous genes, as well as several transgenes (reviewed by Reik, 1992; Gold and Pedersen, 1994), three of which have been extensively studied in the mouse and human. *Igf-2*, the first endogenous gene associated with an imprinted phenotype, is preferentially expressed by the paternal allele (DeChiara et al., 1991) during development. However, contrary to evidence that *Igf-2* expression is imprinted as early as the 2-cell stage (Rappolee et al., 1992), a recent study indicates that

pathenogenetic/gynogenetic embryos containing only maternally derived chromosomes express *Igf-2* mRNA at preimplantation stages (Latham et al., 1994). The *H19* gene maps to the distal region of chromosome 7, approximately 90 Kb downstream of *Igf-2;* despite their proximity*H19* is oppositely imprinted, the paternally inherited allele being transcriptionally silent (Bartolomei et al., 1991). While *H19* encodes one of the most abundant mRNAs in the developing embryo, with high levels in endodermal and mesodermal tissues, expression is restricted to skeletal muscle in the adult. The *Igf-2r* gene maps to chromosome 17 within the T-associated maternal effect *(Tme)* deletion. When the *Tme* locus deletion is maternally inherited *Igf-2r* expression is absent indicating that the paternally inherited *Igf-2r* gene is imprinted in such a way as to resemble an inactive gene. (Barlow et al., 1991). Interestingly, the *Tme* locus and *Igf-2r* are not identical suggesting that a similarly imprinted gene is located within this region.

Erasure of imprinting during gametogenesis is presumed to be a prerequiste for the establishment of parent-specific imprints in the gametes when the parental genomes are separated in the gonads of opposite-gendered individuals. Because the mechanism of imprinting is not known, it is impossible to observe the concomitant changes in the epigenetic modifications that presumably alter the transcriptional potentials of the parental genomes. However, by distinguishing between maternal and paternal transcripts, it is possible to observe changes in the parent-specific expression of known imprinted genes.

Using an interspecific hybrid approach we analyzed the allele-specific expression of *Igf-2*, *Igf-2r*, and *H19* to determine whether modification of the inherited imprint is reflected in altered imprinted expression during gametogenesis. The developmental stages selected for analysis were chosen to delimit the onset of meiosis and spermatogenesis in males and of ooctye growth

and maturation in the females. During testes development at 7, 17, and 30 days post partum (dpp), mRNA pheotyping revealed expression from the maternal allele of *Igf-2*, and the paternal alleles of *H19* and *Igf-2r*. Similarly, the *Igf-2* and *Igf-2r* imprints were also relaxed in the adult ovary, whereas *H19* expression was not detected from either parental allele. These results indicate that imprint switching involves an event that permits expression from both parental alleles, presumably as a consequence or prerequisite of the mechanism of imprinting. While the mechanisms of imprinting are not yet known, we provide functional evidence for the "erasure" of imprinting in the male and female germ line. Furthermore, analysis of the blastocyst-stage embryo suggests that the parent-specific imprinting, albeit necessary for allele-specific expression, is not sufficient to selectively repress the maternal and paternal allele of *Igf-2* and *Igf-2r*, respectively, but requires subsequent imprinting events.

MATERIALS AND METHODS

RNA Isolation

Tongue RNA was isolated from day 1 neonate progeny of *M. musculus* (C57BL/6J) and *M. spretus* (Jackson Laboratories, Bar Harbor, ME). Testes RNA was collected from neonatal, 7, 17 30 and 36 day-old F1 hybrids generated by crossing *M. musculus* females and *M. spretus* males. Poly(A)+ mRNA was prepared using FastTrack™ (Invitrogen, San Diego, CA). mRNAs from pooled ovaries, oocytes and blastocystes were collected from F1 hybrids of the same interspecific cross and prepared using the polyATtract system 1000 from Promega (Madison, WI).

RT-PCR

Each RNA preparation was reverse transcribed into cDNA as described by Rappolee et al. (1988 a,b), and one tenth of the mixture was amplified by PCR using primer pairs designed for specific detection of *Igf-2r* (668-bp), *H19* (755-bp) and *Igf-2* (255-bp) cDNA target sequences. Primer pairs generated by PCR Mate (Applied Biosystems, Foster City, CA) are as follows: *Igf-2r:* 5' ATGATGACAGCGACGAAGACC 3' and 5' AAACCTAGGCACTCAGGGACC 3'; *H19:* 5' GAATTCAAACAGGGCAAGATGGGGTCA 3' and 5' GAATTCGGCGCCACATGGTGTTCAAGAAG 3'; *Igf-2:* 5' GGCCCCGGAGAGACTCTGTGC 3' and 5' GCCCACGGGGTATCTGGGGAA 3' (Dull et al., 1984); Each sample was also amplified using *Gapdh* primers to verify mRNA yield and production of cDNA. The *Gapdh* primer sequences are: 5' TGATGACATCAAGAAGGTGGTGAAG 3' and 5' ATGGCCTACATGGCCTCCAAGGA 3' The absence of PCR product from total mRNA that had not been reverse transcribed indicated the absence of contaminating genomic sequences.

