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INSIGHTS FROM MODEL SYSTEMS
Genomic Imprinting: A Chromatin Connection

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Imprinting represents the difference in function of homologous chromosomes, according to their parental origin. In this review, we consider the epigenetic features that distinguish parental homologues at imprinted chromosome domains, as discerned from a variety of studies, many of which depend on the use of the favored model system, the mouse.

Imprinting and Its Relevance to Human Disease

Genomic imprinting is a mechanism by which one copy of a gene is preferentially silenced according to parental origin (Barlow 1995). It is considered to be epigenetic, since two otherwise identical alleles are regulated differently in the same nucleus and because it is entirely reversible, the silent allele becoming reactivated when passed through the germ line of the opposite parental sex and the nonsilent allele becoming deactivated. The consequences of such monoallelic expression are most graphically illustrated in the mouse, in the developmental failure of embryos containing only the maternal or paternal nuclear genomes (Fundele et al. 1997) and in the phenotypes associated with uniparental inheritance of specific chromosome segments (Cattanach and Beechey 1997). Uniparental disomy (UPD) in the mouse causes phenotypes ranging from early gestational lethalities to effects on fetal growth, neonatal behavior, and viability. Imprinting effects are also encountered in UPD for several human autosomes (Ledbetter and Engel 1995).

Imprinting is now recognized or suspected to be an important factor in a considerable number of human inherited diseases and tumors (Sapienza and Hall 1995; Squire and Weksberg 1996). Prader-Willi syndrome (PWS; MIM 176270) and Angelman syndrome (AS; MIM 105830), neurological disorders mapping to chromosome 15q11-q13, provide perhaps the best paradigm for the involvement of imprinting in disease. PWS most frequently results from the absence of a paternal copy of the 15q11-q13 interval, either by deletions involving the paternal chromosome or by maternal UPD, whereas AS is caused by the lack of a maternal contribution (Nicholls 1994). Several genes (*SNRPN, PAR1, PAR5,* and *IPW*) that are active specifically on the paternal chromosome—and thus are potential PWS candidate genes—have been detected at 15q11-q13. A candidate AS gene (*UBE3A*) has been identified recently, and its mouse homologue has been found to be paternally repressed in specific regions of the brain (Albrecht et al. 1997). In addition to altered functional dosage arising from gross changes in chromosome balance, AS and PWS can be caused by deregulation of imprinted genes, owing to mutations at a so-called imprinting center (Buiting et al. 1995). Such mutations result in the failure to correctly set imprints in the germ line, leading to inheritance of an inappropriate epigenotype across the entire interval.

An epigenotype could also become altered by an epigenetic mistake occurring in the germ line or in somatic tissues. The functional haploid state of imprinted genes causes them to be uniquely sensitive to such effects, as has become particularly apparent from the impact of altered imprinting in tumorigenesis. For example, loss of imprinting—that is, biallelic expression—of the imprinted growth-promoting gene encoding insulin-like growth factor–II (*IGF2*) has been detected as a frequent occurrence in a wide variety of tumors (Squire and Weksberg 1996). In some cases, loss of imprinting has been shown to be associated with an epigenetic lesion at the closely linked *H19* imprinted gene, which participates in *IGF2* gene regulation: methylation of the *H19* promoter leads to its repression and to consequent activation of the normally silent maternal copy of *IGF2* (Squire and Weksberg 1996). Therefore, it is important to understand the mechanisms governing the epigenetic properties at imprinted and other loci and how these mechanisms can be disrupted in pathological states.

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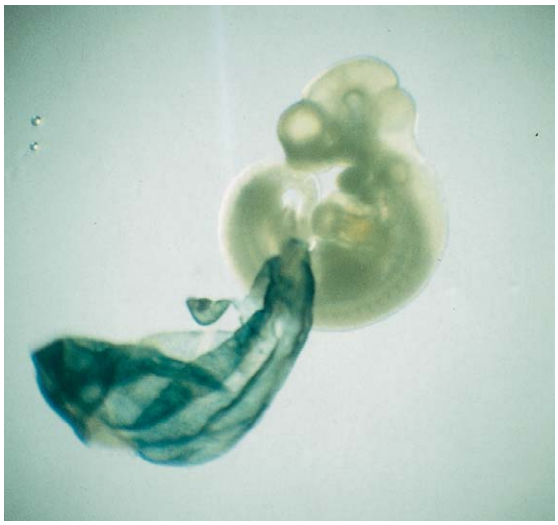
Use of embryonic stem cells to study imprinting

