Review

The silence RNA keeps: *cis* mechanisms of RNA mediated epigenetic silencing in mammals

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One of the fundamental questions of modern biology is to unravel how genes are switched on and off at the right time and in the correct tissues. It is well recognized that gene regulation depends on a dynamic balance between activating and repressing forces, and multiple mechanisms are involved in both gene silencing and activation. Work over the last decade has revealed that in some cases transcriptional silencing of specific genes is mediated by RNAs that specifically recruit repressing complexes to homologous DNA sequences. Examples of both *cis* and *trans* RNA driven transcriptional silencing have been reported. This review focuses on those examples of transcriptional gene silencing in which the RNA component seems to act uniquely in *cis*. Speculative models of how such *cis* acting transcripts may trigger transcriptional silencing are proposed. Future experimental testing of these and other mechanisms is important to gain a fuller understanding of how genes are regulated and to identify instances in which such mechanisms are defective, leading to disease. Understanding the basic molecular basis of these phenomena will provide us with invaluable tools for the future development of targeted therapies and drugs for those diseases in which they are faulty.

Keywords: antisense-RNA; CpG island; cytosine methylation; transcriptional silencing; *cis* effects

1. INTRODUCTION

One of the main goals of molecular biology is to understand how genes are regulated during development and differentiation so that only the right genes are expressed at the correct time and levels in the various tissues. DNA is packaged with histones and other protein in the nucleus of eukaryotes to form a dynamic complex called chromatin. It was soon recognized that this compact structure may constitute a challenge for gene expression by preventing the interaction of transcription factors to their cognate sequences (reviewed in Struhl 1999). Therefore, initially most of the effort was put into understanding how genes are activated from within such a repressive chromatin environment. However, it has now become clear that gene regulation is a dynamic process and that gene silencing as well as gene activation plays an important role in the correct regulation of gene expression.

Silenced and active chromatin can be distinguished by a series of different epigenetic characteristics. Two of the most commonly studied epigenetic marks found at silent chromatin are methylation of particular histone tails residues (predominantly H3-Lys9 and Lys27) and DNA methylation (mainly found in plants and vertebrates; Sims 2003; Freitag & Selker 2005). Recent studies in several model organisms from yeast to human are highlighting the presence of common molecular mechanisms underlying the set up of the epigenetic modifications associated with gene silencing. Of particular interest is the evidence that in some instances transcriptional silencing is mediated by an RNA

component that gives the sequence specificity to precisely target particular DNA regions. Double stranded RNA (dsRNA) molecules are required to direct histone methylation and silencing in the yeast *Schizosaccharomyces pombe* (Hall *et al.* 2002; Volpe *et al.* 2002; Schramke & Allshire 2003) and the ciliate *Tetrahymena thermophila* (Mochizuki *et al.* 2002). Similarly, RNA directed chromatin silencing has been observed in *Arabidopsis* (Chan *et al.* 2004) and *Drosophila* (Pal-Bhadra *et al.* 2002, 2004). In mammals, RNAs have been associated with chromatin silencing in the processes of imprinting (Verona *et al.* 2003) and X-inactivation (Boumil & Lee 2001), and more recently, they have been involved in the methylation and silencing of CpG islands at autosomal and non-imprinted locations (Tufarelli *et al.* 2003; Kawasaki & Taira 2004; Morris *et al.* 2004).

In the majority of the cases of transcriptional gene silencing studied to date, the RNA component is given by dsRNAs similar to the small interfering RNAs responsible for posttranscriptional gene silencing (also known as RNA interference), and indeed the two processes share common components (Matzke & Birchler 2005). A characteristic of chromatin silencing dsRNAs is their ability to act in *trans* (Bender 2004; Kawasaki & Taira 2004; Morris *et al.* 2004). However, some evidence points to the existence of *cis* acting mechanisms of RNA mediated chromatin silencing, suggesting that distinct but related molecular machineries for epigenetic silencing triggered by RNA may be in play (Seitz *et al.* 2003; Sleutels *et al.* 2003; Tufarelli *et al.* 2003; Noma *et al.* 2004).

Since their initial discovery, the field of small RNAs has been growing at a very fast speed and several review

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papers have been published regarding dsRNA directed DNA methylation in plants and other systems (e.g. Morey & Avner 2004; Kawasaki *et al.* 2005; Matzke & Birchler 2005). This review will focus on those examples of *cis* antisense transcription, where the sense and antisense-RNA (AS-RNA) are transcribed from the same locus. In particular, will be discussed those examples of transcriptional gene silencing of CpG islands associated with the presence of AS-RNAs transcribed through the CpG island, as seen at some imprinted loci and at the *Xist/Tsix* locus. In these cases, it is not clear whether a dsRNA component is involved, and based on the available experimental evidence, speculative mechanistic models of how the single stranded AS-RNA may act to trigger transcriptional silencing of the reversely transcribed CpG island *in cis* will be proposed.

2. NATURALLY OCCURRING *CIS*-ANTISENSE TRANSCRIPTS

The discovery of RNAi and of its involvement in posttranscriptional and transcriptional gene silencing has boosted the hunt within the fully sequenced human and mouse genomes for examples of sense/antisense gene pairs with a potential to form dsRNAs.

