REVIEW

Imprinting Mechanisms

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A number of recent studies have provided new insights into mechanisms that regulate genomic imprinting in the mammalian genome. Regions of allele-specific differential methylation (DMRs) are present in all imprinted genes examined. Differential methylation is erased in germ cells at an early stage of their development, and germ-line-specific methylation imprints in DMRs are reestablished around the time of birth. After fertilization, differential methylation is retained in core DMRs despite genome-wide demethylation and de novo methylation during preimplantation and early postimplantation stages. Direct repeats near CG-rich DMRs may be involved in the establishment and maintenance of allele-specific methylation patterns. Imprinted genes tend to be clustered; one important component of clustering is enhancer competition, whereby promoters of linked imprinted genes compete for access to enhancers. Regional organization and spreading of the epigenotype during development is also important and depends on DMRs and imprinting centers. The mechanism of *cis* spreading of DNA methylation is not known, but precedent is provided by the *Xist* RNA, which results in X chromosome inactivation in *cis*. Reading of the somatic imprints could be carried out by transcription factors that are sensitive to methylation, or by methyl–cytosine-binding proteins that are involved in transcriptional repression through chromatin remodeling.

Genomic imprinting is an unusual yet important mechanism of gene regulation by which only one of the parental copies of a gene is expressed (Fig. 1). Although it has been known for some time that DNA methylation is involved in imprinting, the details of how imprints are introduced in the parental germ cells, maintained in embryos, and used to express or repress genes have been elusive. However, recently a number of advances have been made: (1) the definition of *cis*-acting sequences that are important in the control of imprinting; (2) the developmental analysis of how and when imprints are established in germ cells (and when they are erased); and (3) how imprints are maintained, particularly during preimplantation development at which time major changes in methylation occur throughout the genome (Fig. 1). It has also become apparent that imprinted genes tend to be clustered and that aspects of imprinting control are regional and shared between genes in the cluster. Although the reading of the imprint (i.e., the translation of somatic imprint into gene expression pattern) is usually considered to be a more general aspect of epigenetic gene regulation (and therefore not unique to imprinted genes), we describe briefly the known mechanisms that could be involved. Here we consider these recent advances that make the study of

imprinted genes particularly instructive as a major example of epigenetic gene regulation in mammals, with important implications for disease when deregulated. The sections in the first half of this review describe the properties of imprinted genes at key stages of germ cell and embryonic development (Fig. 1). The nature of the molecular elements that combine to initiate and maintain the imprint and also translate it into monoallelic expression form the focus of sections in the second half of the review.

The Germ Line

Erasure

The germ line has the crucial role of erasing existing imprints that are inherited from the previous generation, and establishing the imprints, according to the sex of the germ line, for the next (Fig. 1). Two models have been envisaged for how this might occur (Rossant 1993). In the first, the epigenotype (imprint) of the same sex chromosome is maintained, whereas the one of the opposite sex is reversed. This could be a single step mechanism. In the second, the existing epigenetic modifications are first erased from both parental chromosomes in both germ lines, and imprints are then established in a sex-specific fashion at a later stage. During the past few years, considerable insights have been gained from methylation, expression, and functional studies of

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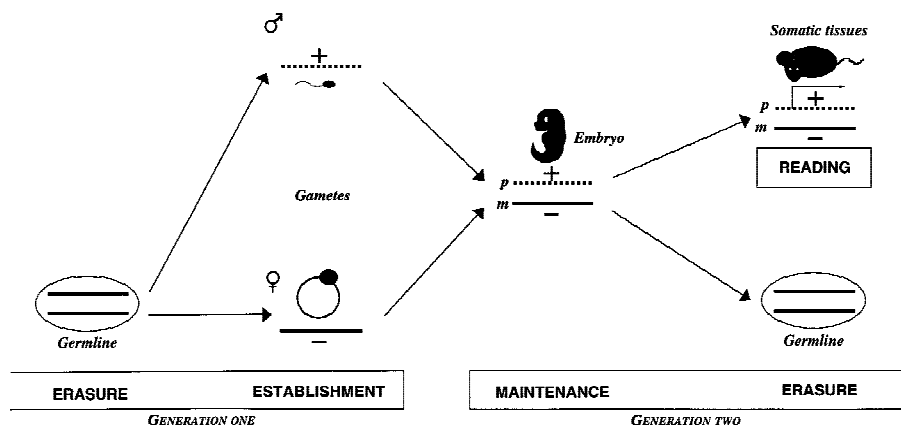FAX 44 (0)1223 836481.

Figure 1 Key stages of genomic imprinting during development. (Erasure) The "imprint" (inherited from the previous generation, generation one) is erased on both parental chromosomes during germ cell development (note that this figure shows complete erasure of all imprints in the germ line, which is one of two models discussed in the text). (Establishment) A new imprint is established (+ or −), according to the sex of the germ line, for the next generation (generation two). (Maintenance) The imprint is stably propagated during mitosis; although not part of the developmental cycle of imprinting, the maternal (m) and paternal (p) imprints are translated into monoallelic expression (indicated by arrow) during development (Reading). Successive cycles of erasure, establishment, and maintenance are a key characteristic of the imprinting process.

both sexes (Sanford et al. 1987; Kafri et al. 1992). This is followed by remethylation from E15.5 in many gene sequences (except CpG islands) with only certain sequences retaining methylation differences between oocyte and sperm genomes (Sanford et al. 1987; Kafri et al. 1992). However, it is possible that the germ line occasionally fails to reprogram epigenetic information. This would lead to epigenetic inheritance through the germ line, which has been observed both for transgene methylation patterns (Sapienza et al. 1989; Allen et al. 1990) and endogenous sequences (Römer et al. 1997).

The global demethylation occurring at early stages of germ cell development also includes imprinted genes (Brandeis et al. 1993). Hence, *Igf2r*, *p57^{Kip2}*, *Peg1*, *Peg3*, *Snrpn*, *U2afrs1*, and *Nnat* are all demethylated by E12.5 (Fig. 2; Brandeis et al. 1993; Tada et al. 1998). *H19* and *Igf2* are not completely demethylated and slightly higher levels of methylation are present in male compared to female PGCs (Tada et al. 1998; H. Sasaki, pers. comm.). This is based on direct examination of PGCs or inferred from the use of embryonic germ (EG) cell lines. Little is known about stages before E12.5 except for one study of E8.5 EG cells that found that the maternal methylation of *Igf2r* region 2 was still present in some cell lines, suggesting that demethylation takes place between E8.5 and E12.5 (Labosky et al. 1994). This timing is partly supported by functional studies. Although chimeras made with E8.5 EG cells develop normally (Labosky et al. 1994), those made with E12.5 EG cells (from both sexes) show enhanced fetal growth, lethality, and skeletal malformations (some of these phenotypes are characteristic of androgenetic chimeras) (Labosky et al. 1994; Tada et al. 1998). In these chimeras, hypomethylation was observed in the normally maternally methylated genes, and *Igf2r* was repressed (which together with expected biallelic expression of paternal genes would contribute to the androgenetic-like phenotype). *H19* and *Igf2* seemed to be 50% methylated in chimeras, suggesting that these imprints were retained at E12.5 when

germ cells. These studies begin to tip the balance toward the second model.

Germ cells develop from a founder population of ~45 cells derived from the epiblast and are determined by day 7.5 of mouse embryonic development (E7.5). Primordial germ cells (PGCs) migrate through extraembryonic regions and the hindgut to their final destination, the gonad primordia in the genital ridge, by E10.5–E11.5 (for review, see Buehr 1997). At E13.5 female germ cells enter the meiotic prophase, whereas male germ cells undergo mitotic arrest. Spermatogonia resume mitosis after birth, which is followed by meiotic differentiation. Oocytes undergo growth after birth, before being ovulated and fertilized.