Species-Specific Restriction Enzyme Digestion of PCR Products

The PCR products were digested with restriction enzymes that produced RFLPs specific to each species to determine their parental origin. All restriction fragments were resolved electrophoretically on 8% polyacrylamide gels. The amplification of nonspecific DNA bands was consistent and did not interfere with our analysis.

Relative quantification of derepressed allele

To determine the relative level of *Igf-2*, *Igf-2r* and *H19* RNAs transcribed from the derepressed parental allele, RT-PCR was performed with known ratios

of *M. musculus* to *M. spretus* RNAs in a total of 1μg. The digested PCR products were electrophoresed in 8% polyacrylamide gels and stained with ethidium bromide; photographed with Polaroid® type 55 positive-negative film and scanned by a laser densitometer (Molecular Dynamics, Mountain View, CA). The band intensities were quantified by Molecular Dynamics ImageQuant™ software to determine the percentage of species-specific fragments relative to the total PCR product. The percentage of PCR product generated by the *M. musculus*-specific fragments (for *Igf2)* and *M. spretus*-specific fragments (for *Igf2r* and *H19)* was then compared with the species-specific fragment ratios to estimate the relative level of maternal and paternal transcripts in the testis, ovary and oocyte at each stage of development.

RESULTS

Imprinted gene expression during gametogenesis

Although nuclear transplantation studies and transgene methylation patterns predict that the inherited imprints are switched during gametogenesis or shortly after ferilization, the concomitant erasure of imprinted gene expression, presumably a prerequiste for these changes, has not been demonstrated. While mRNA phenotyping permits the detection of allele-specific expression, the analysis is limited to those tissues or cells that express the gene of interest. Therefore, we first determined whether *Igf-2*, *Igf-2r* and *H19* were expressed in the developing testes, the neonate and adult ovary as well as in the oocytes. The *Igf-2* primer pair used to detect the presence of *Igf-2* mRNA (cDNA) amplified a 255-bp PCR product. Primer pairs for *Igf 2r* and *H19* were designed to amplify a 668-bp and a 755-bp PCR product, respectively. The identity of PCR products was verified by sequencing. *Igf-2* and *Igf 2r* expression was detected in the

neonate and adult ovary as well as in the primary oocyte. Similarly, the neonate and developing testes also expressed both *Igf-2* and *Igf-2r* (data not shown). In contrast, *H19* expression was tissue-specific with expression observed at all stages of testes development, but restricted to the neonate ovary in the female (data not shown). Thus, mRNA phenotyping was applicable to analysis of allele-specific expression of *Igf-2*, *Igf-2r*, and *H19* during gametogenesis with the exception of *H19* in the adult ovary and oocyte.

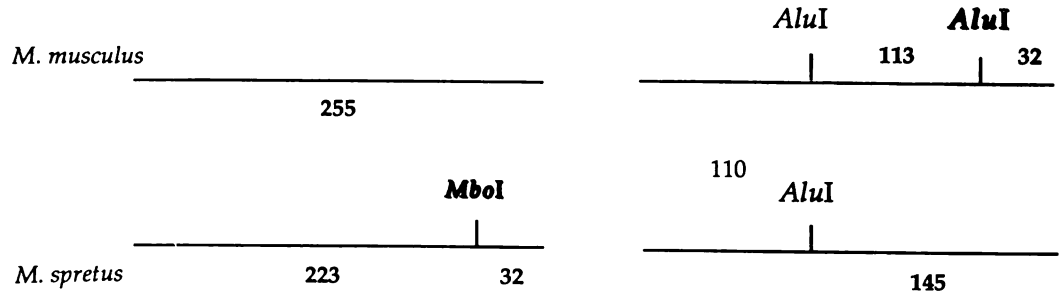
mRNA phenotyping analysis of *Igf-2*, *Igf-2r* and *H19* in the testis

In the mature testis there are a diverse number of cell types, however, the initial temporal appearance of successive cell types during the first waves of spermatogenesis is known. Premeiotic cells, referred to as spermatogonia populate the testis up to 6 days after birth (McLaren, 1984). In the mouse, meiosis begins approximately 7 days post partum (dpp) and requires 11 to 12 days during which the nuclear DNA content in the primary spermatocyte is reduced from a functional 4N level to N level in round spermatids (Goetz et al., 1984). Thus, by examining prepubertal testes it is possible to determine the temporal regulation of allele-specific expression during meiosis and spermatogenesis.