Examples of genes within genes in the human genome have been described in the early 1990s within the human genes neurofibromatosis type I (*NF1*; Cawthon *et al.* 1990, 1991; Viskochil *et al.* 1991), retinoblastoma susceptibility gene (*RB*; Herzog *et al.* 1996), and blood clotting factor VIII (Levinson *et al.* 1990, 1992). In particular, intron 22 of the human blood clotting factor VIII gene contains a bidirectional CpG island from which two internal genes are transcribed in opposite directions, *F8A* (Levinson *et al.* 1990) and *F8B* (Levinson *et al.* 1992). Initial analysis of human chromosome 22 sequence identified two genes that are found within the introns of other expressed genes (Dunham *et al.* 1999). More recently, a study in which an arrangement of genes within genes was sought, referred to as overlapping gene groups, found that a high proportion of these genes are associated with disease (Karlin *et al.* 2002). All these genes, with the exception of *F8B*, are transcribed in the opposite orientation to that of the gene within which they lie, and to date there is no evidence to suggest that they interfere with transcription of the sense gene or vice versa.

More recent genome wide analyses have revealed the existence of *cis* and *trans* sense-antisense gene pairs, depending on whether they are transcribed from the same or different loci, respectively (Lehner *et al.* 2002). The estimates of genes associated with *cis*-antisense transcripts within the human genome have been increasing with the optimization of the criteria used for the computational analysis. The initial prediction based on the study of RefSeq mRNAs and full length EMBL protein coding mRNAs was that ~2% of the human genes are transcribed from both strands (Lehner *et al.* 2002). When the analysis was limited to expressed sequence tags (ESTs) of validated orientation, roughly the same percentage was obtained (Shendure & Church 2002). The prediction went up

to 8% when polyadenylated ESTs spanning introns were considered (Yelin *et al.* 2003). The highest estimate of about 22% sense-antisense gene pairs among human transcripts comes from a recent study considering ESTs containing both a poly(A) signal and poly(A) tail, and ESTs with either of them if they had an annotated protein coding sequence (Chen *et al.* 2004). In mouse, about 15% of the genes appear to have *cis*-antisense transcripts (Kiyosawa *et al.* 2003). Interestingly, human-Fugu and human-mouse comparisons of conserved overlapping sense/antisense pairs have revealed that these gene pairs are evolutionarily conserved and that there is a selective pressure to maintain the distance between such gene pairs constant among species, implying that their presence has affected vertebrate genome evolution (Dahary *et al.* 2005).

Taken together these observations indicate that antisense transcripts are more widespread than previously thought and argue for their role in several processes including transcription, RNA stability, pre-mRNA processing, mRNA transport and translation (Lipman 1997; Kumar & Carmichael 1998; Vanhee-Brossolet & Vaquero 1998; Carmichael 2003). Transcriptional interference, RNA masking, dsRNA dependent mechanisms and antisense-induced DNA methylation have been proposed as possible mechanisms through which *cis*-AS-RNAs can exert their regulatory functions (reviewed in Lavorgna *et al.* 2004). *cis* oppositely transcribed genes can be present in one of four different genomic arrangements, 'tail to tail' (3' end overlap, figure 1a), 'head to head' (5' overlap, figure 1b), one transcript starting within an intron of the other transcript (figure 1c), and one transcript contained within the other (figure 1d; Lehner *et al.* 2002). When genes whose promoters are associated with CpG islands are considered, the latter two configurations (figure 1c,d) are consistent with a role of AS-RNA in DNA methylation and silencing of the sense gene promoter, while figure 1b would represent genes that share a bidirectional CpG island. Interestingly, the relative orientations of the sense-antisense gene pairs in figure 1c,d have been observed at some imprinted loci, at the *Xist/Tsix* locus and at few autosomal loci, where the presence of *cis*-antisense transcripts correlates with methylation and silencing of the sense gene CpG island. These examples will be discussed in detail in the following sections.

3. ANTISENSE-RNAs SPANNING CpG ISLAND

As outlined above, *cis*-antisense transcripts in conjunction with silencing and methylation of the sense gene have been initially observed in mammals in the processes of imprinting and X-inactivation. It has been proposed that such antisense transcripts may be involved in silencing and methylation of the sense gene, but their exact role in these instances is not known. For example, it has been shown that imprinting of the *Igf2r* gene is lost when expression of its AS-RNA is abrogated by removing the intronic CpG island from which antisense transcription is initiated (Wutz *et al.* 1997). Similarly, targeted deletion of the *Tsix* gene, the *Xist* antisense gene, leads to non-random X

Figure 1. Relative organization of *cis* sense/antisense gene pairs as described by Lehner *et al.* 2002. In each pair, the gene drawn above the line is transcribed from left to right and that drawn below the line from right to left. Transcription orientation is shown by bent arrows. Same shaded boxes represent exons of the same gene. The two oppositely transcribed genes can overlap at the 3' end (a) or they can overlap at the 5' end, being divergently transcribed from a common region (b). Alternatively, they can each contain the promoter region of the other gene within one of their introns (c), or they can have a 'gene within gene' arrangement with one gene completely contained within the other (d). For the purposes of this review that focuses mainly on genes associated with CpG islands, hatched boxes of arbitrary extent above each gene promoter indicate putative CpG islands. Note that in (b) a bidirectional CpG island is shared by the two oppositely transcribed genes.

inactivation (Lee & Lu 1999). However, the molecular mechanisms by which these *cis*-antisense transcripts act remain a matter for speculation.