Although it is clear that DNA methylation has a crucial role to play in imprinting, it is not established whether methylation is the primary imprinting signal that needs to be removed and reestablished in the germ line. However, it appears that methylation changes in imprinted genes in the germ line are at least temporally associated with altered functional properties of the germ cells (see below). Therefore, even if methylation is not the primary imprinting signal, it is likely to be related to such a signal.

Global demethylation and methylation events occur in germ cells. By E12.5–E13.5 all nonimprinted sequences tested so far are demethylated in

| | | Igf2 | | Igf2r | H19 | Snrpn | | U2af1-rs1 | Xist |
| | | DMR1 | DMR2 | | | DMR1 | DMR2 | | |

FEMALE
d 8.5
d 12.5
d 15.5
d 18.5
d 21.5
Oocyte

MALE
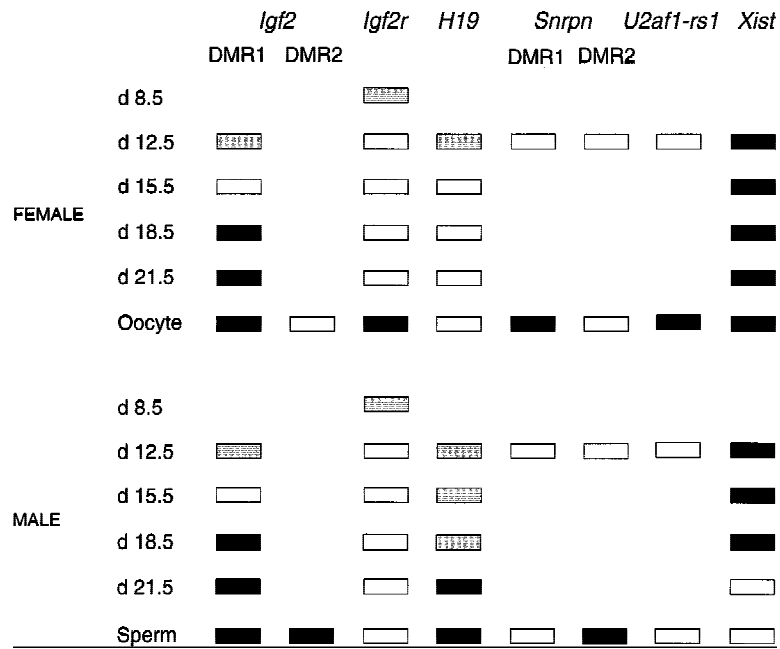d 8.5
d 12.5
d 15.5
d 18.5
d 21.5
Sperm

Figure 2 Methylation patterns in imprinted genes in the germ line. The stages of germ cell development analyzed for each imprinted gene are indicated. (Solid box) Complete methylation; (shaded box) partial methylation; (open box) no methylation. (See text for details and references.) (DMR) Differentially methylated region.

the EG cells were derived (Tada et al. 1998). Whether the paternal copy of *H19* becomes demethylated (erased imprint) in male germ cells at any stage is not clear (see below). However, *Igf2, H19, Igf2r,* and *Snrpn* are all biallelically expressed in both germ lines beginning from E11.5 (Szabo and Mann 1995; Villar et al. 1995), suggesting that imprints are indeed largely erased (or not recognized) at these stages.

Fusion experiments between EG cells and thymic lymphocytes show that EG cells have a dominant demethylating activity that acts on imprinted as well as nonimprinted genes and repeat sequences (Tada et al. 1997). The only sequences so far detected that might escape this global demethylation in early germ cells seem to be the 5′ region of *Xist,* which remains methylated in both sexes (Fig. 2; Ariel et al. 1995; Razin and Shemer 1995) and the paternal copy of *H19* in male germ cells as mentioned above (H. Sasaki, pers. comm.).

Establishment

The timing of methylation establishment in imprinted genes is quite clear for the female, but less clear for the male germ line (Fig. 2). In the female, oocytes in dictyate stage arrest (from E13.5) are apparently not methylated until after birth, when methylation occurs during oocyte growth. This has been established for repeat sequences (Howlett and Reik 1991), *Igf2r* (Brandeis et al. 1993; Stöger et al. 1993), imprinted transgenes (Chaillet et al. 1991; Ueda et al. 1992), and inferred for *Peg1, Peg3,* and *Snrpn* from functional studies (see below). These methylation events are coincident with the presence of high levels of DNA methyltransferase (Dnmt1) in the nucleus of the growing oocyte (Mertineit et al. 1998). Whether other imprinted genes that remain unmethylated in the oocyte (e.g., *H19*) are specifically protected from de novo methylation at these stages is not known. That such protection may be necessary is indicated by the fact that the $p57^{Kip2}$ gene, which is normally paternally methylated, and is demethylated in EG cells, became de novo methylated in EG cell chimeras (Tada et al. 1998). As pointed out above, the establishment of imprinted gene methylation in the male germ line is less clearly defined (Fig. 2). However, *H19* and *Igf2* methylation is apparently established around the time of birth (Brandeis et al. 1993; H. Sasaki, pers. comm.). This is again coincident with high levels of Dnmt1 protein in nuclei of spermatogonia (Mertineit et al. 1998). Whether the grandpaternal copy of *H19* becomes completely demethylated before this de novo methylation occurs is not clear (H. Sasaki, pers. comm.). It has been proposed that *H19* is targeted specifically for de novo methylation in male germ cells, rather than being protected from it in female ones (Surani 1998).

Two sets of functional studies have examined germ cells by nuclear transplantation at presumed stages of erasure and before establishment. Gynogenetic embryos have been prepared with the second maternal genome originating from ungrown oocytes (Kono et al. 1996; Obata et al. 1998). These embryos show improved development when compared to gynogenetic ones, and express the (presumably unmethylated) *Peg1, Peg3,* and *Snrpn* genes. In contrast, *Igf2r* and $p57^{Kip2}$ were repressed in the genome originating from the immature oocyte, showing again that they need to be methylated for activity. The nucleus from male germ cells from E15.5 to E16.5 was also transplanted, and chimeras were obtained in which the transplanted nucleus participated in embryonic and extraembryonic development to E10.5 (Kato and Tsunoda 1995). Inas-

much as androgenetic cells are not expected to contribute to embryonic development in chimeras (they contribute to extraembryonic tissues), this may indicate that these germ cells are still at a stage before acquiring the paternal imprints.

Genetic observations of ''imprinting mutations'' in the Prader-Willi/Angelman syndromes (PWS/AS) have indicated previously that such mutations may interfere with the required switch in epigenotype in the germ lines (Horsthemke et al. 1997). It has been proposed that such mutations prevent the chromosome of the opposite parental sex switching epigenotype, but do not affect the epigenotype of the same sex chromosome (Horsthemke et al. 1997). This would appear to lend support to the ''same sex, no reprogramming'' hypothesis. However, as argued previously, these observations are not conclusive and do not currently allow the distinction between the models (Kelsey and Reik 1997; Reik and Walter 1998).

The Embryo

Ontogeny of Allelic Methylation

The ontogeny of DNA methylation at imprinted loci after fertilization, as in the germ line, has to be seen in the context of the genome as a whole and the global changes in methylation that occur during early embryonic development. Much of the methylation arriving with the gametes, including the gross difference between the maternal and paternal genomes, is erased rapidly during preimplantation development. The higher methylation levels that characterize adult somatic tissues begin to be laid down after implantation, with the onset of general de novo methylation (Monk et al. 1987; Sanford et al. 1987; Howlett and Reik 1991; Kafri et al. 1992; Yoder et al. 1997a). The essential role of methylation in development is indicated by the midgestational failure of mouse embryos that lack the product of the DNA methyltransferase gene *Dnmt1* (Li et al. 1992; Lei et al. 1996); abnormalities include the deregulation of imprinted gene expression (Li et al. 1993).