Embryonic stem (ES) cells are totipotent cells derived from the inner cell mass of blastocysts, and they constitute an excellent model for early embryogenesis (Pedersen 1994). Uniparental, intraspecific, and interspecific hybrid ES cell lines are particularly useful to imprinting studies, since they allow parental chromosomes to be distinguished. Parthenogenetic and androgenetic ES cell lines, for example, have been used in differentiation studies that analyze the developmental regulation of expression, methylation, and chromatin conformation of imprinted genes (Szabó and Mann 1994; Feil et al. 1995, 1997). A particular strength of the ES-cell system is that in vitro studies can be transferred to the in vivo context, since manipulated ES cells can be returned to mouse blastocysts and can populate all lineages in the developing embryo. For some purposes, it is desirable to produce embryos that are entirely ES-cell derived.



Completely stem cell–derived fetuses can be obtained by introduction of diploid ES cells into tetraploid host blastocysts. On subsequent postimplantation development, the embryo proper becomes depleted of tetraploid cells (Nagy et al. 1993). The figure shows an 11-d-old fetus derived by injection of diploid ES cells into a tetraploid blastocyst from a mouse strain that has a ubiquitously expressed *LacZ* marker gene. Note that blue-stained tetraploid cells are present only in the yolk sac: the embryo proper is entirely stem-cell derived. We use this technology to assess whether epigenetic mutations in ES cells can affect imprinted gene expression during fetal development. Imprinted genes play key roles in the regulation of growth and differentiation, and this system therefore constitutes a model for epigenetic mechanisms involved in human disease.

The Mouse as a Model Organism for the Study of Imprinting

Since the discovery of imprinting, the mouse has remained the model organism of choice, and, because the imprinting of specific genes is almost invariably conserved between humans and mice, insights gained are directly relevant to human studies. The strengths of the mouse system are many. First, samples can be obtained throughout development, including from the germ line and early embryonic stages when critical decisions are likely to be made, and from all possible tissues, which is of particular importance in cases of tissue-specific imprinting (e.g., *UBE3A*). Second, genotype can be readily selected or manipulated in several ways. Hybrid genotypes can be obtained from intra- or interspecific crosses, allowing one to distinguish parental alleles on the basis of sequence polymorphism. Alternatively, uniparental genotypes can be made for the genome as a whole: parthenogenetic embryos are produced by activation of unfertilized oocytes, whereas androgenetic embryos contain only the paternal nuclear genome (however, neither progresses beyond midgestation [Fundele et al. 1997]). At the level of individual chromosomes, UPDs can be recovered with predictable frequencies, by breeding of translocation carriers marked with recessive phenotypes (Cattanach and Beechey 1997). Finally, embryonic stem (ES) cells (see sidebar), in addition to representing a source of early embryonic material, offer the possibility to perform high-resolution manipulation of genotype, by gene targeting or chromosome engineering. These interventions enable functional experiments to be performed, such as the evaluation of candidate imprinted genes, the testing of models of imprinting mechanisms, or the analysis of the consequences of alteration of the epigenotype.

Evaluation of Components of the Imprinting Mechanism

When mechanisms for imprinting are considered, three events need to be taken into account: setting of the imprint(s) in the germ line; translation of the imprint into the functional difference of the parental alleles, after fertilization; and maintenance—that is, memory of the parental origin in somatic lineages. It also should be appreciated that imprinting may encompass a domain of many hundreds of kilobases, containing multiple imprinted and perhaps nonimprinted genes. One approach toward elucidation of mechanisms has been to examine the epigenetic properties that distinguish the maternal and paternal alleles and then to trace the ontogeny of such differences, with the ultimate aim being functional testing.

This process is most advanced for DNA methylation (see Robertson and Jones 1997 [in this issue]), which we discuss only very briefly here. Methylation has long been considered an important component of the imprinting mechanism. Arguments in its favor are the following: all imprinted genes examined display differences in methylation patterns of the maternal and paternal alleles

(Neumann and Barlow 1996); methylation states are heritable through cell division; and methylation can affect gene transcription directly or indirectly (Tate and Bird 1993). It is clear that methylation in male and female gametes differs much more widely than just at imprinted loci, and the genome as a whole experiences extensive reprogramming of methylation during preimplantation development (Yoder et al. 1997). However, at some imprinted sequences, the differences in methylation inherited from the gametes appear to survive this reprogramming (Neumann and Barlow 1996), and such methylation may well coincide with putative imprinting signals. Other allelic methylation differences at imprinted loci arise later in development, perhaps to stabilize monoallelic expression or as a reflection of it, and this may involve spreading from discrete elements. For example, deletion of the PWS/AS imprinting center leads to altered methylation of loci over a distance of at least 1 Mb (Buiting et al. 1995). Finally, reduction of methylation in mouse embryos, through targeted disruption of the DNA methyltransferase locus, causes the deregulation of imprinted genes in a manner that is predictable from their methylation patterns (Li et al. 1993). These observations establish that methylation is involved at least in the somatic maintenance of imprinting.