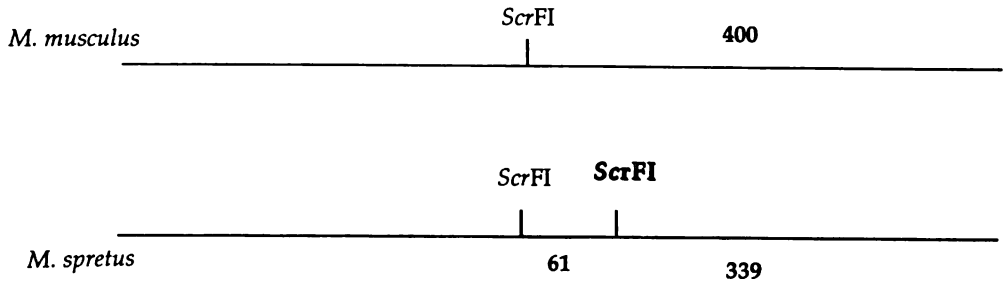
We predicted that the consequence of altered imprinting patterns in the male germ line would be reflected in changes in the expression of imprinted genes, *Igf-2*, *H19* and *Igf-2r*. To identify the parent-of-origin of specific transcripts we used mRNA phenotyping, as previously described(Villar and Pedersen, 1994), to determine the imprinted expression of *Igf-2*, *Igf-2r*, and *H19* genes from hybrid progeny derived by an interspecific cross between *M. musculus* and *M. spretus*. Sequence differences between these two species generated diagnostic

Figure 4-1. Restriction map of PCR products: A, *Igf-2* digested with *Alu*I and *Mbo*I; B, *Igf-2r* digested with *Scr*FI; C, *H19* digested with *Bsr*I. Unique enzyme restriction fragments are in bold outline.

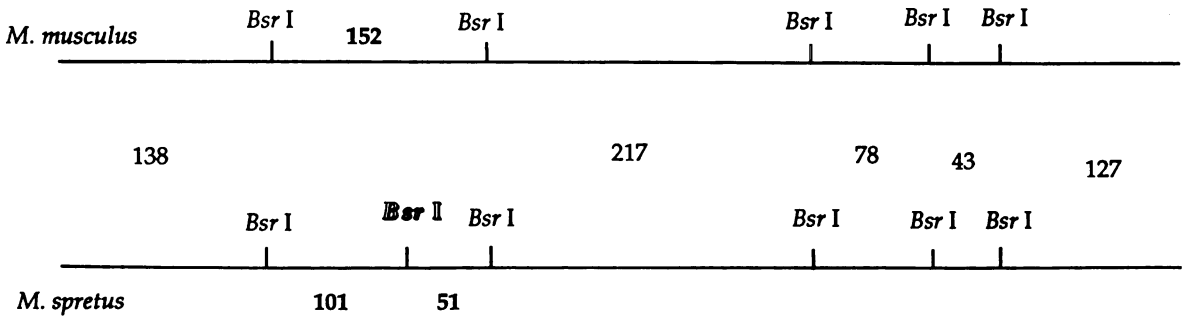
A *Igf-2* PCR product (255 bp)
(nucleotides 119-374 in cDNA)



B *Igf-2r* PCR product (668 bp)
(nucleotides 79-747 in cDNA)



C *H19* PCR product (755 bp)
(nucleotides 279-1034 in cDNA)



restriction fragment length polymorphisms (RFLPs) that were used as parental allele-specific markers. Single-basepair differences generated a *M. spretus*-specific *Mbo*I site and *M musculus*-specific *Alu*I site in *Igf-2*, a *M. spretus*-specific *ScrF*I site in *Igf-2r* and a *M. spretus*-specific *Bsr*I site in *H19*, in addition to other restriction sites common to both species. We controlled for the possibility of genomic contamination by running concurrent PCR reactions of RNA samples that had not been reversed transcribed. These controls invariably gave negative results (data not shown). In addition, the primers were designed to flank an intron, so that any contaminating DNA would yield a unique larger PCR product. The *Igf-2* PCR product was digested with *Mbo*I to produce restriction fragments unique to *M. musculus* (255-bp) and *M. spretus* (223-bp and 32-bp); or with *Alu*I to produce *M. musculus*-specific (110-bp,113-bp, and 32-bp) and *M. spretus*-specific (110-bp and 145-bp) fragments (Fig. 4-1A). The *Igf-2r* PCR product was digested with *ScrF*I to produce restriction fragments corresponding to the *M. musculus* (400-bp and 264-bp) and *M. spretus* (339-bp, 264-bp and 61-bp) (Fig. 4-1B). RFLP analysis of the *H19* PCR product yielded *M. musculus* (217-bp, 152-bp, 138-bp, 127-bp, 78-bp, and 43-bp) and *M. spretus* (217-bp, 138-bp, 127-bp, 101-bp, 78-bp, 51-bp and 43-bp) fragments after *Bsr*I digestion (Fig. 4-1C).

In the neonatal premeiotic testes, the mRNA phenotypes of *Igf-2r* and *H19* corresponded to the *M. musculus*, i.e., the maternal allele, whereas the restriction fragments of *Igf-2* resembled the mRNA phenotype of the *M. spretus*, i.e., the paternal allele (Fig. 4-2). Therefore, at this stage of development, the monoallelic expression of *Igf-2*, *Igf-2r* and *H19* in testes, containing only mitotic germ cells, resembled the imprinted pattern of expression of these genes observed in somatic tissues. At 7 dpp, when the first wave of spermatogonia enter meiosis, expression from the somatically silent alleles of *Igf-2*, *Igf-2r* and *H19* was detected. As the testes matured, 17 dpp mice provided a means to examine the