The enzymology of the embryonic demethylation and remethylation events is beginning to be understood. The principal DNA methyltransferase is Dnmt1, in addition to its activity as a maintenance methylase, through its action on hemimethylated DNA at replication, Dnmt1 has also been suggested to be the predominant de novo methylation activity in mouse embryos (Yoder et al. 1997b). Although present in abundance as a maternal gene product in early embryos, the protein is cytoplasmic during the first cleavage stages, except for a brief appearance in the nucleus at the eight-cell stage, and becomes localized in nuclei only at implantation (Carlson et al. 1992; Trasler et al. 1996; Mertineit et al. 1998). Therefore, generalized demethylation could come about by a passive dilution of methyl groups at each cell division (Howlett and Reik 1991). In addition, demethylation in early embryos may occur by an active process (Kafri et al. 1993). A demethylating activity has been characterized partially in vitro, in extracts of myoblast and embryonic carcinoma (EC) cells, and is mediated by an RNA component, whose activity, sequence specificity, or cell-type specificity may be modulated by protein factors (Weiss et al. 1996). De novo methylase activity, as measured by methylation of newly integrated retroviral DNA, is readily detected in mouse embryonic stem (ES) cells (which derive from the inner cell mass of the blastocyst) and in undifferentiated EC cells, but is down-regulated in differentiated EC cells and postimplantation embryos (Jähner and Jaenisch 1984; Lei et al. 1996). Undifferentiated ES cells in which the *Dnmt1* gene has been homozygously deleted retain a low level of genomic methylation and the ability to methylate retroviral DNA (Lei et al. 1996), indicating the existence of methyltransferase activities in addition to Dnmt1.

If the methylation that distinguishes parental alleles in the gametes does have a role in determining allele-specific expression, or at least coincides with functionally important sequences, it might be anticipated to resist the early demethylation/remethylation cycle of the remainder of the genome. Evidence for such methylation differences has been found at several imprinted loci. The best characterized gametic imprint is at the mouse *H19* gene, located 2–4 kb upstream of the start site, where the paternal, repressed allele is heavily methylated, and the maternal allele hypomethylated in mid-gestation embryos (Bartolomei et al. 1993; Ferguson-Smith et al. 1993; Tremblay et al. 1995). Differential methylation extends over 59 CpG dinucleotides, as shown by bisulfite genomic sequencing (Olek and Walter 1997; Tremblay et al. 1997). The difference between sperm and oocyte is almost absolute, but soon after fertilization (by the eight-cell stage) some modification of the pattern inherited from the gametes has occurred, with limited de novo methylation on the maternal allele and demethylation of the paternal allele toward the upstream extreme of the region (Fig. 3). However, a strong methylation difference is maintained across much of the region throughout preimplantation
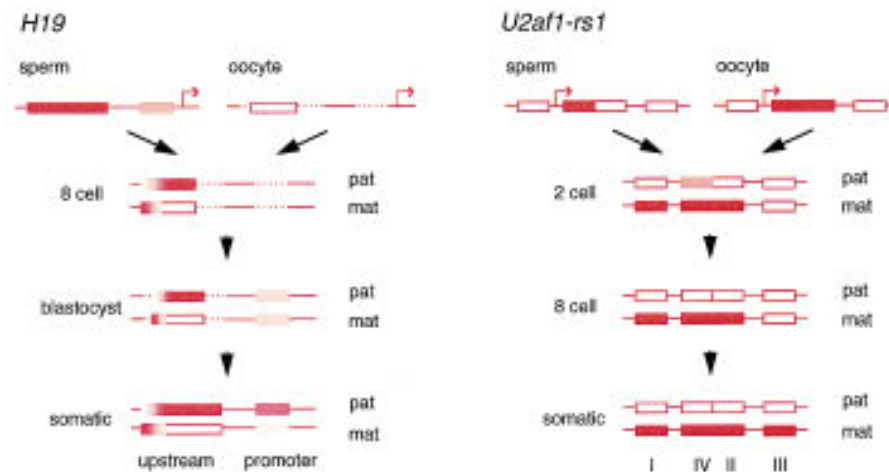
Figure 3 The ontogeny of allelic methylation at the *H19* and *U2af1-rs1* genes. (Solid bars) Complete methylation (>90%); (open bars) absence of methylation (<10%); (shaded bars) intermediate levels. Dashed lines represent regions not analyzed at the indicated stages. The locations of the transcription start sites are indicated by the arrows in the *top* line (which are not intended to represent actual expression status). The scheme for *H19* represents a composite of two bisulfite genomic sequencing studies (Olek and Walter 1997; Tremblay et al. 1997). The upstream differentially methylated region comprises 59 CpG dinucleotides, of which 27 were assayed in oocytes and eight-cell embryos (Olek and Walter 1997) and 14 in blastocysts (Tremblay et al. 1997); the promoter proximal region comprises 9 CpG dinucleotides. The methylation of maternal and paternal alleles in two-cell embryos did not differ from the gametes in the regions analyzed (Olek and Walter 1997). The scheme for *U2af1-rs1* derives from a PCR analysis of the methylation of restriction sites by Shibata et al. (1997). Each region contains multiple sites for *Hpa*II and *Hha*I. Therefore, precise levels of methylation at individual sites cannot be inferred. Methylation at the one-cell stage did not differ from the gametes.

and later development. In contrast, closer to the promoter, differential methylation is less pronounced. Both parental alleles are relatively hypomethylated in the blastocyst, although methylation was high in sperm. The higher level of methylation of the paternal allele is attained in the embryo postimplantation (Tremblay et al. 1997).

Rapid postzygotic changes in methylation have also been described in some detail at the mouse *U2af1-rs1* gene, although without the resolution of the bisulfite approach (Shibata et al. 1997). A gametic difference exists in the tandem repeat containing 5′ UTR (region II, Fig. 3). Exclusive maternal allele methylation is retained throughout preimplantation development and into somatic tissues. A flanking part of the 5′ UTR (region IV) is methylated in both gametes and in one-cell embryos, but methylation is lost from the paternal allele from the two-cell stage. In contrast, the promoter (region I) is unmethylated biallelically to the one-cell stage, and methylation specifically on the maternal allele is detected first at the two-cell stage. Further methyl-

ation of the maternal allele occurs after implantation, exemplified by region III within the gene.

These two examples reveal a very dynamic pattern of methylation at imprinted genes during preimplantation development. More limited PCR analysis has shown the existence of gametic imprints at the *Igf2r, Snrpn,* and *Grf1/ Cdc25Mm* genes (Brandeis et al. 1993; Stöger et al. 1993; Shemer et al. 1996, 1997; Shibata et al. 1998), with some indication of similar remodeling. There are several implications of such dynamic behavior. First, a "core" of differential methylation exists, which is retained throughout development (described henceforth as a core differentially methylated region, core DMR). Remodeling of methylation occurs at sites flanking the core DMR from very early stages, which ultimately stabilize to produce the allelic methylation of somatic tissues. Second, the two alleles may be undergoing simultaneously opposing modification processes. In this, differential methylation runs counter to two trends; methylation on one allele is protected from the general demethylation occurring over the rest of the genome, and hypomethylation of the other must resist the subsequent global remethylation. It is possible that an unusually high density of ^{Me}CpG at a CpG island-like sequence (nonimprinted CpG islands in contrast remain hypomethylated) is sufficient to resist demethylation (Howell et al. 1998). In addition, the protection of CpG islands from de novo methylation afforded by the transcription factor Sp1 offers some precedent for the resistance of the unmethylated allele to modification (Brandeis et al. 1994; Macleod et al. 1994). If methylation is lost from a core DMR, as occurs in *Dnmt1*$^{-/-}$ ES cells, returning methyltransferase activity cannot restore it, without passage through the germ line (Tucker et al. 1996). This might indicate that once methylation has been erased from the normally methylated allele, remethylation is prevented in somatic tissues by the same

factors that protect against methylation of the normally unmethylated allele.