The precise roles of methylation in imprinting, however, are not fully understood. Parental allele methylation differences are generally restricted to discrete sequences and do not involve entire imprinted domains. Methylation may well function in concert with other components of the epigenotype (e.g., chromatin) in exerting an effect on gene activity (Tate and Bird 1993). Other epigenetic mechanisms of gene regulation can operate independently of methylation and may contribute to imprinting (Hendrich and Willard 1995). Below, we consider in some detail observations indicating that differences in chromatin structure do exist between homologues of imprinted chromosomes. Some of these concepts arise from studies of X-chromosome inactivation in female mammalian cells.

Chromosome Replication and Meiotic Recombination

The timing of replication reflects chromatin conformation and gene expression, and mammalian genes that are abundantly expressed often replicate earlier in the S phase than less active genes (Holmquist et al. 1987). In the case of the X chromosomes in female cells, in which practically one whole chromosome is transcriptionally silent, the entire inactive X chromosome is late replicating. Like X inactivation, this late replication is imprinted in the extraembryonic tissues (Riggs and Pfeifer 1992).

Asynchronous replication at imprinted autosomal domains was first demonstrated by direct cytogenetic analysis of the PWS/AS region on human chromosome 15q11.2 (Izumikawa et al. 1991). Most subsequent studies have used FISH to interphase nuclei and have revealed differential replication timing of the paternal and maternal chromosomes at the imprinted *IGF2-H19, IGF2R,* and PWS/AS regions, in mouse and human cell lines (Kitsberg et al. 1993; Knoll et al. 1994). In keeping with the association with gene activity, replication asynchrony at the PWS/AS region is greatest in neuroblast cells, in which the genes in the region are presumed to be more highly expressed (Gunaratne et al. 1995). Asynchronous replication detected by FISH appears to involve large domains that can extend beyond the region containing imprinted genes and that can be divided into regions of preferential paternal or maternal early replication (Kitsberg et al. 1993; Knoll et al. 1994; Bickmore and Carothers 1995). Interestingly, at chromosome 15q11-q13, differential replication requires a normal biparental contribution (LaSalle and Lalande 1995). Whether the FISH studies are indicative solely of replication asynchrony or whether they reflect structural effects on the timing of separation of chromatids after replication remains to be resolved (Bickmore and Carothers 1995; Kawame et al. 1995). In this respect, it is intriguing that replication asynchrony has been detected, by an independent method, at *SNRPN* but not at other imprinted loci (Kawame et al. 1995). Whatever the basis for the FISH observations, they are likely to reflect differences in chromatin conformation of the parental chromosomes in somatic cells.

Differences in chromatin structure at imprinted loci in the germ line have been inferred from rates of meiotic recombination. On average, the frequency of female germ-line recombination is 50% higher than that of male germ-line recombination. This might reflect the sexual dimorphism in cytogenetic chromosome length and, hence, chromatin compaction, observed at meiotic prophase stages. In the imprinted *IGF2-H19* and PWS/AS domains, regions showing strong excesses of paternal and maternal recombination have indeed been detected (Pàldi et al. 1995; Robinson and Lalande 1995).

Allelic Nuclease Sensitivity in Imprinted Genes

A more direct indication of chromatin organization is provided by nuclease-sensitivity assays of genes in isolated nuclei. These assays can identify nuclease-hypersensitive sites, small nucleosome-free regions usually associated with *cis*-regulatory sequences of active or potentially active genes, which are typically about two orders of magnitude more sensitive than bulk chromatin. In addition, these assays can identify chromosome domains with increased generalized sensitivity, which frequently extend beyond the (potentially) active gene (Gross and Garrard 1988).