allele-specific expression of *Igf-2*, *Igf-2r* and *H19* in a population of cells undergoing events up to and including meiosis. At this stage of development, we observed an increase in the relative level of expression from the paternal allele of *Igf-2r* and *H19*, and the maternal allele of *Igf-2*. By 30 dpp, when the haploid round spermatid differentiates to the mature spermatozoon, the first phase of spermiogenesis has almost been completed with the germ cell compontent of the testis greater than 80% (Norris et al., 1994). In the mature testis, the level of expression from the somatically repressed alleles of *Igf-2*, *Igf-2r*, and *H19* continued to increase relative to the other parental allele, presumably because of the subsequent waves of germ cells passing through spermatogenesis (Fig. 4-2). While it is possible that erasure of the imprints observed in the testes is due to the somatic component rather than the germ cells, it is not likely given the general maintanence of imprinting in other somatic tissues (Villar and Pedersen, 1994a, 1994b), as well as the premeiotic testes. To determine the relative expression of the parental alleles during spermatogenesis, we performed RT-PCR by using known ratios of *M. musculus* and *M. spretus* RNA in a total of 1 μg RNA (Fig. 4-3). Densitometric analysis was performed by using ImageQuant™ software to determine the percentage of species-specific fragments relative to the total PCR product. The level of maternal expression relative to paternal expression for *Igf-2;* and the level of paternal expression relative to maternal expresson for *Igf-2r* and *H19* are summarized in table I. In each case, expression from the somatically silent allele ranged from 10-40% of the total PCR product or approximately 1/9 to 2/3 that of the expression from opposite parental allele.

mRNA phenotyping analysis of *Igf-2* and *Igf-2r* in the ovary

In the mouse, oogenesis begins with the formation of the primordial germ cells in the 8-day-old embryo (McLaren, 1984). By 14 days post fertilization,

Figure 4-2. mRNA phenotyping analysis of *Igf-2, H19* and *Igf-2r* PCR products from tongue RNA of *M. musculus, M. spretus* , and testes mRNA of F1 hybrid progeny derived from an interspecies cross between *M. musculus* females and *M. spretus* males. Allele-specific *Igf-2, H19* and *Igf-2r* mRNA from 1, 7, 17 and 30 day-old mice was detected by means of restriction enzyme digestion to generate species-specific RFLPs. A, *Igf-2* PCR products digested with *Mbo* I; B, *Igf-2r* PCR products digested with *Scr*FI; C, *H19* PCR products digested with *Bsr*I: tongue from *M. musculus* (lane 1) and *M. spretus* (lane 2); control in which equal amounts (0.5 μg) of tongue RNA from *M. musculus* and *M. spretus* were used in the PCR (lane 3) 1 day (lane 5); 7 day (lane 6); 17 day; (lane 7); 30 day (lane 8) from F1 hybrids (*M. musculus* females X *M. spretus* males) The molecular size marker (lane 4) is DNA molecular weight marker V pBR322 DNA-*Hae* III (Boehringer Mannheim, Indianapolis, IN). Arrows indicate enzyme restriction fragments of derepressed allele (bp).