Somatic Maintenance of Allelic Methylation

As implied above, gametic imprints represent only a part of the differential methylation at imprinted loci in somatic tissues. At the *H19* gene, for example, methylation of the paternal, repressed allele extends to the promoter and encompasses the body of the gene during postimplantation development (Bartolomei et al. 1993; Ferguson-Smith et al. 1993; Sasaki et al. 1995). Likewise, at the *Igf2r* gene, the repressed promoter of the paternal allele becomes methylated only late in gestation (Stöger et al. 1993). Such differential methylation may thus be regarded as a secondary effect, possibly in response to transcriptional inactivity, as it arises after monoallelic expression has become established.

Differential methylation need not involve the whole gene or the entirety of a region containing a cluster of imprinted genes. For the small intronless *U2af1-rs1* gene, methylation does extend over the entire length of the repressed maternal allele, with the paternal copy being hypomethylated (Shibata et al. 1996; Feil et al. 1997). Generally, however, parental allele-specific methylation appears to be restricted to discrete elements. At *Igf2r*, a gene spanning ~90 kb, DMRs occur at the promoter and the gametic imprint in intron 2, elements separated by ~30 kb (Stöger et al. 1993). At the *Igf2/H19* cluster, differential methylation is confined to the *H19* gene (Bartolomei et al. 1993; Ferguson-Smith et al. 1993) and three elements in *Igf2*: DMR0 and DMR1 in upstream introns and DMR2 around the final intron (Feil et al. 1994; Moore et al. 1997). The CpG island at *Igf2* promoter P2 is unmethylated biallelically (Sasaki et al. 1992) and the 70-kb region between *H19* and *Igf2* has equal levels of methylation on both chromosomes (Koide et al. 1994). However, differential methylation of these elements does depend on the integrity of the presumed imprinting signals associated with core DMRs. Deletion of the *H19* gene, including the upstream region, leads to altered methylation at the three *Igf2* DMRs on the same parental allele (Forné et al. 1997; Moore et al. 1997). In the Prader-Willi syndrome (PWS), imprinting mutations including deletion of the DMR at the ''imprinting center'' at the *SNRPN* CpG island result in altered methylation in *cis* of loci over a region of at least 1 Mb (Horsthemke 1997). One model foresees that such ''secondary'' DMRs may represent staging posts in the spreading of the imprinted epigenotype across a cluster of imprinted genes outward from an imprinting center represented by the core DMR (Reik and Walter 1998).

In general, methylation effects have shown little tissue-specific variation. Differential methylation of the *H19* gene occurs in liver and brain, despite expression in the former and not in the latter, and is not altered after the postnatal repression of the gene (Bartolomei et al. 1993). Other imprinted genes that show pronounced tissue-specific variation in expression levels generally have constitutive differential methylation. At the *Igf2* gene, in contrast, tissue-specific DMRs have been described. DMR0 is methylated on the repressed maternal allele specifically in placenta (Moore et al. 1997); methylation at DMR2 is restricted to the paternal allele in liver and other endoderm-derived tissues (Feil et al. 1994). It is possible that such methylation reflects tissue-specific transcription effects; DMR0, for example, is located close to noncoding upstream exons restricted to placental transcripts. It remains to be seen whether genes that are widely expressed but subject to highly tissue-specific imprinting, such as *Gnas* and *Ube3a* (Williamson et al. 1996; Albrecht et al. 1997), will show equally tissue-specific differential methylation, or whether they also contain core DMRs that could serve as a memory of parental origin in all tissues.

Allelic methylation differences are generally regarded to be stable in somatic tissues once established in the embryonic phase. Cultured fibroblasts are reluctant to express the maternal *Igf2* allele, unless challenged with agents such as 5-azadeoxycytidine (an inhibitor of methyltransferase) or sustained growth arrest (Eversole-Cire et al. 1993; Ungaro et al. 1997). Changes in epigenotype leading to relaxation of imprinting are also encountered in pathological situations, where they might be seen to offer a selective advantage on cellular phenotype. Biallelic methylation of the *H19* promoter occurs frequently in Wilms' tumor of the kidney. The silencing of the normally active maternal *H19* promoter may lead to derepression of the maternal *IGF2* allele, through competition with the shared enhancers (Moulton et al. 1994; Steenman et al. 1994), resulting in a growth advantage from enhanced IGF2 expression. ''Relaxation'' of *IGF2* and *H19* imprinting is a frequent occurrence in many tumor types.

Cis-Acting Signals

Tandem Repeats

What is the evidence that DMRs define *cis*-acting

sequences involved in the establishment or mainte-
nance of imprinting? *H19* transgenes having dele-
tions of the core DMR fail to show correct imprint-
ing, but other interventions within H19 transgenes
also impair imprinting (Pfeifer et al. 1996; Elson and
Bartolomei 1997). When assayed in *Drosophila*
(whose genome is unmethylated) this region func-
tions as a *cis*-acting silencer (Lyko et al. 1997). De-
letion of the core DMR (region 2) of the *Igf2r* gene in
the context of transgenic yeast artificial chromo-
somes (YACs) also eliminates imprinting of the
transgene (Wutz et al. 1997). The PWS ''imprinting
mutations'' are small deletions including the DMR
at the CpG island of the *SNRPN* locus, which may
lead to incorrect resetting of the imprint in the pa-
ternal germ line (Horsthemke 1997). A similar dele-
tion at the mouse *Snrpn* gene has now also been
shown to block the switch from maternal to pater-
nal epigenotype (Yang et al. 1998). Does this knowl-
edge help to identify key *cis*-acting sequences? One
provocative finding has been the association of
short tandem repeats with DMRs.

Neumann et al. (1995) first proposed that direct
tandem repeats, embedded in CG-rich sequences
and associated with regions of differential methyl-
ation, may constitute a feature common and possi-
bly unique to imprinted genes. This hypothesis was
based on the concept that the imprinting mecha-
nism may have evolved from the host defense func-
tion of DNA methylation (Barlow 1993). For ex-
ample, an insertion of a retrovirus-like intracisternal
A-particle (IAP) upstream of the mouse agouti locus
can induce imprinting. The resulting ectopic expres-
sion of agouti is silenced specifically with paternal
transmission, correlating with hypermethylation of
the IAP long terminal repeat sequence (Michaud et
al. 1994). Therefore, sequences with properties of
''foreign DNA'' may have been recognized by com-
ponents of the methylation system to attract allele-
specific modification and given rise to imprinting
(Barlow 1993).

Many, if not all, imprinted genes contain tan-
dem direct repeats. However, comparison of such
direct repeats reveals few obvious common features.
No sequence homology can be found between re-
peats in different genes (the consensus sequences
tend, however, to be G-rich); the repeat units can be
of different lengths, and the number of times they
are repeated is variable; their location with respect
to the gene also differs (upstream, 5′ UTR, intronic;
some being transcribed, others not) as does their
relationship with CpG islands and allele-specific
methylation patterns (they are found within, or at
variable distances from CpG islands or DMRs). A

comparison between mouse and human regarding a
possible role for the repeats is not conclusive. The
IGF2 gene in humans lacks a repeat in the homolo-
gous region to the mouse and yet is imprinted
(Moore et al. 1997). A block of repeats may, how-
ever, exist further upstream of *IGF2,* as reported for
the *H19* gene (Jinno et al. 1996). On the other hand,
the tandem repeat region and differential methyl-
ation in the *U2af1-rs1* 5′ UTR is specific to this gene;
related *U2af1-rs* loci in the mouse and human ge-
nomes lack this sequence feature and are bialleli-
cally expressed (Shibata et al. 1997). Finally, the
IGF2R gene is polymorphically imprinted in hu-
mans (Xu et al. 1993) but contains numerous large
direct repeats and is differentially methylated (Smr-
zka et al. 1995). What experimental evidence sup-
ports a role for these repeats in the establishment or
maintenance of imprinting, given this diversity of
properties?