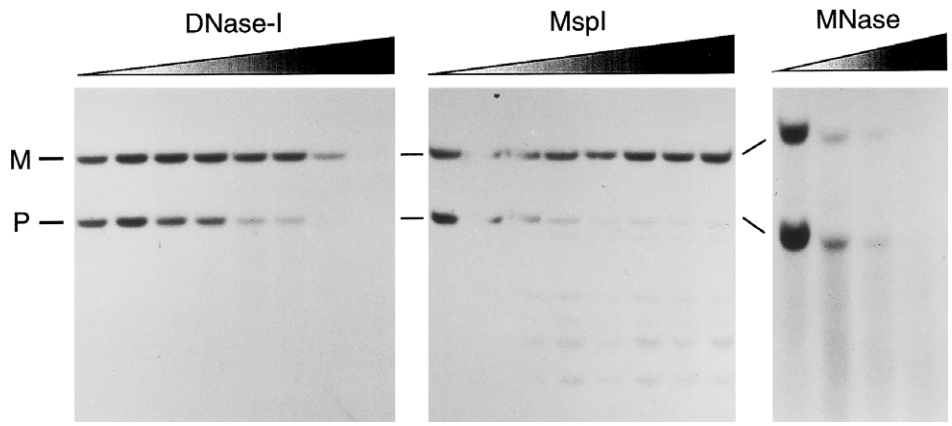


Figure 1 Allele-specific nuclease sensitivity in the imprinted *U2af1-rs1* gene. The use of nuclei from hybrid mice allows the sensitivity of both parental alleles to be compared in the same assay. Liver nuclei were incubated with increasing concentrations of DNase-I, *Msp*I, or MNase. After extraction, the DNA samples were analyzed by Southern blotting, and an RFLP in the 3′ part of the gene was used to differentiate the paternal (P) and maternal (M) chromosomes. In liver and in other tissues, the paternal chromosome is more sensitive to DNase-I and *Msp*I than is the maternal chromosome (the paternal fragment disappears first). No major differences in sensitivity to MNase were detected: the maternal fragment (M), which is twice as long as the paternal fragment (P), is about twofold more digested by MNase than is the paternal fragment. (Modified from Feil et al. 1997.)

So far, nuclease-sensitivity assays have been performed on the imprinted *Igf2, H19,* and *U2af1-rs1* genes in the mouse, and it is for the latter that the most pronounced differences between the parental chromosomes have been detected. The studies of the paternally expressed *U2af1-rs1* gene were performed by use of material from *Mus m. domesticus* × *M. spretus* hybrids, and RFLPs between the two mouse species were used to differentiate maternal and paternal chromosomes (Feil et al. 1997). In all expressing and nonexpressing tissues analyzed, a chromosomal region comprising the entire *U2af1-rs1* gene displayed greater generalized sensitivity to DNase-I on the paternal chromosome than on the maternal chromosome (fig. 1). This imprinted region, which is also constitutively methylated on the maternal chromosome and unmethylated on the paternal chromosome, displays an even greater allelic difference in its sensitivity to the endonuclease *Msp*I. These findings are comparable to those of studies of the X-linked *HPRT* and *PGK-1* genes, in which DNase-I and *Msp*I sensitivity is greatest on the active X chromosome (Wolf and Migeon 1985; Riley et al. 1986; Antequera et al. 1989). In contrast, studies of *U2af1-rs1* did not detect major differences between the parental chromosomes, in their sensitivity to micrococcal nuclease (MNase), which preferentially digests DNA between nucleosomes.

In addition to generalized DNase-I sensitivity, three paternal chromosome–specific DNase-I–hypersensitive sites were detected in the *U2af1-rs1* gene (Shibata et al. 1996; Feil et al. 1997). Two are in the 5′ UTR and appear to be constitutive, being fully established in ES cells, which represent the blastocyst stage. A third paternal site, in the promoter, is most readily detected in tissues with high levels of *U2af1-rs1* expression and arises during in vitro differentiation of hybrid ES cells, concomitant with an increase in gene expression.

How representative of other imprinted genes is this situation? In the case of the maternally expressed *H19* gene, Bartolomei et al. (1993) found sensitivity to *Eco*RI specifically on the maternal promoter. There is, however, no evidence for pronounced sensitivity differences in the body of the *H19* gene, and a cluster of DNase-I–hypersensitive sites downstream of the gene is present on both parental chromosomes (Koide et al. 1994; R. Feil, unpublished data). In contrast, and rather unexpectedly, at the neighboring paternally expressed *Igf2* gene, DNase-I–hypersensitive sites associated with the promoters are present on both parental chromosomes (Sasaki et al. 1992; Feil et al. 1995).