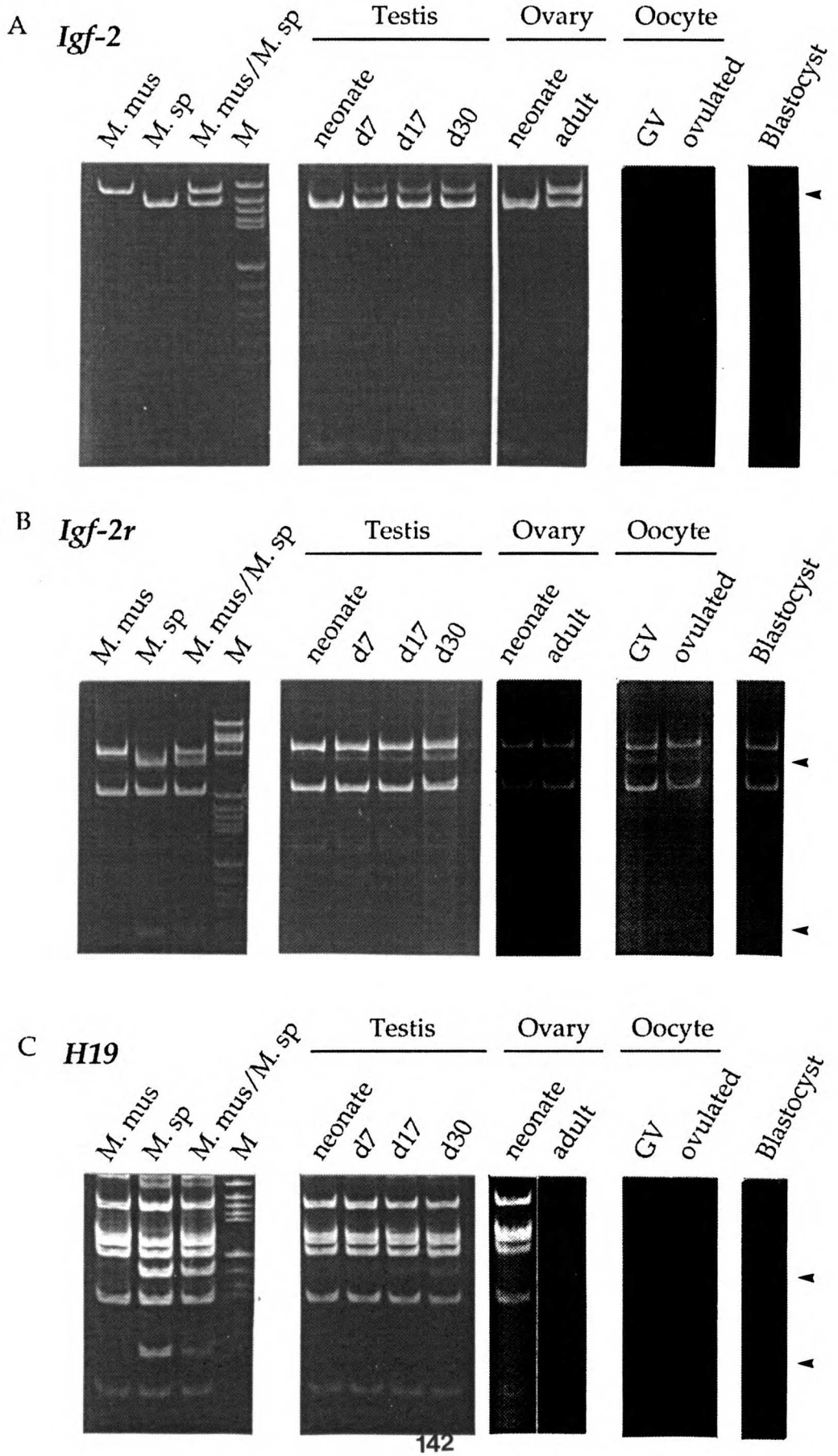

A
Igf-2
Testis
Ovary
Oocyte
M. mus
M. sp
M. mus/M. sp
M
neonate
d7
d17
d30
neonate
adult
GV
ovulated
Blastocyst
B
Igf-2r
Testis
Ovary
Oocyte
M. mus
M. sp
M. mus/M. sp
M
neonate
d7
d17
d30
neonate
adult
GV
ovulated
Blastocyst
C
H19
Testis
Ovary
Oocyte
M. mus
M. sp
M. mus/M. sp
M
neonate
d7
d17
d30
neonate
adult
GV
ovulated
Blastocyst

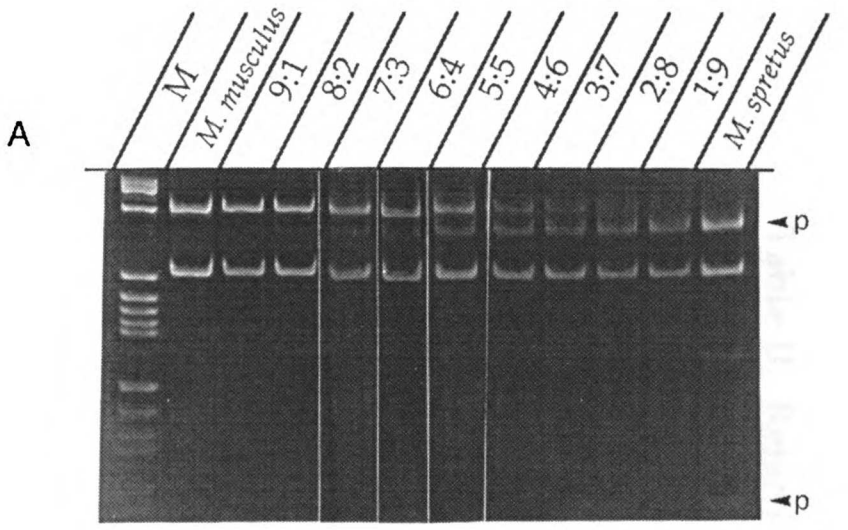
Table I. Relative expression of the parental alleles during gametogenesis

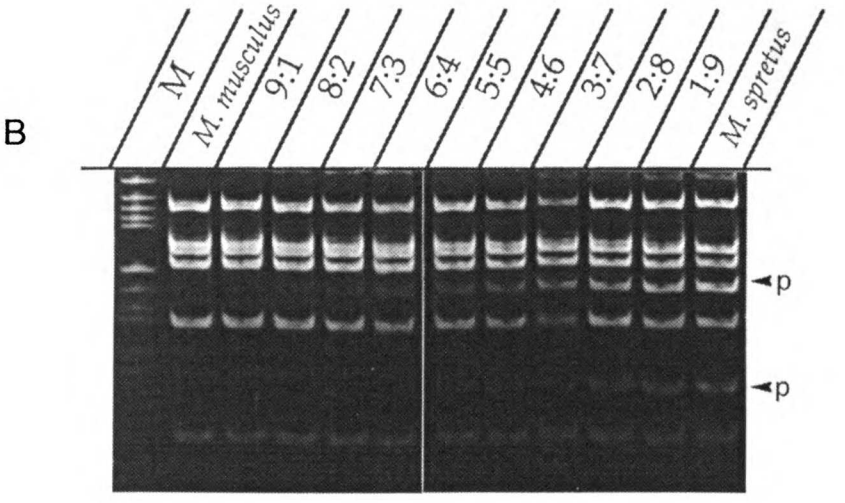
	Neonate Testis		d.7		d.17		d.30	
	M	P	M	P	M	P	M	P
Igf-2	–	+++	(+)	+++	+	+++	++	+++
Igf-2r	+++	–	+++	(+)	+++	+	+++	++
H19	+++	–	+++	(+)	+++	+	+++	++