The first example of AS-RNA associated with methylation of the sense gene CpG island was described at the imprinted *Igf2r* locus in 1997 by Wutz *et al.* Following this observation, AS-RNAs were soon identified at other imprinted loci, at the *Xist* locus on the X chromosome, and for few non-imprinted genes. Some of the experimentally identified examples of *cis* sense-antisense gene pairs in which transcription of at least one of the two genes runs through the CpG island of the conversely transcribed gene are listed in table 1.

(a) AS-RNAs in imprinting

It is clear at first glance from table 1 that the majority of AS-RNA transcripts are found at imprinted loci, though it has to be noted that the first *cis*-antisense described is at an imprinted locus biasing the initial search for such transcripts in imprinted regions of the genome. Interestingly, all the antisense transcripts described at imprinted loci are non-coding RNAs transcribed from the paternal allele, although their expression does not always correspond to silencing of the sense gene on the same allele. For example, both of the genes within the gene pairs *MEST*(*Peg1*)/*MESTIT1*, *Igf2*/*Igf2as* and *Zfp127*(*MKRN3*)/*Zfp127as* are expressed from the paternal allele (table 1 and references therein). The mechanisms of action of these antisense transcripts are not known, but it has been suggested that antisense transcription through the *Igf2* promoters may regulate tissue specific levels of *Igf2* (Constancia *et al.* 1998). Here will be described three

gene pairs, *Igf2r*/*Air*, *Nesp*/*Nespas* and *Kvlt1*/*Lit1*, showing mutually exclusive transcription.

The *Igf2r*/*Air* and *Nesp*/*Nespas* gene pairs are examples of the configuration represented in figure 1c whereby each gene starts within an intron of the oppositely transcribed gene, and show a correlation between antisense transcription and silencing and methylation of the sense gene. A common feature of these gene pairs is that both genes are associated with CpG islands showing reciprocal methylation and referred to as differentially methylated regions (or DMRs; figure 2a,b). There are two DMRs in the *Igf2r* locus, DMR1 associated with the promoter of the *Igf2r* and DMR2 found in intron 2 of the *Igf2r* gene and associated with the promoter of the antisense transcript *Air* (figure 2a). While DMR2 is methylated in the maternal germ line and remains so in all tissues (Stoger *et al.* 1993), methylation of DMR1 on the paternal allele occurs during implantation following repression mediated by *Air* transcription (Sleutels *et al.* 2002). The first evidence for a role of antisense transcription in imprinting of *Igf2r* came from a study reporting that imprinting of the *Igf2r* gene is lost when expression of its AS-RNA is abrogated by removing the intronic CpG island from which antisense transcription is initiated (Wutz *et al.* 1997). Transgenic experiments using fragments containing the *Air* promoter highlighted the possible requirement of oppositely firing CpG islands for imprinted DNA methylation of the *Air* CpG island (Sleutels & Barlow 2001). However, deletion of the *Igf2r* promoter leading to lack of sense gene expression did not affect imprinting of *Air* and other non-overlapping genes, suggesting that the AS-RNA acts in *cis* to silence the locus and arguing against the involvement of dsRNAs to establish imprinting of these

Table 1. Examples of sense/antisense gene pairs encompassing CpG islands.

S/AS gene pairs	<i>Hs</i> chromosome	<i>Mm</i> chromosome	sense expression	antisense expression	reference
<i>Igf2r/Air</i> ^a	6q ^b	17A	maternal	paternal	Wutz <i>et al.</i> 1997
<i>MEST(Peg1)/MESTIT1</i>	7q32	6A	paternal	paternal	Nakabayashi <i>et al.</i> 2002
<i>Copg2/Copg2AS2</i>	7q32 ^b	6D	maternal	paternal	Lee <i>et al.</i> 2000; Yun <i>et al.</i> 2003
<i>Igf2/Igf2as</i>	11p	7F	paternal	paternal	Rivkin <i>et al.</i> 1993; Moore <i>et al.</i> 1997
<i>Kcnq1(or KvLQT1)/LIT1</i> (also <i>Kvlt1ot</i> or <i>KCNQT1OT1</i>) ^a	11p	7F	maternal	paternal	Lee <i>et al.</i> 1999b; Mitsuya <i>et al.</i> 1999; Smilnich <i>et al.</i> 1999
<i>Dio3/Dio3AS</i>	14q32	12F1	maternal	paternal	Hernandez <i>et al.</i> 2002
<i>Ube3A/Ube3A-AS</i>	15p	7C	maternal	paternal	Rougelle <i>et al.</i> 1998; Runte <i>et al.</i> 2001
<i>Zfp127(MKRN3)/Zfp127as</i>	15p	7B	paternal	paternal	Jong <i>et al.</i> 1999a,b
<i>Nespl/Nespas</i>	20q	2H	maternal	paternal	Li <i>et al.</i> 2000; Hayward & Bonthron 2000; Wroe <i>et al.</i> 2000
<i>WT1/WT1AS</i>	11p13	2E	maternal	paternal	Campbell <i>et al.</i> 1994; Eccles <i>et al.</i> 1994; Malik <i>et al.</i> 1995, 2000; Dallosso <i>et al.</i> 2004
<i>Xist/Tsix</i>	X	X	inactive-X	active-X	Lee <i>et al.</i> 1999a; Debrand <i>et al.</i> 1999; Mise <i>et al.</i> 1999
<i>HFE/HFE-AS</i> ^c	6p	13A	not analysed	not analysed	Thenie <i>et al.</i> 2001
<i>HBA2/HBA2AS</i> ^d	16p	NA	monoallelic	monoallelic	Tufarelli <i>et al.</i> 2003