Nature of Chromatin Structural Differences

What constitutes the chromatin differences at the imprinted *U2af1-rs1* gene? Despite the differential sensitivity to DNase-I and *Msp*I, the apparently comparable sensitivity of the parental chromosomes toward MNase seems to indicate that both alleles are similarly packaged into nucleosomes but does not rule out other differences in nucleosomal organization. For example, it has been found that, on the inactive X chromosome in female cells, the bulk of core histone H4 is underacetylated (Jeppesen and Turner 1993). Also, core-histone acetylation in the chicken β-globin locus colocalizes with a region of generalized DNase-I sensitivity (Hebbes et al. 1994). Considered together with other data on autosomal genes (Tsukiyama and Wu 1997), this has given rise to a model

in which hyperacetylation of specific lysine residues of core histones marks the position of potentially active genes (Jeppesen 1997). Although the precise role of acetylation is unclear, a recent study suggested that acetylation is involved in the decondensation of higher-order chromatin structure (van Holde and Zlatanova 1996). In addition, it is generally recognized that chromatin in active genes is partially depleted of linker histone H1, which is regarded as a general repressor of gene activity (Kamakaka and Thomas 1990; van Holde and Zlatanova 1996). Methylation also may play a role in the determination of chromatin, and, in the case of the *U2af1-rs1* gene, hypermethylation invariably was accompanied by nuclease resistance in a wide variety of tissues and developmental stages. Interestingly, linker histone H1 may be preferentially associated with methylated DNA sequences (McArthur and Thomas 1996). Other, nonhistone chromatin proteins, the methyl-CpG–binding proteins (MeCPs), have a strong preference for methylated DNA, and their binding is determined by the density of CpG methylation rather than by sequence (Tate and Bird 1993). Some of these proteins are involved in repression of transcription; for example, the abundant MeCP2 has been shown to contain a transcriptional repression domain (Nan et al. 1997). It may be significant that the methylated maternal *U2af1-rs1* domain is highly resistant to *Msp*I in nuclei (fig. 1), but it remains to be determined to what extent this can be directly accounted for by MeCPs or by other heterochromatin-associated proteins (Hendrich and Willard 1995). Many of these questions now can be addressed allele specifically in mouse model systems.

What Comes Next?

We are still some way from being able to incorporate into a general model all the epigenetic properties that differentiate parental alleles of imprinted genes. It remains unclear, for example, how much of the imprinted epigenotype encompasses an entire imprinted domain. Differential methylation is frequently restricted to discrete elements, and nuclease-sensitivity studies localized differences in chromatin organization at least at some imprinted genes; but, so far, only parental chromosome-specific replication timing appears to involve entire imprinted domains. At the same time, our general understanding of chromatin is steadily increasing (van Holde and Zlatanova 1996; Jeppesen 1997; Tsukiyama and Wu 1997), and it has become clear that many regulatory mechanisms and chromatin components are evolutionarily conserved (Hendrich and Willard 1995). That this may be true for aspects of imprinting is suggested by the recent finding that a putative imprinting control center upstream of the mouse *H19* gene can function as a silencer in *Drosophila* (Lyko et al. 1997).

Transgenic and gene-targeting studies in the mouse will enable identification of *cis*-acting elements involved in the establishment and maintenance of allelic epigenotypes in imprinted genes and will test the hypothesized involvement of direct repeat sequences (Neumann and Barlow 1996). These and in vitro–manipulation experiments will start to answer the important question "What comes first?"—to elucidate the hierarchy of the various epigenetic properties and their roles in the regulation of monoallelic expression. Inhibitors of histone deacetylation, for example, have been found to alter the differential replication timing in imprinted domains (Bickmore and Carothers 1995), and it would be interesting to determine whether they affect higher-order structural features of chromatin and allelic gene expression as well. A greater challenge will be to follow the development of the components of the imprinted epigenotype during the critical embryonic stages when the imprints inherited from the gametes must be interpreted into the functional differences of the parental alleles. This has been feasible for methylation (Neumann and Barlow 1996). In contrast, chromatin and functional studies frequently require larger cell numbers and are, therefore, likely to rely on model systems, such as ES cells and embryonic germ cells. Analysis of genetically altered ES cells—for example, those that do not express the maintenance methyltransferase (Li et al. 1993)—may provide useful additional information. Such studies of and investigations into the precise nature of the chromatin conformational differences detected in nuclease-sensitivity assays may reveal whether chromatin and DNA methylation function independently. These are exciting times for the exploration of the connection between chromatin and genomic imprinting. Future research in mouse and other model systems promises to lead to new insights into the complex regulation of imprinting. Undoubtedly, this will enhance our understanding of the pathological deregulation of imprinting in humans, which is associated with cancer and a growing number of imprinting disorders.

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