Imprinting of the *RSVIgmyc* mouse transgene,
which expresses the c-*myc* oncogene derived from a
translocation, seems to require a *cis*-acting signal
that is principally derived from the tandemly re-
peated sequences that make up the 3′ portion of the
murine immunoglobulin α (IgA) heavy-chain
switch region (Chaillet et al. 1995). Sequence con-
text also seems to be important for imprinting, as
the sequence elements of *RSVIgmyc* are not im-
printed in their normal endogenous location. A re-
cent analysis of additional mutations of the *RSVIg-
myc* transgene, however, indicates that the IgA re-
peat sequences are not absolutely required for
transgene imprinting (Howell et al. 1998). Similar
results have also been observed with *H19* trans-
genes; deletion of the G-rich repeat upstream of *H19*
eliminates transgene imprinting (M. Bartolomei,
pers. comm.). However, a larger transgene carrying
this deletion did, in fact, show imprinted expression
(M. Bartolomei, pers. comm.). These observations
suggest that the repeats, although potentially im-
portant for attracting allele-specific methylation
and expression, are not sufficient and must interact
with other sequence features or context. Further-
more, although deletion of the repeat-containing
region from an *Igf2r* YAC abolishes imprinting,
shorter transgenes that contain the repeats fail to
attract parental-specific methylation (Wutz et al.
1997).

These results suggest the possibility of a redun-
dant role for different sequence elements in govern-
ing imprinting. Different sequence elements may be
able to compensate for the loss of others. A model
can be envisaged in which imprinted genes are char-
acterized by a set of combinatorial *cis* elements

spread out in regions of the gene. Ultimately, it would be their organization or context (rather than sequence) that confers imprinting potential. These elements may constitute a network of responsive elements, under the hierarchical control of imprinting centers.

Although the role of direct repeats needs to be better defined by more precise genetic experiments, their involvement can be envisaged in a number of ways. Because organizational conservation rather than sequence homology is observed, secondary DNA structure may play an important role. Repeats may form alternative secondary structures that attract de novo methylation either in the gametes or soon after fertilization. Alternatively, the repeats could nucleate specific chromatin structures and the observed methylation patterns would be a consequence of this. Either way, germ-line-specific factors that protect one allele from these modifications need to be involved (Fig. 4); for instance, methylation at Alu repeats is blocked in sperm by an Alu-binding protein (SABP) (Chesnokov and Schmid 1995).

We propose a model in which the allele-specific methylation patterns at imprinted loci are established in regions where opposing de novo methylation signals (emanating from repetitive elements) and demethylation signals (induced by CpG-rich environment) interact (Fig. 4). *Trans*-acting factors dictate the allele-specific patterns by either blocking the de novo methylation or demethylation pathways (Fig. 4). Although core DMRs are methylated (or not) in the germ line and these states are then inherited after fertilization, it should be pointed out that repeats could have a role in either establishment (in the germ line) or in maintenance of allelic methylation (in embryos), or in both processes.

There is precedent for the role of repeat sequences in gene silencing and possibly in inducing regional methylation. Hence, repeat-induced gene silencing has been observed in organisms as diverse as fungi, insects, plants, and mammals, in-cluding those in which there is no methylation (Trends Genet. 1997). A de novo methylation center has been identified in the mouse adenine phosphoribosyltransferase gene (*aprt*) that imparts a methylation signal to upstream and downstream *Hpa*II sites (Mummaneni et al. 1993). The bulk of the methylation center signal seems to emanate from tandem B1 repetitive elements (Turker and Bestor 1997) and it has been postulated that Sp1-binding sites in the promoter region are necessary to stop the spreading of the de novo methylation (Mummaneni et al. 1995). Hypothetically, repeat elements may also function as recognition ''nuclei'' by protein complexes such as the methyltransferase, PCNA–p21–methyltransferase complexes, or even protein complexes involved in chromosomal repression, as in telomeric silencing mediated by RAP1–SIR protein complexes in yeast (Moretti et al. 1994).

Some DMRs acquire their allelic methylation after fertilization. The local spacing and cooperativity between these DMRs and core DMRs (i.e., somatic
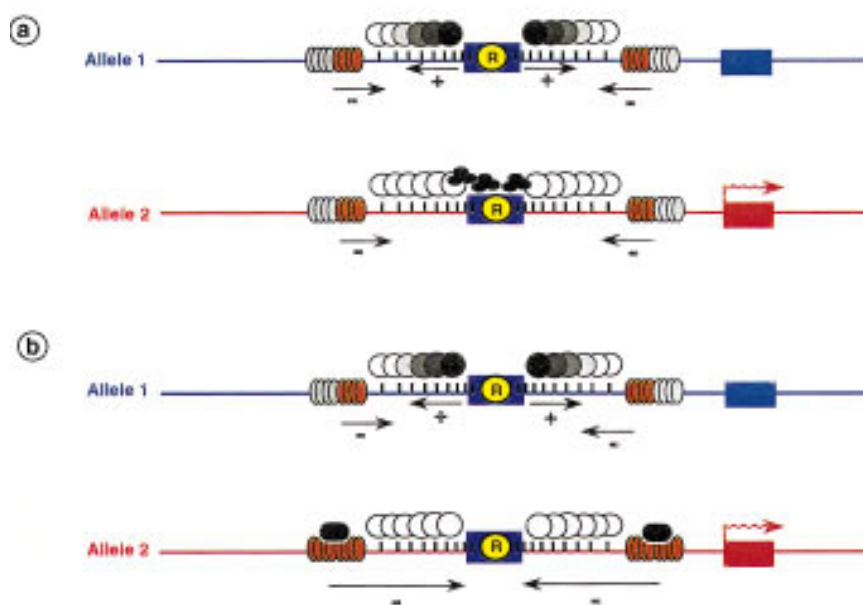

Figure 4 Hypothetical role for tandem repeats in the establishment of allele-specific methylation patterns at imprinted loci. These patterns are the result of the interaction of opposing positive (+) methylation and negative (−) demethylation signals. (*a*) The repeats (R) may act as methylation centers (see text), attracting or inducing the spread of methylation over a stretch of DNA (circles emanating from the repeat). The extent of methylation may be limited by counteracting demethylation signals (represented as gray/red disks) induced by CpG-rich environment (vertical bars). *Trans*-acting factors (trefoil structures) interfere with methylation or its spreading (allele 2) [a gradient of methylation is represented by differently shaded circles; (white circles) lack of methylation; (solid circles) complete methylation]. (*b*) *Trans*-acting factors (black ovals) may act alternatively in the demethylation pathway resulting in a dominant demethylation signal, spreading over the entire region (allele 2).

and germ-line methylation imprints) may be important for the propagation of the imprinting signal and stabilization of specific epigenotypes in imprinting clusters (Reik and Walter 1998). The disruption of any of these elements could result in failure to maintain or establish a parental epigenotype.

