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PROSPECTS

Regulation of Imprinting: A Multi-Tiered Process

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Abstract Although most mammalian genes are expressed from both alleles, there is a small group of special genes which are imprinted so that only one of the parental alleles is actually expressed in target cells. This epigenetic process involves regulation at a number of different stages of development and is very complex. In principle, imprinted gene regions must be marked in *cis* in the gametes using epigenetic features capable of being maintained through cell division and able to direct multigenic monoallelic expression in differentiated cells of the mature organism. The difference between alleles must be erased during early gametogenesis to allow the imprint to be reset in the mature gametes. In this review we will summarize what is currently known about the molecular mechanisms which mediate these steps. J. Cell. Biochem. 88: 400–407, 2003. © 2002 Wiley-Liss, Inc.

Key words: DNA methylation; replication timing; antisense RNA

One of the major players in the imprinting process is DNA methylation. Early studies demonstrated that specific transgenic sequences often behave in a parent-of-origin manner, becoming methylated during oogenesis, yet emerging from spermatogenesis in an unmethylated form, and this difference was preserved throughout development [Chaillet et al., 1995]. These experiments showed that mice harbor sophisticated molecular machinery capable of imprinting DNA, and it is now well known that this represents a general phenomenon. Indeed, a large body of evidence attests to the fact that all endogenous imprinted genes are associated with distinct sequence regions differentially methylated either on the maternal or paternal allele [Ferguson-Smith and Surani, 2001]. In a sense, methylation represents an ideal marker for imprinting, since it can be established by de novo methylation in one of the gametes, and once this occurs, the differential pattern will be automatically preserved by means of the maintenance methylase present in every cell type.

CIS ACTING ELEMENTS

Although the precise features of how imprinted genes get specifically methylated in the gametes has not been fully worked out, it is now clear that this process utilizes novel types of *cis* acting sequences and *trans* acting factors. The *Igf2r* gene provides a good model system for identifying some of these *cis* acting sequences. In the embryo and adult organism, *Igf2r* is expressed exclusively on the maternal allele. The major epigenetic mark associated with this gene sequence is located in the first intron, about 25 kb downstream from the Igf2r promoter and is composed of a 3 kb CpG island sequence which is methylated differentially on the maternal allele (Fig. 1). This region evidently acts as a repressor to inhibit the paternal allele, but methylation on the maternal allele abrogates this inhibitory function, causing the maternal allele to be active [Wutz et al., 1997].

In order to appreciate how this imprinting mechanism works, it should be pointed out that all differentially-methylated regions (DMRs) associated with imprinted genes undergo erasure during early gametogenesis. In the mouse,

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germ cells deriving from the epiblast initially go through a process of migration leading them to the developing gonad at about 11 dpc. It is during this period that all of the DMRs undergo erasure and thus emerge in a fully unmethylated form, setting the stage for gamete specific re-methylation [Reik and Walter, 2001]. In the case of the *Igf2r* gene, this step occurs towards the end of oogenesis, during oocyte maturation [Brandeis et al., 1993].

In order to study this de novo methylation, Birger et al. [1999] used plasmids containing segments of the Igf2r DMR and injected them unmethylated into either the maternal or paternal pronucleus of fertilized eggs. The state of methylation was then evaluated by PCR in blastocysts that subsequently developed in vitro. In a striking manner, only when injected into the maternal pronucleus did the DNA undergo de novo methylation. By carrying out reverse genetics with smaller fragments and by introducing point mutations, these authors succeeded in defining a bipartate element composed of one element capable of directing de novo methylation in both gametic nuclei, and a second sequence which specifically inhibits this process, but only in the paternally derived nucleus.

It should be noted that these sister elements are not only capable of generating differential methylation of the Igf2r DMR, but can also imprint another unrelated DMR from the Snrpn gene. Since imprinted methylation patterns are not found on other CpG island sequences, it appears that this de novo system is composed of two components, one which induces methylation and a second which defines the target region. This study is particularly informative, since all of the analyses were restricted to preimplantation embryos, thus enabling the authors to identify the sequence elements required for the establishment of differential methylation without interference from *cis* acting sequences that may be involved in maintenance of the signal.