^a These antisense transcripts originate within the imprinting control element (ICE) of the locus.

^b It is not clear whether these genes are imprinted in human.

^c This S/AS gene pair was analysed by RNase protection and it is unknown whether the transcripts are monoallelic or mutually exclusive.

^d This S/AS gene pair is only present in cells from a patient with alpha thalassaemia because of an interstitial deletion which brings the *HBA2* gene within the transcriptional unit of a ubiquitous oppositely transcribed gene, *LUC7L*. (NA, not applicable; the mouse alpha globin and *Luc7L* genes are on different chromosomes (Tufarelli *et al.* 2001, 2004).)

genes (Sleutels *et al.* 2003). Truncation of *Air* transcripts by the insertion of a polyA site results in loss of imprinting of the overlapping *Igf2r* gene but also of other non-overlapping genes, confirming the involvement of the *Air* RNA in silencing (Sleutels *et al.* 2002). Although it is clear that transcription of the sense *Igf2r* RNA is not required for silencing and methylation of the *Air* CpG island on the maternal allele, the question of how the *Air* RNA silences the overlapping *Igf2r* and other non-overlapping imprinted genes within the cluster (*Slc22a2* and *Slc22a3*) has not yet been answered. The more plausible model suggests that, similarly to the *Xist* RNA on the inactive X-chromosome, the *Air* RNA silences overlapping and non-overlapping genes *in cis* by coating a specific chromosomal region probably through the recruitment of repressor complexes (Rougelle & Heard 2002; Sleutels *et al.* 2003; Morey & Avner 2004). It is, however, possible that two mechanisms are at play in this region and that silencing and methylation of the *Igf2r* CpG island is dependent on the transcriptional overlap, while silencing of the non-overlapping genes is independent of it (Sleutels *et al.* 2003).

In addition to the differential CpG island methylation, chromatin immunoprecipitation (ChIP) studies have revealed a different pattern of histone modifications associated with the two DMRs in tissues where *Igf2r* expression is imprinted (Hu *et al.* 2000; Fournier *et al.* 2002; Yang *et al.* 2003; Vu *et al.* 2004). In these tissues, DMR1 on the maternal allele is enriched in histone modifications that have been associated with

active genes, such as histone H3 acetylated at residues K9 and K14 and methylated at residue K4, and acetylated histone H4, whereas on the paternal allele enrichment is found with antibodies to methylated K9 in histone H3, a mark for silent chromatin. The reciprocal pattern is observed at DMR2, with the paternal and maternal alleles enriched in modifications consistent with transcriptionally active or silent chromatin, respectively (Hu *et al.* 2000; Fournier *et al.* 2002; Yang *et al.* 2003; Vu *et al.* 2004). While the *Air* transcript is paternally expressed in all tissues analysed, biallelic expression of *Igf2r* is observed in brain (Hu *et al.* 1999). In brain, the *Air* promoter maintains the parent of origin specific patterns of DNA methylation and histone modifications, whereas the promoters of *Igf2r* on both alleles become indistinguishable, both showing high levels of histone acetylation (H3 and H4) and H3-K4 methylation, and low levels of H3-K9 methylation. (Fournier *et al.* 2002; Yang *et al.* 2003; Vu *et al.* 2004).

The other imprinted gene pair showing reciprocal methylation of the CpG island DMRs associated with the promoters of the two genes is the *Nespl/Nespas* pair within the *Gnas/GNAS* locus on mouse chromosome 2H and human chromosome 20q (figure 2b). This is a complex imprinted cluster, including maternally, paternally and biallelic expressed transcripts with shared exons, and three DMRs, one associated with the maternally expressed *Nespl* transcript, one with the paternally and divergently transcribed *Nespas/Gnasxl* RNAs and one with the paternally expressed *ex1A*