RNAs, Competition, and Regional Control of Epigenotype

Transcripts originating from the noncoding or antisense strand, in a parental allele-specific manner, have been found in three imprinted genes so far. An antisense transcript (with no apparent ORF) is expressed abundantly from the paternal allele of *Igf2r,* initiating within region 2. This antisense is thought to be responsible for monoallelic expression of *Igf2r* by repressing specifically the paternal allele of *Igf2r* in *cis* (Wutz et al. 1997), an example of ''expression competition'' (Fig. 5a) in which the antisense is perceived as an ''imprintor'' and the *Igf2r* gene as its imprinted target (Barlow 1997). Deletion of the antisense promoter or enhancer leads to nonimprinted expression of *Igf2r.* Although no developmental details have yet been reported, it is conceivable that antisense expression coincides with the onset of monoallelic expression of *Igf2r* at E5–E6 (Lerchner and Barlow 1997). This would explain the lack of correlation between the onset of repression and promoter methylation (region 1) on the paternal allele (Stöger et al. 1993). Transcription could be repressed first by the antisense and then methyl-

ation would ''lock'' the repressed state later in development. The antisense may repress the paternal *Igf2r* allele in *cis* in several ways, such as physically coating the paternal allele (in a similar way to *Xist;* see below), occlusion of the *Igf2r* promoter by transcribing through it in the opposite direction, or competition for transcription factors or enhancers (Reik and Constância 1997).

An imprinted antisense RNA has also been found to overlap the maternally expressed *UBE3A* gene and is thought to regulate tissue specificity of *UBE3A* imprinting (Rougeulle et al. 1998). The antisense RNA is expressed exclusively in the brain from the paternal chromosome, thus apparently limiting expression of the *UBE3A* gene to the maternal chromosome in this tissue. In other tissues, where the antisense RNA is not detectable, *UBE3A* is expressed biallelicaly (Rougeulle et al. 1998).

Several imprinted antisense transcripts (*Igf2as-a, b,* and *c*) have been described in the upstream region of the *Igf2* gene (Moore et al. 1997), but in contrast to *Igf2r* they are expressed from the same allele as *Igf2* and at low levels. The role of these antisense transcripts is unclear, but overlapping antisense transcription may influence the activity of promoters 0 and 1, because of their proximity to these two regions, and regulate *Igf2* levels in a tissue-specific fashion.

H19 is an example of an imprinted, untranslated RNA involved, through enhancer competition, in regulating the closely linked but recipro-
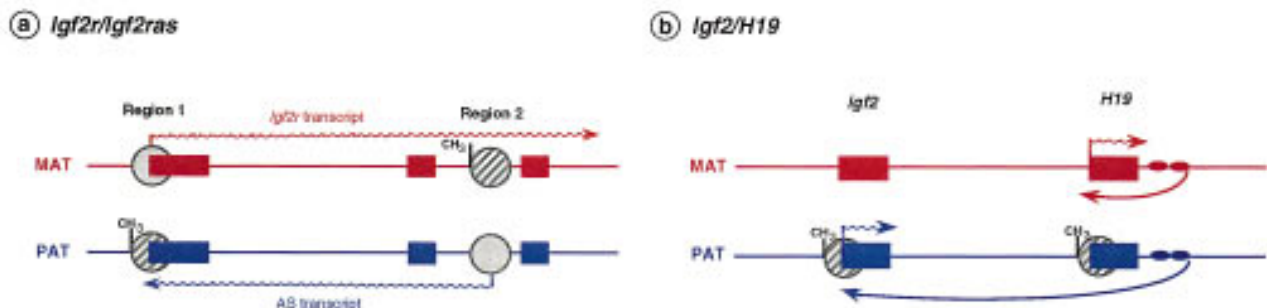

Figure 5 Expression competition mechanisms at the *Igf2r/Igf2ras* and *Igf2/H19* loci. (*a*) Exons of *Igf2r* are depicted as solid boxes, with arrows indicating the transcribed alleles; regions 1 and 2 are sites of differential methylation (hatched circles with CH₃: methylation) arising during embryonic development and in the egg, respectively. When the antisense transcript (AS) is expressed, the sense (*Igf2r*) is not and vice versa. The methylation mark at region 2 may function by regulating a putative antisense promoter that competes with *Igf2r* for expression (Barlow 1997); (*b*) *H19* and *Igf2* genes are indicated as solid boxes. The downstream *H19* endodermal-specific enhancers are represented by filled ovals. DMRs are represented by hatched circles; at the *Igf2* gene the expressed allele is hypermethylated at two intronic regions (for simplification represented as a single region; there is also a maternally methylated region that is not shown here); methylation of the paternal *H19* promoter blocks interaction with downstream enhancers on the paternal chromosome, which are then free to interact with the paternal *Igf2* promoter (long arrow). Proper imprinting at the *Igf2/H19* loci seems, therefore, to depend on promoter competition for the downstream *H19* enhancers.

cally imprinted genes *Igf2* and *Ins2* (Leighton et al. 1995a). The mechanism appears to be competition for shared enhancer elements located downstream of *H19* (Bartolomei et al. 1993; Leighton et al. 1995b) (Fig. 5b). When the maternal *H19* promoter is activated by the downstream enhancer, the *Igf2* gene is not expressed from the maternal allele. Paternal expression of the *Igf2* gene is observed when the enhancer is unable to interact with the *H19* paternal promoter because it is methylated (Fig. 5). Proper imprinting of *Igf2* and *H19* on the maternal chromosome seems to be dependent on the position of the enhancers relative to the two genes. Mice carrying an extra set of the *H19* endoderm-specific enhancers between *Igf2* and *H19* show expression of the normally silent *Igf2* gene in liver, consistent with relief from competition (Webber et al. 1998). When a single set of enhancers is located equidistantly from *Igf2* and *H19* on the maternal chromosome, *Igf2* is expressed instead of *H19,* suggesting that the strength of the *H19* promoter is not the main determinant in the competition for enhancers (Webber et al. 1998). The core DMR associated with the *H19* promoter may determine methylation of the *H19* promoter but, in addition, may also play a role in allowing the 3′ enhancers access to the *Igf2* promoters.

Unusual RNAs are also found in the upstream region of the *SNRPN* gene and may be involved in imprinting control for the entire PWS/AS region. Deletions and a splice mutation affecting these upstream transcripts are associated with AS, as they lead to an apparent failure to switch the paternal epigenotype in the maternal germ line (Dittrich et al. 1996). In contrast, deletion of the *SNRPN* CpG island region and promoter leads to an apparent inability to switch the maternal regional epigenotype in the paternal germ line, and hence to PWS. However, these observations could also be explained by extending the expression or enhancer competition model to the PWS/AS situation (Barlow 1997; Tilghman et al. 1998). Promoter competition between the *SNRPN* and *UBE3A* genes for a neuronal enhancer has been proposed recently (Tilghman et al. 1998). Methylation inactivating the *SNRPN* promoter on the maternal chromosome would lead to activation of the *UBE3A* gene.

A common feature of the enhancer competition model is that deletion of DMRs, promoter, or enhancers would imbalance the competition system. However, this model does not explain how regional methylation can be altered by DMR or promoter deletions (Sutcliffe et al. 1994; Buiting et al. 1995; Forné et al. 1997; Moore et al. 1997; Wutz et al.

1997). It has been suggested that *cis*-acting RNAs that interact with DMRs could be involved in this regional spreading (Reik and Walter 1998).