Maternal allele expression (M)
Paternal allele expression (P)

some of the primordial germ cells have migrated from the allantois and colonized the genital ridge of the presumptive gonad. The oogonia undergo a last round of DNA synthesis prior to entering meiotic prophase at 14 days post fertilization. The primary oocytes enter the dictyate stage at 5 dpp and remain arrested in the first meiotic prophase until just prior to ovulation. In the neonate ovary, the mRNA phenotypes of *Igf-2r* corresponded to the *M. musculus* maternal allele, whereas the restriction fragments of *Igf-2* corresponded to the *M. spretus* paternal allele (Fig 4-2). Therefore, at this stage of development, we observed maintenance of imprinting in the neonate ovary, similar to somatic tissues. In contrast, both parental alleles of *Igf-2* and *Igf-2r* were expressed in the adult ovary. To determine whether the relaxation of imprinting was due to the germ cell component rather than the somatic component, we examined the allele-specific expression of *Igf-2* and *Igf-2r* in germinal vesicle oocytes isolated from adult ovaries and ovulated oocytes. mRNA phenotyping revealed expression from both parental alleles of *Igf-2* and *Igf-2r* demostrating relaxation of their opposite imprints. Because the female germ cells enter meiosis during gestation and arrest in prophase of meiosis shortly after birth, it is possible that the erasure of the parental imprint has occurred prior to this stage of gametogenesis, as was observed in the testes, perhaps between the onset of meiosis and completion of oocyte growth. To determine the relative level of transcription from the somatically silent allele in the ovary and oocytes, we compared the level of maternal expression relative to paternal expression for *Igf-2;* and the level of paternal expression relative to maternal expresson for *Igf-2r.* Densitometric analysis was performed by using ImageQuant™ software to determine the percentage of species-specific fragments relative to the total PCR product (Fig. 4-3). These results are summarized in table II. In the adult ovary, expression from

Figure 4-3. mRNA phenotyping analysis of *Igf-2r*, *H19*, and *Igf-2* PCR products from tissue RNA with decreasing ratios of *M. musculus* to *M. spretus*. mRNA phenotyping analysis of *Igf-2r*, *H19*, and *Igf-2* PCR. These control amplifications were compared to the experimental RFLPs to estimate the amount of RNA generated by each parent. Allele-specific expression was detected by means of restriction enzyme digestion to generate species-specific RFLPs: (A) *Igf-2r* digested with *Scr*FI; (B) *H19* digested with *Bsr*I; (C) *Igf-2* digested with *Alu*I. The relative quantitation of expression from the maternal allele of *Igf-2* (indicated by arrows) and paternal allele of *H19* and *Igf-2r* (indicated by arrows) was estimated by determining the amount of species-specific restriction fragments as a percentage of the total PCR product. Densitometric analysis was performed by using ImageQuant™ software to determine the percentage of species-specific fragments relative to the total PCR product. The molecular size markers are identified in the legend to Fig. 4-2. Restriction enzyme fragments in bp.


A
M
M. musculus
9:1
8:2
7:3
6:4
5:5
4:6
3:7
2:8
1:9
M. spretus
p
p


B
M
M. musculus
9:1
8:2
7:3
6:4
5:5
4:6
3:7
2:8
1:9
M. spretus
p
p

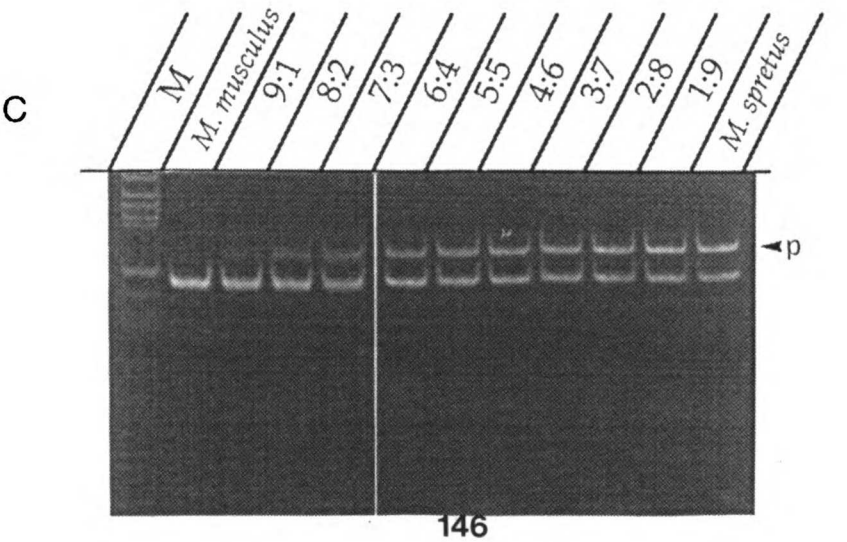

C
M
M. musculus
9:1
8:2
7:3
6:4
5:5
4:6
3:7
2:8
1:9
M. spretus
p

Table II. Relative expression of the parental alleles during gametogenesis

	Neonate ovary		Adult ovary		GV oocyte		Ovulated oocyte	
	M	P	M	P	M	P	M	P
Igf-2	–	+++	++	+++	+++	+++	+++	+++
Igf-2r	+++	–	+++	++	+++	+++	+++	+++
H19	+++	–	—	—	—	—	—	—