Figure 2. Some sense/antisense gene pairs encompassing CpG islands and showing mutually exclusive expression. Schematic diagrams (not to scale) of the organization of the mouse imprinted gene pairs (a) *Igf2r/Air*, (b) *Nesp/Nespas* and (c) *Kcnq1/Kcnq1ot1*. Paternally (black), maternally (grey) and biallelically (empty boxes) expressed genes are indicated. Exons are omitted for simplicity, except those in the *Gnas* locus in which different transcripts share common exons (b). Dotted lines in (b) indicate some of the splicing events. In (d) is shown the organization of the *Xist* (grey)/*Tsix* (black) locus with individual exons omitted for simplicity. CpG islands showing differential methylation are depicted as hatched boxes. Only the names of the genes referred to in the text are annotated. Bent arrows indicate the orientation of transcription.

transcript (Holmes *et al.* 2003). The maternally expressed *Nesp* transcript encodes the neuroendocrine secretory protein *NESP55* (Ischia *et al.* 1997). Its paternally expressed antisense, *Nespas*, is a non-coding RNA expressed as a long unspliced transcript and as a number of alternative splicing isoforms, both in human and in mouse (Hayward & Bonthron 2000; Li *et al.* 2000; Wroe *et al.* 2000; Williamson *et al.* 2002). In contrast to what is observed with deletions of the *Igf2r* DMR, deletions of the *NESP* promoter DMR in two independent families with pseudohypoparathyroidism type Ib were shown to cause loss of maternal *GNAS* imprints (Bastepe *et al.* 2005). The *NESP* DMR is methylated during postimplantation, while the *Nespas* DMR is methylated during oogenesis (Liu *et al.* 2000; Coombes *et al.* 2003), suggesting that the *Nesp* DMR contains a *cis* acting element essential for maintenance but not establishment of the maternal imprints. Nevertheless, these experiments cannot rule out the possibility that transcription of *Nesp* and/or the *Nesp* RNA itself is also important in maintaining the maternal imprints.

Although expression of *Nesp* and *Nespas* is non-complementary in mid-gestation mouse embryos and in adult interspecific mice (Li *et al.* 2000; Ball *et al.* 2001), reciprocal imprinting is observed in heart, suggesting a potential role of the antisense in regulating expression of the sense gene (Wroe *et al.* 2000). It would be interesting to further investigate the potential

role of *Nespas* in regulating the sense gene by performing experiments in which the *Nespas* DMR is deleted or the transcripts itself truncated through the introduction of polyadenylation and transcription termination site, similarly to those reported for the *Igf2r/Air* gene pair.

The *Lit1/LIT1* (or *Kvlt1ot* or *Kcnq1ot1*) gene is a non-coding RNA transcribed antisense to *Kvlt1/KvLQT1* (or *Kcnq1*) within the *KCNQ1OT1* (or *Kcnq1*) imprinted subdomain on human 11p15.5 and mouse 7F (Lee *et al.* 1999b; Mitsuya *et al.* 1999; Smilnich *et al.* 1999; table 1 and figure 2c). This gene constitutes an example of a gene within a gene (figure 1d), as it does not extend to the promoter of the sense gene but is all contained within it (Mitsuya *et al.* 1999). The DMR of the locus (KvDMR1) corresponds to the CpG islands associated with the paternally expressed *Lit1/LIT1* gene, which becomes methylated during oogenesis and is maintained differentially methylated in zygote and embryonic stem (ES) cells (Engemann *et al.* 2000). Targeted deletion of the human and mouse DMRs on the paternal allele results in lack of antisense *Lit1* RNA expression and reactivation in *cis* of several imprinted genes normally exclusively maternally expressed (Horike *et al.* 2000; Fitzpatrick *et al.* 2002). More recent experiments using transient transfection assays suggest that, similarly to what is observed with the *Air* DMR, imprinting centre function of the KvDMR1 also requires the AS-RNA, as both deletion

of promoter elements required for *Lit1* expression and truncation of the *Lit1* RNA by introduction of a polyadenylation site cause loss of bidirectional silencing (Thakur *et al.* 2004). Newly published studies have revealed that two differentially methylated regions (KvDMR1 on the maternal allele and the CpG islands of *Cdkn1c* on the paternal chromosome) are enriched in histone H3 trimethylated at residue K9 (Umlauf *et al.* 2004). Silencing of genes on the paternal allele does not require DNA methylation but displays repressive histone modifications such as trimethylation of residue K27 and dimethylation of residue K9 on histone H3 (Lewis *et al.* 2004; Umlauf *et al.* 2004). Consistently with this Eed-Ezh2 polycomb complex components are found associated with the paternally silenced genes, probably recruited by the *Lit1* RNA (Umlauf *et al.* 2004). Indeed, expression *in cis* of the *Lit1* RNA is required for establishment of the repressive histone modifications at a critical early stage in embryogenesis (Lewis *et al.* 2004). Interestingly Eed-Ezh2 is not found at the methylated KvDMR1 on the maternal chromosome despite the presence of both trimethyl H3-K27 and trimethyl H3-K9, suggesting that other complexes may be involved in methylation of H3-K27 and K9 of this DMR (Umlauf *et al.* 2004). All these data indicate that the non-coding *Lit1* RNA is necessary for *cis* silencing of genes on the paternal chromosome, probably through mechanisms that include the coating *in cis* of regions of the paternal chromosome. It is not clear, however, if transcriptional overlap of the *Kvlt1* gene across KvDMR1, the CpG island of the *Lit1* gene, is required for its silencing and methylation on the maternal allele.