A precedent for the role of an untranslated RNA in regional silencing in *cis* is the *Xist* gene. The *Xist* RNA is expressed from the inactive X chromosome in female mammals and is essential for inactivation of genes along the X chromosome (Penny et al. 1996; Marahrens et al. 1997). The accumulation of *Xist* transcripts along the length of the inactive chromosome (Clemson et al. 1996; Lee and Jaenisch 1997) is thought to be required for the nucleation and spread of heterochromatin from the X inactivation center (Xic) (Panning et al. 1997). Inactivation of the paternal X chromosome occurs in extraembryonic tissues and consistent with this the paternal *Xist* copy is expressed preferentially in female preimplantation embryos (Kay et al. 1993). However, in embryonic lineages, in which X inactivation is random, the imprinted expression needs to be reprogrammed. This may involve expression of unstable *Xist* transcripts from both X chromosomes (Panning et al. 1997; Sheardown et al. 1997), which could be brought about by using alternative promoters (Sheardown et al. 1997). The future inactive X chromosome then stabilizes *Xist* RNA by an unknown mechanism (possibly involving a promoter switch), whereas the active X chromosome fails to accumulate *Xist.* This initial decision is "locked" by subsequent silencing of *Xist,* at the transcriptional level, on the active X chromosome by DNA methylation (Panning and Jaenisch 1996; Panning et al. 1997).

Reading the Imprint

The different methylation states of the two alleles have to be translated into monoallelic expression. This is termed the reading of the imprint. The reading mechanism requires the involvement of various *trans*-acting factors, some of which can detect the methylation status of the DNA environment and some of which interact with these factors to control the transcriptional competence of the particular allele. Methylation, although of key importance to the imprinting process, is involved primarily in the general control of gene expression. The typical observation is that methylating a gene results in its silencing. In fact, the majority of CpG dinucleotides in the mammalian genome are methylated; this has been suggested to be the default state required to shut down unwanted and potentially harmful transcriptional noise (Bird 1995), including that of the transposable element-based repeats scattered

throughout the genome (Yoder et al. 1997a). Hence, unmethylated regions are relatively rare and usually encountered in the form of CpG islands associated with the promoters of expressed housekeeping genes (Bird 1995). If such regions become erroneously methylated (for example, in tumors) then the associated gene is repressed. This is the case for the CpG islands of tumor suppressors VHL, p16, and Rb (Laird and Jaenisch 1996). Certain transgenes also show a tight inverse correlation between methylation levels and expression (Allen et al. 1990; Chaillet et al. 1991).

Is it possible to treat monoallelic expression through imprinting as just a special case of methylation silencing? It could be postulated that the "hard work" of distinguishing the two parental alleles has already been accomplished by the imposition of the differential methylation pattern. The next step is the "blind" transcriptional response to the methylation pattern, which only requires the factors responsible for the initiation or inhibition of transcription to associate with the appropriate allele. This would be determined not only by the availability of the appropriate transcription factors for the enhancer/promoter elements, but also how these transcription factors respond to the methylation patterns that may exist in those regions.

Reading Factors

DNA-binding factors can respond in a number of ways to the methylation of their target sites. Some seem relatively unaffected by the methylation status of their binding sites (Tate and Bird 1993). These include Sp1, MTF-1, Krox-20, CTF/NF1, and TCR-ATF. Table 1 lists those factors whose binding affinities are altered by the methylation of their target sequences and Figure 6 illustrates the potential effects of methylation on *trans*-acting factor binding and subsequent transcriptional control.

The silencing of transcription brought about by methylation has been divided into two categories: (1) that resulting from the inability of methylation-sensitive transcription factors to bind to their target sequences and contribute to gene activation, and (2) that resulting from the binding of methylation-dependent silencer proteins that may act by nucleating chromatin structures in the gene region. The *Xist* gene possesses a transcription-promoting element that binds factors belonging to both of these categories (Huntriss et al. 1997). When unmethylated, a factor binds and may activate expression of the gene. However, with methylation, the activator is no longer able to bind and is replaced by a meth-

ylation-dependent binding protein that may act to silence gene expression.

Two proteins of the second category, MeCP1 and MeCP2, appear to be crucial in general methylation-dependent silencing. MeCP2 is an abundant protein that possesses domains that bind methylated DNA and repress transcription (probably indirectly). Immunofluorescence studies show that the MeCP2 protein is tightly associated with chromosomes, especially in heterochromatic regions (Nan et al. 1997). Interestingly, MeCP2 may carry out part of its inhibitory role by becoming a key part of the chromatin complex itself as it has been shown that MeCP2 can replace the linker histone H1 in the compacted DNA of silenced regions. MeCP1 is a complex of several proteins including one, PCM1, which has a methyl-binding domain homologous to MeCP2 and a cysteine-rich domain homologous to those found in the DNMT1 and HRX proteins (Cross et al. 1997). The complex is widely expressed at a low level and requires a greater density of methylated cytosines than does MeCP2 before binding occurs. Binding of MeCPs to methylated DNA results in reduced accessibility of chromatin to endonucleases (DNase I, restriction enzymes). Consistent with an involvement of such proteins in allelic silencing, some imprinted genes show markedly reduced DNase accessibility in regions of extensive methylation (Bartolomei et al. 1993; Ferguson-Smith et al. 1993; Feil and Kelsey 1997; Feil et al. 1997). However, the link between such proteins and the establishment of silencing through the formation of specific chromatin structures is still unknown (Kass et al. 1997). A key process may be the balancing act between the acetylation and deacetylation of histones within nucleosomes (Pazin and Kadonaga 1997). Deacetylation of these proteins, carried out by "histone deacetylases" (in mammals there are two, HD1 and HD2) in conjunction with another protein, Sin3, is associated with the silencing of genes such as *Xenopus TRβA* (Wong et al. 1998). It has been suggested that the specificity of the deacetylation process may be dictated by DNA-bound factors that serve to anchor the activity in appropriate areas. Indeed, it has been shown recently that MeCP-dependent silencing acts through deacetylation of histones (Jones et al. 1998; Nan et al. 1998).

In terms of imprinting, a recent paper has described the use of trichostatin A, a specific inhibitor of histone deacetylase, to examine the developmental onset of silencing of the paternal *H19* allele in mouse trophectodermal cells (Svensson et al. 1998). Previous work had shown that there are variable

Table 1. Methylation-Sensitive and -Dependent DNA-Binding Proteins and their Putative Functions

DNA-binding factor	Effect of methylation of target sequence (binding)	Factor mol. weight	Target sequence	Comments	Reference
Myc/Myn CREB/ATF NFKB AP-2 MLTF/USF EBP-80 VBP1	inhibits	N.A.	N.A.	a selection of general transcription factors with binding inhibited by methylation	Tate and Bird (1993)
Xist promoter BP1	inhibits	unknown	GCGCCGCGG	putative factor that binds unmethylated sequence upstream of *Xist* transcription start site and has a positive effect on expression (see *Xist* promoter BP2 below)	Huntriss et al. (1997)
AP-1	permits	N.A.	TGACTCG	methylation of the X-sequence in the first exon of the metastasis-associated gene *mts-1* induces Fos/Jun binding resulting in transcriptional silencing	Tulchinsky et al. (1996)
MMBP-1 MMBP-2 MMBP-3	permits permits permits	42 kD 63 kD 50–60 kD	CACGTG	three factors isolated from C2C12 cells that bind the methylated form of the c-Myc binding site (expressed only in actively proliferating cells)	Suetake et al. (1993/95)
DBPm	permits	50 kD	35-mer (see Ref.)	isolated from pea seed. Plant methylation plays roles in transposable element silencing and general control of development and flowering	Ehrlich et al. (1993)

Table 1. (*Continued*)