Many imprinted genes are organized in conserved clusters. A typical example is the Prader-Willi/Angelman syndrome (PWS/AS) domain on human chromosome 15q11–q13 and its orthologue on mouse chromosome 7C-D1 (Fig. 1). The 2 Mb PWS/AS domain contains a group of genes which are paternally expressed, and only a few which are expressed exclusively from the maternal allele [Nicholls et al., 1998]. Genetic aberrations in this domain result in two clinically distinct neurobehavioral disorders. PWS is a result of molecular defects which bring about silencing of the paternally expressed genes, while AS comes about because of molecular defects which cause a loss of expression of genes on the maternal copy of this domain.

Studies of spontaneous minideletions in the 15q11–q13 domain in man and induced deletions of the orthologous region on chromosome 7 of the mouse have led to the proposal that the imprinting process is coordinated by an imprinting center (IC) located upstream of the SNRPN gene [Bielinska et al., 2000]. One region of this IC is required for establishing and maintaining the paternal imprint, and is defined by a series of PWS families in which minideletions are observed on the paternal allele. The shortest region of deletion overlap (PWS-SRO) for this region maps to a 4.3-kb sequence which encompasses the SNRPN promoter and exon 1 [Ohta et al., 1999a]. In these PWS families the paternally expressed genes are all methylated and silenced. On the other hand, families with AS carry minideletions on the maternal chromosome which all overlap a 880-bp sequence (AS-SRO) located 35 kb upstream of the SNRPN gene [Buiting et al., 1999]. Defects in the AS-SRO affect the maternal imprint exclusively. These genetic data support the idea that both the AS-SRO and the PWS-SRO work cooperatively to bring about the full imprinting phenotype, and this has been nicely confirmed by transgenic experiments showing that sequence elements from these two regions are sufficient to reproduce imprinting in mice [Shemer et al., 2000].

It now appears that the AS-SRO and PWS-SRO operate in a stepwise manner to establish imprinting during early development (Fig. 1). The AS-SRO probably acquires its differential epigenetic makeup during gametogenesis prior to the PWS-SRO, which emerges from both gametes unmethylated in its CpG island sequences [El-Maarri et al., 2001]. The conformationally active AS-SRO on the maternal allele apparently acts in *cis* as a repressor to bring about both de novo methylation of the adjacent PWS-SRO and its assembly into a closed chromatin structure [Perk et al., 2002]. In contrast, the PWS-SRO on the paternal allele remains unmethylated presumably because its corresponding AS-SRO is in the off conformation. Finally, the open PWS-SRO on the paternal allele operates in *cis* in a secondary manner

to bring about structural and transcriptional activation over the entire PWS/AS domain.

Although other imprinted genes containing CpG island DMRs are differentially methylated on the maternal allele, there are also many imprinted domains which undergo specific methylation exclusively on the paternal allele [Reik and Walter, 2001]. The *H19* and *Igf2* genes, for example, are located in one cluster on mouse chromosome 7 and are oppositely imprinted with Igf2 being expressed paternally and H19, maternally (Fig. 1). In this case, the IC is made up of a 2 kb DMR located upstream to the H19 promoter, and genetic studies have shown that this sequence is absolutely required for imprinting [Tremblay et al., 1995]. Gamete specific methylation of this H19 region during spermatogenesis seems to be directed by *cis* acting elements located within the DMR itself (unpublished observation).

Better evidence for *cis* acting elements which control the establishment of paternal specific modification comes from studies of the *Rasgrf1* gene, which carries a paternally methylated DMR about 35 kb upstream to the promoter. This region contains a multiply repeated 41-bp element, and when deleted, the entire DMR fails to undergo methylation [Yoon et al., 2002]. These studies did not investigate whether these sequences are required for actually directing de novo methylation during spermatogenesis or whether they help define the target domain, but in any event, it appears clear that like methylation in the oocyte, this process requires *cis* acting elements.