Maternal allele expression (M)
Paternal allele expression (P)

the somatically silent allele was 40 % of the total PCR product or approximately 2/3 that of the expression from opposite parental allele. Both the germinal vesicle and ovulated oocytes expressed the parental alleles equally.

Biallelic expression of *Igf-2* and *Igf-2r* in the blastocyst

The developmental potential of the male and female gamete is limited due to epigenetic modifications acquired during transmission through the germline. To determine whether the acquisition of the physical imprint during gametogenesis results in the selective inactivation of the maternal or paternal allele, I analysed the allele-specific expression of *Igf-2*, *Igf-2r* and *H19* in the blastocyst derived from the same interspecies cross as desribed above. The mRNA phenotyping approach was applicable to *Igf-2* and *Igf-2r*, but not to *H19*, as it was not expressed at this developmental stage. The *Igf-2* PCR product generated from the mRNA of hybrid blastocysts was digested with either*Alu*I (data not shown) and *Mbo*I to produce restriction fragments corresponding to the *M. musculus* and *M. spretus* cleavage products (Fig. 4-2). RFLP analysis of the *Igf-2r* PCR product from these same mRNAs after *Scr*FI digestion also yielded both *M. musculus* fragments. Both parental alleles of *Igf-2* and *Igf-2r* were expressed equally suggesting that the aquisition of the parental imprints during gametogenesis does not result in allele-specific inactivation at this stage of preimplantation.

DISCUSSION

The relative importance of the male and female gamete for subsequent development and the nature of the interaction between parental genomes, remain basic questions in developmental biology. Despite the fact that the egg and sperm each carry a complete set of genetic blueprints, the uniparental

inheritance of whole or portions of particular autosomal chromosomes results in abnormal development (Searle and Beechey, 1979; Cattanach and Kirk 1985; Beechey and Searle, 1987; Gold and Pedersen, 1994; Villar and Pedersen, in press). This phenomenon has been attributed to 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. To investigate the developmental regulation of germ line imprinting in the male and female, we analysed the allele-specific expression of *Igf-2, Igf-2r, and H19* using mRNA phenotyping, an approach applicable to the developmental and tissue-specific analysis of imprinted genes. *Igf-2* and *H19* map approximately 90 kb apart on the distal region of chromosome 7 (Zemel et al., 1992), whereas *Igf-2r* maps to chromosome 17. Transmission of *Igf-2* through the female germ line results in its repression while *Igf-2r* and *H19* gene expression are silenced when transmitted through the male germ line.

mRNA phenotyping analysis of neonatal testis and ovary RNAs confirmed the exclusive maternal expression of *Igf-2r* and *H19* and exclusive paternal expression of *Igf-2*, similar to somatic tissues. In the prepubertal male, examination of 7 dpp testes mRNA revealed that expression from both parental alleles correlated with the onset of meiosis and continued during testis maturation. In the adult ovary and oocyte, *Igf-2* and *Igf-2r* were also expressed by both parental alleles. However, because female germ cells enter meiosis during gestation and arrest in prophase of meiosis, it is not clear whether the relaxation of the parental imprint occured prior to or during the maturation of the germinal vesicle stage. Assuming that the germ cells of the fetal gonad actively transcribe *Igf-2* and *Igf-2r*, further studies will reveal whether the imprint is erased during the onset of meiosis in the female germ line.

Although the mechanism of imprinting is unknown, it most likely involves the differential protection and/or enhancement of hypersensitive sites that affect promoter function. The differential chromatin condensation of the maternal and paternal pronuclei observed in mouse zygotes may be a reflection of such epigenetic modifications (Ciemerych and Czolowska, 1993).

Several lines of evidence have strongly implicated DNA methylation in regulating expression of specific genes, of chromosome domains, and of whole chromosomes (Doerfler, 1983; Naveh-Many and Cedar, 1981; Monk, 1986). Global changes in DNA methylation has been observed in developing germ cells in the mouse (Kafri et al., 1992). Interestingly, our data demonstrating the temporal regulation of relaxation or "erasure" of imprinted expression during male gametogenesis correlates with the decrease in the level of global methylation observed from meiotic cells to elongated spermatids (del Mazo et al., 1994).