DNA-binding Factor	Effect of methylation of target sequence (binding)	Factor mol. weight	Target sequence	Comments	Reference
Xist promoter BP2	permits	100 kD	GCGCCGCGG	when methylated the target sequence binds this transcriptional silencer, replacing (outcompeting?) the *Xist* promoter BP1 described above	Huntriss et al. (1997)
HMBP	permits	300-kD complex	43-mer (see Ref.)	methylation of 3 SP1-like sites in the 5′ LTR of HIV can lower expression of the viral-encoded genes (role in AIDS latency?); HMBP, expressed in T lymphocytes, is thought to participate in this inhibition as it only binds the methylated target site	Joel et al. (1993)
RFX1, 2, and 3	permits	2 × 105-kD complex	GCCGTCATGGCGCC	(also known as EF-C, MDBP, NF-X, etc), family of 5 + conserved DNA binding proteins. Involved in MHCII and viral gene expression. RFX1, 2, and 3 shown to prefer some target sites when methylated; transcriptional activators	Zhang et al. (1993) Emery et al. (1996)
MDBP-2-H1	permits	47 kD	methylated DNA	member of the histone H1 family found in avian liver; preference for association with methylated DNA; shown to be truncated form of normal H1; general significance unknown	Schwarz et al. (1977)
MeCP1	permits	400/800 kD	several methyl-CpGs	PCM1 (36/80 kD) is a constituent of this large complex	Cross et al. (1997)
MeCP2	permits	80 kD	2 methyl-CpGs	abundant but insufficient in quantity to bind to all genome's methylated CpGs	Nan et al. (1997)

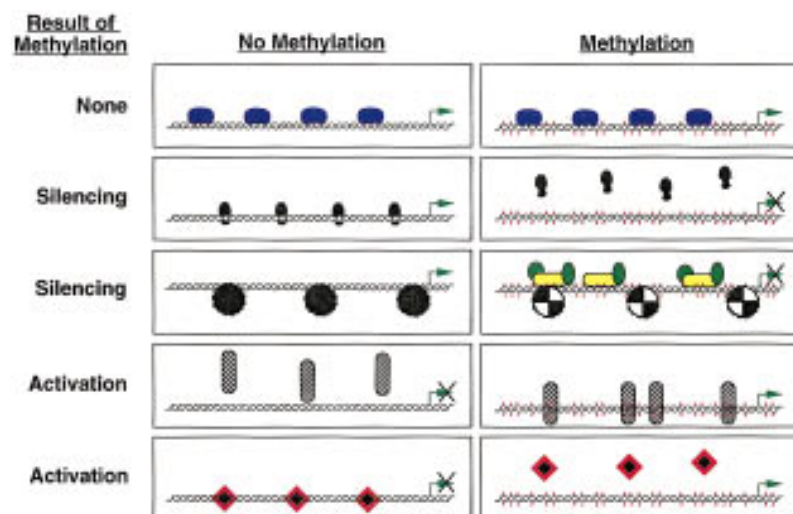Modified from Tate and Bird (1993).

Figure 6 Four mechanisms by which methylation can alter transcription. Methylation may have no effect on expression (*top*). Methylation-sensitive transcription factors cannot activate a methylated promoter (*second* pair of panels). Transcriptionally competent promoters with nucleosomes (dark circles) can acquire a protein complex with methylation (*third* pair of panels). This may include MeCPs and deacetylases, which alter nucleosome function (as shown by quartered circles) resulting in silencing. Some transcription factors require their target sites to be methylated before they can bind and promote gene expression (*fourth* pair of panels). A hypothetical situation where a repressor is dislodged by methylation (*bottom*). The last two models could explain the instances of expression from methylated alleles (see text).

amounts of biallelic *H19* expression in early embryos. The drug treatment slowed the rate of paternal allele silencing, which resulted in increased numbers of patches of biallelic *H19* expression beyond the normal stages. From these results we can conclude that the presence of biallelic expression from imprinted genes early in development may be the result of the immaturity of the silencing complex as well as the unrefined nature of the methylation pattern. Other routes to heterochromatization exist that do not require changes in histone acetylation. The polycomb group of genes has been shown to play important chromatin-mediated silencing effects in *Drosophila* (position effect variegation) and the regulation of mammalian genes such as the *Hox* clusters (Pirrotta 1997). The full repertoire of chromatin or chromatin-organizing genes potentially involved in the control of monoallelic expression remains to be cataloged.

The two categories of silencing described above are a useful guide but cannot explain all observations. For example, the actively transcribed paternal allele of the *Igf2* gene is the allele that shows higher methylation levels (at DMR1 and DMR2). Assuming that these regions play an important role in the con-

trol of monoallelic expression, it becomes necessary to explain this apparent reversal in the effect of methylation on transcription. It could be proposed that either a methylation-dependent activator protein is binding to the paternal DMRs (acting in the same way as *RFX1/RFX2/RFX3*), or that a methylation-sensitive silencer protein is associated with the repressed maternal allele.

Reading Disorders

This review has already mentioned diseases arising from the loss of the correct imprint or from the failure to switch the imprint to reflect the parental origin. Is it possible that there exist defects in the reading process that have pathological consequences? Targeted disruption of the methyl-cytosine-binding protein gene *MeCP2* has an early embryonic lethal phenotype leading to speculation that this is a critical component in the conversion of methylation into silencing that cannot be replaced by compensatory or parallel pathways (Tate et al. 1996). It will be interesting to determine whether expression of imprinted genes is altered in these knock-out mice. Are there less severe phenotypes arising from other reading machinery defects? There are several instances when a seemingly allele-specific methylation pattern is not translated into genuine monoallelic expression. This is the case for the human *Igf2r* gene where only some individuals (genotypes) show monoallelic expression. A possible cause of this variability could be that functional polymorphisms are present in the human genes responsible for the reading of the imprint as opposed to functional alterations in the imprint itself.

Conclusions

Major reprogramming of the imprints occurs in the germ lines of developing embryos. Both sex germ cells undergo global demethylation including imprinted and nonimprinted genes. The bulk of this demethylation probably occurs between E8.5 and E12.5. Functional studies on germ cells from this stage in chimeras or nuclear transplantations show that this demethylation is indeed associated with substantial reprogramming of imprinted genes. It is not yet clear whether all imprinted genes undergo

complete reprogramming. New methylation imprints are established in the female germ line during oocyte growth after birth, and in males probably around the time of birth. In addition to germ-line-specific de novo methylation, protection from methylation may also be required.

Regions of differential methylation have been identified at all imprinted genes. Their developmental kinetics appear to be diverse and complex, some being stable and potentially associated with gametic imprinting signals, others arising later in development and more likely to be involved in maintenance of imprinting or spreading of an imprinted epigenotype in a cluster of imprinted genes. Genome-wide alterations in methylation occur in the preimplantation and early postimplantation embryo. However, methylation of core DMR regions of imprinted genes remains unaffected. This requires protective mechanisms that not only prevent the methylated allele from becoming demethylated but also prevent the demethylated allele from de novo methylation. High methylation densities may play a role by causing resistance to demethylation. Multiple *cis*-acting sequences are probably required for the creation and maintenance of the methylation state. The precise methylation patterns are elaborated during postimplantation development.

Diverse sequence and structural motifs are associated with imprinted genes. DMRs are CpG-rich and associated with direct repeats; although this association with imprinted loci is strong, evidence for a functional role is, at present, inconclusive. One model for control of methylation at imprinted loci may involve the interplay between de novo methylation caused by repeats and demethylation induced by the CpG-rich environment. Other regulation mechanisms such as antisense transcription and enhancer competition play an important role in the imprinting process, probably both in individual genes and in imprinting clusters.

The mechanisms underlying the translation of the imprint into monoallelic gene expression are largely unknown. The methylated allele of most imprinted genes is the inactive one. Repression could be brought about by the inability of transcription factors to bind or alternatively, by the binding of MeCPs and associated silencing factors. Expression from the methylated allele is rare but may involve the displacement of repressors or the binding of methylation-dependent activating factors.

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