TRANS ACTING FACTORS

Little is known about the enzymes and factors used for marking imprinted genes during gametogenesis. Several different DNA methyl transferases have been characterized. Dnmt1, for example, is highly specific for hemimethylated DNA and, as such is considered to be the primary enzyme responsible for the maintenance of methylation following replication. This protein has only a limited ability to carry out de novo methylation, and even when it is completely absent during oogenesis, de novo methylation of imprinted genes proceeds normally [Howell et al., 2001]. In contrast, two other family members, Dnmt3a and Dnmt3b, are capable of carrying out de novo methylation on DNA substrates in vitro. The targeted deletion of each gene individually leads to a lack of DNA methylation at specific gene loci, but removal of both genes causes a general deficiency in de novo methylation. On the basis of these findings it seems likely that it is these two genes which bring about genome wide de novo methylation in the implantation embryo after methylation erasure in the morula and blastula. Genetic experiments indicate that Dnmt3a and 3b also mediate de novo methylation of imprinted genes during gametogenesis [Okano et al., 1999].

The importance of methylase motifs in the process of imprinting is reflected in the startling observation that yet another family member, Dnmt3L, plays a role in gamete specific methylation, despite its lack of methyl transferase activity in vitro. Indeed, deletion of this gene in female mice leads to a complete lack of methylation on all known maternally modified imprinted genes in offspring, while deletions of this gene in the male has no effect [Bourc'his et al., 2001; Hata et al., 2002]. It thus appears that Dnmt3L may be directly involved in the marking of maternal genes, perhaps operating by specifically recruiting Dnmt3a and 3b to the correct imprinted gene sequences. Protein factors present during oogenesis and spermatogenesis are also required to coordinate maternal specific methylation at the *Igf2r* gene domain [Birger et al., 1999], but it is not known whether these play a general role in imprinting.

Recent genetic studies on complete hydatiform moles (CHM) have helped identify a novel factor that appears to be critically important for directing the marking process which takes place during gametogenesis. These moles usually come about sporadically as a result of losing the maternal genome following fertilization, and it is thought that the resulting cancellation of imprinting may actually be the main factor responsible for its extraembryonic-like phenotype. Although very rare, some patients exhibit repeated occurrences of CHM. In an elegant example of biological logic Judson et al. [2002] suggested that these cases may be due to a genetic defect in the imprinting process and then succeeded in demonstrating that although the genome of these CHMs are biparental, all of the maternally methylated imprinted genes lack DNA methylation. In contrast, there was no effect on paternally methylated domains [Judson et al., 2002]. These results clearly suggest that patients with this defect lack some gene (other than DNMT1) involved in

the methylation of maternally imprinted gene domains. When taken together, these studies indicate that the identification and methylation of imprinted genes during gametogenesis is a complex process which involves several different types of *cis* acting elements and multiple *trans* acting factors.

METHYLATION-INDEPENDENT EPIGENETIC STRUCTURES

Both from correlative data and from experiments involving methyltransferase mutants, it is clear that DNA methylation plays an important role in the control and maintenance of imprinting. However, a growing amount of evidence now suggests that other epigenetic mechanisms must also be involved in this process. There are a number of indications that methylation alone is not sufficient to explain imprinting. Thus, although the expression of many imprinted genes is directly affected by decreased methylation in Dnmt1 knockout mice, several exceptional genes continue to show an imprinted phenotype even in the absence of DNA methylation [Reik and Walter, 2001]. At the molecular level, genetic studies have pinpointed a 1 kb region upstream of the SNRPN gene which is absolutely essential for normal imprinting on the maternal allele. Despite strong biological evidence that this region is specifically marked during oogenesis, several laboratories have demonstrated that it lacks any differential methylation. This state is evidently compensated for by a striking differential chromatin structure where the maternal allele is more accessible to DNase I and is preferentially packaged with acetylated histones. Despite the lack of methylation, this structure is normally maintained in dividing cells, suggesting that DNA methylation is not required either to generate or maintain a differential epigenetic state [Perk et al.].