The study of mouse imprinted transgenes has provided additional information regarding the parent of origin effects including developmental changes in methylation (reviewed by Gold and Pedersen, 1994). In general, as the transgene passes from one generation to another its methylation pattern is reversed during successive generations depending on the parent-of-origin, with paternal inheritance correlating with undermethlyation and expression and maternal inheritance correlating with methylation and transgene repression (Chaillet, 1994). Although this would seem to contradict the observation that in unique and some repetitive sequences, sperm DNA is more methylated than oocyte DNA (Kafri et al., 1992; Monk, et al., 1987; Sanford et al., 1984), 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. For example, the differentially expressed RSV-Ig-

myc transgene is highly methylated when inherited from the female and unmethylated when paternally inherited (Chaillet et al., 1991). Subsequently, the methylation patterns are erased in the primary germ cells and established during gametogenesis to reflect the parent of origin. On the other hand, examination of the mouse *Xist* gene, expresssed from the inactive X-chromosome, reveals that the temporal regulation of demethylation of the paternal allele in the germ line correlates with the onset of meiosis (Norris et al., 1994).

With the identification of parent-specific methylation patterns evidence is also accumulating for the role of methylation in differential gene expression in germ cells (Ariel et al., 1994), however, the function of such differential methylation is not understood. An upstream site within the promoter region of *Igf-2* is differentially methylated in sperm and maintained throughout embryogenesis, a prerequisite for a role in the mechanism of imprinting (Brandeis et al., 1993; Sasaki et al., 1993). The differential methylation pattern of *H19* observed in the neonate and adult are also observed in the sperm (Brandeis et al., 1993), however, it remains to be determined which sites maintain the imprint during the genome-wide demethylation that occurs during preimplantation development (Kafri et al., 1992). In the case of *Igf-2r*, there is a methylation site in the body of the gene inherited from the female gamete, whereas, the promoter region of the inactive paternal allele is methylated in sperm (Stoger et al., 1993). Although it is not clear whether the imprint responsible for the differential expression of the parental alleles functions to permit or to suppress gene expression, our data indicate that the relaxation of imprinting corelates with the onset of meiosis in the male. Comparing the methylation status in the testis before the beginning of the stage at which biparental expression of *Igf-2*, *Igf-2r*, and *H19* is initiated with subsequent stages

of spermatogenesis may be useful in elucidating the role of methylation in imprinting.

Although the reason for the evolutionary genesis of genomic imprinting remains speculative, the functionally hemizygous state of a select number of genes appears not only normal but is required for proper mammalian development as evidenced by human ovarian teratomas and gestational trophoblastic neoplasia (Bagshawe and Lawler, 1982; Ariel et al., 1994). Porter and Gilks (1992) have proposed that the behavioral differences of testicular and ovarian germ cell tumors are due to differences in imprinting, with the male pattern promoting growth and the female pattern controlling growth. This hypothesis not only presumes that imprinted genes are expressed by the germ cells, it also predicts a relationship between germ cell tumorogenesis and alterations in the pattern of imprinting. The fact that the testis and ovary can relax their respective patterns of imprinting without any apparent consequence, raises the question of how the germ cells undergoing imprint switching avoid potential oncogenic activity. Of interest is the possibility that germ cells possess a mechanism the precludes tumorigenesis, the failure of which results in abnormal growth of germ cell origin. The allele-specific expression patterns of *Igf-2* and *H19* are not observed in testicular and ovarian germ cell tumours (Porter and Gilks, 1992). Complete hydatidiform moles containing chromosomes of paternal origin co-express *Igf-2* and *H19* in the same androgenetic cells, whereas ovarian teratomas containing chromosomes of maternal origin lack both *Igf-2* and *H19* expression (Mutter et al., 1993). Our results suggest that the failure to manifest the normal imprint pattern in the hydatidiform mole may be a consequence of the relaxation of imprinting in the maturing testis. However, a different mechanism must be invoked to explain the repression of *Igf-2* and *H19* in the teratomas. Although loss of *H19* expression could be due to the absence of

tissues that produce this RNA in the mature teratoma, this explanation does not account for the lack of *Igf-2* expression. In fact, the inactivation of *H19* has been demonstrated to result in the loss of *Igf-2* imprinting, as seen in Wilms' tumor (Steenman et al., 1994). Bartolemei et al., (1992) have suggested that the opposite imprinting of *Igf-2* and *H19* is a consequence of a competitive *cis* interaction between these genes for common transcription factors. If these factors are contributed exclusively by the paternal genome, we propose that the deregulation of the *Igf-2* and *H19* imprints in the teratomas may be due in part to the absence of transcription factors that are themselves imprinted. Therefore, it is possible that biparental interactions are required not only for the expression of *Igf-2* and *H19* but also for proper mainfestation of the *Igf-2* and *H19* imprints.

In summary, my results indicate that imprint switching involves an event that permits expression from both parental alleles presumably as a consequence or prerequisite of the mechanism of imprinting, a phenomenon that persists in the blastocyst-stage embryo. Further analysis of the developmental stage(s) involved in the "erasure" and acquisition of the parent-specific imprint in the testis and ovary may provide an approach for identifying the mechanism of imprinting and lead to diagnostic or therapeutic interventions for human ovarian teratomas and gestational trophoblastic neoplasia.

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