(b) *The Xist/Tsix sense-antisense gene pair*

Xist/Tsix is a well characterized mouse sense/antisense gene pair expressing non-coding RNAs that are involved in X-chromosome inactivation, the process that leads to the silencing of one X-chromosome in mammalian female cells to achieve dosage compensation of X-linked genes between males and females (figure 2d; Willard & Carrel 2001). *Xist* and *Tsix* show a reciprocal pattern of expression and the CpG islands associated with the promoters of *Xist* and *Tsix* are methylated in a reciprocal manner (Courtier *et al.* 1995). The initiation of X-inactivation, which ultimately results in the methylation of many X-encoded CpG islands, requires the expression *in cis* of the non-coding RNA *Xist* which spreads and coats the inactive X chromosome (Lee & Jaenisch 1997). Although spreading of silencing on the inactive X chromosome requires the presence of the *Xist* RNA and its ability to coat *in cis* the inactive X chromosome (reviewed in Park & Kuroda 2001), initiation of X-inactivation and X chromosome choice rely on the ability to regulate the levels of expression of the *Xist* RNA itself. In mouse, *Xist* expression appears to be regulated by the expression of its AS-RNA *Tsix* (Lee & Lu 1999; Lee *et al.* 1999a,b). Consistent with this, deletion of the *Tsix* CpG island results in inactivation of the targeted allele in all cells, suggesting a role for *Tsix* in X-chromosome choice (Lee & Lu 1999). Further, truncation of the *Tsix* RNA by the insertion of a transcription termination site results in inactivation of the targeted allele, supporting

the idea that antisense transcription and/or RNA play a primary role in downregulating *Xist* expression (Luikenuis *et al.* 2001; Shibata & Lee 2004). Constitutive expression of *Tsix* driven by constitutive or inducible promoters prevents the targeted allele from being inactivated, indicating that to allow accumulation of *Xist* on the future inactive X chromosome *Tsix* needs to be down-regulated (Stavropoulos *et al.* 2001; Luikenuis *et al.* 2001). Interestingly, *Tsix* mRNA expressed from the same locus but not overlapping *Xist* cannot rescue the *Tsix* knockout phenotype, indicating that transcription through *Xist* is necessary for its down regulation (Shibata & Lee 2004). Concomitant expression of *Tsix* and low levels of unstable *Xist* RNA prior to X-inactivation (Panning *et al.* 1997; Sheardown *et al.* 1997) have suggested a possible involvement of double stranded RNA in X-chromosome choice (Boumil & Lee 2001). Indeed, deletions upstream of the *Xist* promoter cause preferential inactivation of the X chromosome carrying the targeted allele but do not affect *Tsix* levels (Newall *et al.* 2001; Nesterova *et al.* 2003), supporting a model in which RNAi related mechanisms triggered by *Xist/Tsix* double stranded RNA may act *in cis* to repress *Xist* transcription (Nesterova *et al.* 2003).

Analyses of the chromatin structure at the *Xist* locus have been performed using allele specific assays in female cells. DNaseI sensitivity studies have revealed an increase in sensitivity limited to the transcribed *Xist* locus on the inactive X (McCabe *et al.* 1999). ChIP assays have shown that on the inactive X expressing *Xist*, the promoter region is marked by acetylated H3 while H3-K4 methylation and H4 acetylation span the whole gene (Goto *et al.* 2002). Conversely, the repressed *Xist* promoter and gene on the active X is enriched in H3 methylated at residues K9 and K4 (Goto *et al.* 2002). Therefore, the chromatin modifications found reflect the expression status of *Xist* on the active and inactive alleles. Newly published data show that *Xist* expression in mouse cells displaying either imprinted or random X inactivation is accompanied by enrichment in H3-K4 methylation and RNAPII preinitiation complex (PIC) at the *Xist* promoter on the expressed allele (Navarro *et al.* 2005). In contrast, in ES cells, in which both X chromosomes are active, low levels of *Xist* expression can be explained by low levels of PIC at its promoter (Navarro *et al.* 2005). Truncation of the *Tsix* transcript in ES cells does not lead to PIC enrichment at the *Xist* promoter or to increased levels of *Xist* expression, indicating that in these cells *Tsix* transcription is not required to down-regulate *Xist* expression, and the authors propose that *Tsix* transcription renders the two alleles epigenetically equivalent prior to the onset of random X-inactivation (Navarro *et al.* 2005). However, a role for *Tsix* RNA in silencing and methylation of the *Xist* CpG island upon ES cell differentiation cannot be ruled out.

In conclusion, the data summarized here are consistent with the proposed potential dsRNA mediated mechanisms, but they do not exclude a possible co-transcriptional action of the single stranded *Tsix* RNA in silencing the *Xist* CpG island.