In several instances, DNA methylation does not adequately correlate with gene expression at imprinted gene domains. During early spermatogenesis, for example, all methylation signals are erased in order to equalize the alleles so that they can both be re-marked as being of paternal origin [Reik and Walter, 2001]. Despite this erasure, the paternal allele still undergoes remethylation prior to the maternal allele during late spermatogenesis [Davis et al., 1999]. In the case of the *Igf2r* gene, the differential methylation pattern derived from the gametes is actually erased in the 2–4 cell embryo before being re-established at the 8-cell stage [Shemer et al., 1996]. These examples clearly indicate that both alleles can still maintain their parental identities despite the absence of differential methylation.

One of the most outstanding features of imprinting is that differential methylation patterns are maintained during preimplantation development where most methyl groups in the genome are erased [Brandeis et al., 1993]. This observation implies that these sequences must be specifically recognized as being imprinted, and strongly suggests the involvement of additional epigenetic markers. Although the mechanism for this maintenance has not been worked out, nuclear localization control of the protein Dnmt1 during a single replication cycle at the 8-cell stage appears to play a role in this process [Ratnam et al., 2002].

ASYNCHRONOUS REPLICATION TIMING

A prime candidate as an auxiliary imprinting signal is asynchronous replication timing. There is a close correlation between gene expression and its time of replication in S phase. Constitutively expressed housekeeping genes all replicate in the first half of S phase, while many tissue specific genes are developmentally regulated to replicate late in most cell types, yet early in the tissue of expression [Dimitrova and Gilbert, 1999]. The cause and effect relationship between replication timing and transcription has not yet been fully worked out, but it is now thought that late replication itself brings about the re-packaging of DNA into a closed chromatin structure, thereby repressing expression [Zhang et al., 2002]. Like DNA methylation, replication timing can be maintained in *cis* through cell division [Simon et al., 1999]. This is exemplified by the active and inactive X chromosomes in females, which replicate at different times in S phase even though they reside in the same nucleus.

In a manner similar to the X chromosome, all imprinted genes are located in large domains which undergo replication in an asynchronous manner with the paternal allele being early replicating and the maternal allele late replicating in all cells of the organism [Kitsberg et al., 1993; Knoll et al., 1994] (Fig. 1). Like methylation, this intrinsic property is erased (equalized)

during early gametogenesis and then reset in a parental specific manner during later stages of germ line development at about the time of meiosis. It is also maintained in an allele specific manner through early embryogenesis and later stages of development [Simon et al., 1999].

COORDINATION OF IMPRINTED GENES

While imprinting signals are established at key *cis* acting centers in the gametes and maintained during development, the actual coordination of gene expression in the imprinted domain is carried out by secondary mechanisms which take their cue from the original gametic markings. The imprinted Igf2-H19 domain represents a good example of this process. Both of these gene sequences are imprinted, but one of them (*Igf2*) is expressed exclusively on the paternal allele while the other is only transcribed from the maternal allele (H19), and this reciprocal pattern appears to be generated through the involvement of multiple molecular mechanisms.

The primary imprinting signal is located on a 2-kb region upstream to the *H19* gene which becomes methylated exclusively on the paternal allele during spermatogenesis [Thorvaldsen et al., 1998]. The fact that the maternal allele is unmethylated on this regulatory region causes the nearby H19 promoter to be in an open conformation, thus allowing it to be activated by downstream long-range enhancers [Davies et al., 2002]. This same upstream region also contains boundary elements which bind the protein factor, CTCF, thereby preventing these same enhancers from activating the far upstream Igf2 promoter. Methylation on the paternal allele acts as a simple switch to reverse these interactions, both by causing H19 promoter modification, and by inhibiting the binding of CTCF. This in essence cancels out the boundary function and allows the enhancers to activate the paternal *Igf2* gene [Bell and Felsenfeld, 2000; Hark et al., 2000]. It should be noted that a similar mechanism is involved in coordinating gene expression at the Dlk1-GH2 imprinted domain, as well [Schnare et al., 2000].

ANTISENSE RNA

Many imprinted gene regions are characterized by the presence of allele specific antisense RNA [Reik and Walter, 2001], and there is now good evidence suggesting that these transcripts actually play a role in controlling gene expression. A typical example of how antisense RNA participates in imprinting can be observed in the PWS/AS domain (Fig. 1). In this region on chromosome 15, a large number of genes are expressed exclusively from the paternal allele, and only two, ATPIOC and UBE3A, are transcribed specifically from the maternal allele. One of the transcripts produced from the paternal allele is antisense to UBE3A, and as such, is thought to inhibit its transcription. As a result, only the maternal allele, which lacks the antisense transcript, can express UBE3A [Chamberlain and Brannan, 2001].

The Igf2r domain on chromosome 17 in the mouse provided a good model system for testing the involvement of antisense RNA. The expression of this gene is regulated by an intronic DMR located 27 kb downstream of the start of transcription which acts as a repressor to inhibit transcription on the paternal allele. Methylation of this region on the paternal allele prevents this repression, allowing transcription of the *Igf2r* gene [Wutz et al., 1997]. Since this DMR is also the source of an extended antisense transcript, it has been suggested that the RNA itself mediates repression (Fig. 1). In order to test this idea, Sleutels et al. [2002] engineered a transgenic construct containing an RNA stop signal, and this mutation actually relieves repression of the *Igf2r* gene. Since truncation of this RNA also brings about the activation of two other nearby imprinted genes which do not overlap the antisense transcripts, this repression mechanism cannot be explained exclusively on the basis of steric interference. Using a similar approach, it has also been demonstrated that transcription of Tsix from the maternal allele serves as an antisense transcript to Xist, and it is this mechanism which brings about imprinted, paternal specific inactivation of the X chromosome in extraembryonic tissues of the mouse [Luikenhuis et al., 2001]. Since antisense transcripts have been discovered within almost every imprinted domain [Reik and Walter, 2001], it is likely that this represents a common mechanism for mediating imprinted expression. It should be pointed out, however, that in all of these cases, the use of antisense transcripts for coordinating imprinted gene activity must represent a secondary mechanism, since this molecular strategy will only work if the antisense transcript itself is imprinted to begin with.