Figure 3. A disease associated deletion leads to expression of aberrant antisense RNA transcripts through a tissue specific CpG island. The diagrams show the relative organization of the erythroid specific alpha globin gene (*HBA*, light grey box) and the ubiquitous *LUC7L* (dark grey box) gene in the wild type allele and in the deleted allele. The deletion (striped box) removes the transcription termination site of the *LUC7L* gene and leads to the expression of antisense transcripts running through the *HBA* CpG island (dashed arrow). This results in silencing of the *HBA* gene *in cis* of the deletion and full methylation of its associated CpG island (black bar). Unmethylated (grey) and methylated (black) CpG islands are drawn as bars. Only the names of the genes described in the text are shown. Diagrams are not to scale.

(c) AS-RNA at non-imprinted autosomal loci

Few experimentally identified cases of non-imprinted overlapping gene pairs spanning the promoter regions of the oppositely transcribed genes have been reported (table 1). For example, the presence of a non-coding polyadenylated AS-RNA transcript encompassing exon 1 and up to 1 kb upstream of the haemochromatosis gene *HFE* has been described, and it has been shown that the AS-RNA transcripts can interfere *in vitro* with the expression of sense gene product (Thenie *et al.* 2001). However, it is not known whether the AS-RNA is acting to regulate *HFE* expression at a transcriptional or translational level.

AS-RNA transcription through the CpG island of a non-imprinted and autosomal tissue specific gene can lead to its silencing and methylation (Tufarelli *et al.* 2003). This phenomenon was observed in a patient with an inherited form of anaemia (α thalassaemia), in whom the decrease in expression of the erythroid specific alpha globin gene (*HBA*) is due to changes in chromatin structure, including the complete methylation of the alpha globin CpG island (Barbour *et al.* 2000). In this patient, a small interstitial deletion results in the juxtaposition of the intact erythroid specific alpha globin gene to a truncated, widely expressed gene (*LUC7L*), transcribed in the opposite orientation to that of the alpha globin gene (Tufarelli *et al.* 2001; figure 3). This results in a 'gene within gene' (figure 2d) configuration of the *HBA* gene on the deleted allele. In this patient and in a transgenic mouse model, methylation of the alpha globin CpG island is dependent upon the presence of AS-RNA transcripts (*HBA-AS*) transcribed *in cis* of the methylated gene from the promoter of the truncated *LUC7L* gene (Tufarelli *et al.* 2003). The *cis* nature of this AS-RNA mediated DNA methylation event is supported by the observation that the normal allele of the patient is not methylated (Barbour *et al.* 2000). In addition, F1 double hemizygous mice, generated by mating

transgenic mice carrying constructs reproducing the AS-RNA mediated DNA methylation observed in the patient and mice containing a normal human alpha globin gene, display methylation only at the alpha globin gene *in cis* of the antisense transcripts (Tufarelli *et al.* 2003). The use of a differentiating mouse ES cell system revealed that methylation of the alpha globin CpG island is observed uniquely in the presence of AS-RNA transcripts and requires induction of differentiation in embryoid bodies (Tufarelli *et al.* 2003). The identification of AS-RNA mediated DNA methylation as a novel mechanism of human genetic disease indicates that this phenomenon is not specific to some imprinted genes or X-inactivation but it may be a more general mechanism by which associated genes become silenced during development, cell differentiation and malignancy progression. Moreover, the ES cell data clearly show that the machinery responsible for establishing DNA methylation via AS-RNA is normally present in the cells. Although a role for double stranded RNA cannot be excluded as both sense and AS-RNAs are expressed in undifferentiated ES cells, the observed *cis* effect points to a possible co-transcriptional effect triggered by the single stranded AS-RNA.

4. DISCUSSION

There are several examples of naturally occurring AS-RNAs in higher eukaryotes, and in some situations, like the cases described above, overlapping transcription across CpG island promoter regions has been associated with their transcriptional silencing and methylation. It has been proposed that the RNA transcripts may be directly involved in the silencing process, but their mechanism of action remains largely unknown. RNA-directed DNA methylation, or RdDM, is a well-known process in plants where expression of dsRNA spanning the promoter region of genes leads to silencing and methylation of the

Figure 4. Models of antisense RNA mediated CpG island methylation *in cis*. The newly transcribed antisense RNA (dark grey arrow) driven by the antisense promoter (bent dark grey arrow) could interact with the DNA locus (black) from which it is transcribed to form specialized structures, such as an RNA/DNA triple helix (a), or an RNA/DNA hybrid and a DNA loop (b). Alternatively, both antisense and sense (light grey arrow) RNAs could form an RNA/DNA hybrid with their respective template strands (c). These unusual structures could recruit (dashed arrows) by unknown mechanism the DNA methyltransferases and/or other repressive complexes (oval), leading to methylation (filled lollipops) and silencing (crossed arrow) of the sense promoter (bent light grey arrow) without preventing continued expression of the antisense RNA. Note that these models do not address the order of the silencing events, i.e. whether histone modifications precede or follow DNA methylation, but can accommodate either.