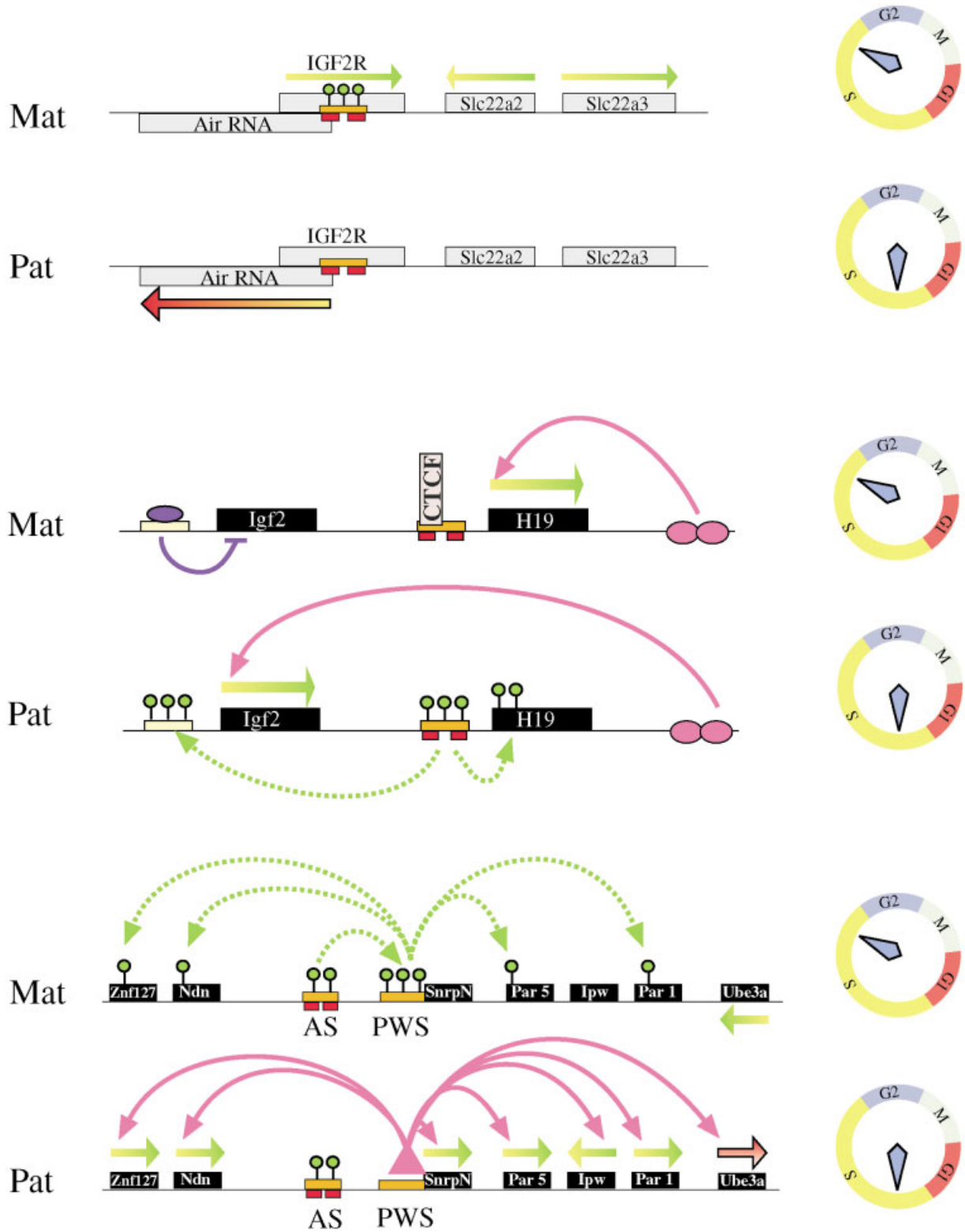

Fig. 1. Regulation of imprinting. The figure, which is not drawn to scale, provides a summary of common regulatory mechanisms used for three different imprinted domains. *Cis* acting elements (squares) generate the primary imprinting signal by causing differential methylation (lollipops) on specific regions (DMRs). These regions (light rectangle) can either cause secondary methylation (dotted arrow) or prevent the binding of specific proteins (e.g., CTCF) when methylated, apparently serve as enhancers (arrow), when unmethylated. Allele specific repression is mediated through interactions between elements on the DNA and repressor factors (oval) [Eden et al., 2001]. Gradient arrows indicate sense or antisense (framed) transcription. The clock shows the relative replication timing in S phase for each allele. It should be noted that although the AS element is not differentially methylated, it has a differentially open structure which directs the PWS element to become methylated on the maternal allele. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

IMPRINTED DOMAINS

While boundary elements and antisense RNA represent interesting examples of how *cis* acting gametic signals can be used to direct expression patterns in imprinted domains, it is clear that many additional mechanisms are involved in this process. In almost all cases of imprinting, the expression of multiple genes in large domains must be coordinated. Thus, a single primary mark at the IC has to be able to communicate with numerous loci on the same allele, even at great distances. In the PWS/AS domain, for example, the CpG island sequence located upstream to the SNRPN serves as a signal for bringing about secondary methylation on multiple small designated regions along the maternal allele (Fig. 1). It is not yet known how this is actually carried out, but it is reasonable to assume that this process must involve *cis* acting elements capable of initiating de novo methylation, as well as other sequences which mark the appropriate DNA regions as targets. On the unmethylated paternal allele, it is thought that sequences upstream to the SNRPN gene act as powerful enhancers which can activate appropriate genes on the same allele, some located as far as 2 Mb from this center. Similar mechanisms utilizing both activator and repressor elements must also be operable at other imprinting domains [Eden et al., 2001] Figure 1.

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