homologous DNA sequences with mechanics highly related to those of RNA interference (e.g. [Mette *et al.* 2000](#); see [Mathieu & Bender 2004](#) for a recent review). RdDM in plants acts *in trans*, i.e. it does not require co-expression of sense and AS-RNA at the target site, and leads to methylation of all cytosines, not only those in the CpG or CpNpG sequences ([Pelissier *et al.* 1999](#); reviewed in [Matzke *et al.* 2001](#)). It has been proposed that similar dsRNA mechanisms act in mammalian cells, and indeed recent reports have shown that introduction of short interfering RNAs (siRNAs) homologous to endogenous CpG island promoter sequences can lead to methylation and silencing *in trans* of the endogenous target promoters (e.g. [Kawasaki & Taira 2004](#); [Morris *et al.* 2004](#)). However, the sense/antisense-RNA examples described in this review seem to function strictly *in cis* to silence and methylate CpG islands. Nevertheless, the involvement of dsRNAs in some or all of these cases cannot be excluded. It is possible that in mammalian cells the dsRNAs generated by these loci are very unstable or cannot diffuse to the other allele, or can act through the interaction with the nascent AS-RNAs that are expressed by one allele only. Interestingly, in *S. Pombe* nascent RNAs at the centromeric repeats recruit complexes containing components of the RNAi machinery through the interaction with RNA guides derived from siRNAs processed from dsRNAs expressed by the centromeric repeats themselves ([Motamedi *et al.* 2004](#); [Noma *et al.* 2004](#)). The *cis* interaction of the complexes with the nascent RNA is critical for establishment of a silent chromatin state at centromeres ([Motamedi *et al.* 2004](#)), but it cannot be excluded that the recruited complexes at a given centromere may contain guide RNAs originating at different centromeres. Although it is important to determine whether this or similar mechanisms are at work in the mammalian systems reported here, their *cis* action would imply a

thermodynamic component given by the sequences of the siRNAs, so that in the complexes only the sense strand is retained allowing the recognition of exclusively the nascent antisense RNA.

It is, therefore, tempting to speculate the existence of alternative mechanisms, that do not require the formation of dsRNAs, to explain how AS-RNAs running through a CpG island can trigger its *cis* silencing and methylation. One of the first questions to address is whether antisense transcription *per se* rather than the RNA itself can be causing the silencing. While this cannot be excluded, both sense and antisense intergenic transcription have been associated with gene activation ([Gribnau *et al.* 2000](#); [Bolland *et al.* 2004](#)). In these cases, intergenic transcription is developmentally regulated and it has been proposed that transcription may be involved in the establishment of an open chromatin environment ([Gribnau *et al.* 2000](#)). As RNA polII complexes contain histone acetyltransferase activities, it seems unlikely that they would favour the formation of repressive chromatin when running through a CpG island. After all it is only transcription from the methylated CpG island that is blocked, as antisense transcription continues to occur through the methylated region. However, it is still possible that transcription is required to render the locus accessible to the DNA and histone methyltransferase containing complexes that may be recruited by the AS-RNA to lock the sense CpG island in a transcriptionally silenced state (see below).

Several options can be envisaged to explain RNA mediated *cis*-silencing in alternative to double stranded RNA mechanisms. The models put forward in [figure 4](#) are based on the assumption that, at least in some instances, the observed *cis* effects are due to the ability of AS-RNA to co-transcriptionally interact with the locus from which it is transcribed. These models do not attempt to explain the *cis* action of the *Xist* RNA, but

rather those CpG island silencing examples that seem to require the presence of AS-RNA which are complementary in sequence to the silenced CpG island, including the silencing of the *Xist* CpG island itself. It is proposed that transcription of AS-RNA could result in the formation of an RNA/DNA triple helix (figure 4a). Alternatively, the newly transcribed AS-RNA strand and its template DNA strand could form an RNA/DNA hybrid with the non-template strand forming a single stranded DNA loop (figure 4b). It is also possible that both sense and antisense-RNA could interact with their respective template strands to form RNA/DNA hybrids (figure 4c). Such unusual structures might attract DNA methyltransferases (Smith *et al.* 1991; Laayoun & Smith 1995), guiding them to the target sequence. These series of events could also play a part in the chromatin modification observed at the oppositely transcribed CpG islands through the recruitment of chromatin remodelling complexes and methyl CpG binding proteins (Jones *et al.* 1998; Nan *et al.* 1998; Razin 1998; Ng & Bird 1999; Wade *et al.* 1999; Zhang *et al.* 1999; see Freitag & Selker 2005 for a recent review). However, these models do not address the temporal order in which DNA methylation and histone modifications appear at the silenced locus, but can accommodate alternative possibilities, with DNA methylation preceding or following histone modifications depending on the local context.

Bioinformatics tools have revealed a surprising number of sense/antisense gene pairs in the genome with a potential to be regulated by the *cis* mechanisms described above, suggesting that these mechanisms may play a more general role in normal regulation of gene expression and are not restricted to imprinted genes or to pathological situations. There are examples of random monoallelically expressed genes (e.g. immunoglobulin genes, T cell receptors genes, olfactory genes, etc., Chess 1998), but the mechanisms leading to their monoallelic expression remain elusive (see Goldmit & Bergman 2004 for a review). It is possible that in some cases monoallelic expression is achieved through the mechanisms described here. However, monoallelic gene expression is not necessarily the inevitable output of AS-RNA mediated silencing *in cis*, but these mechanisms may account for developmental stage and/or tissue specific gene silencing at some loci.

In summary, the available observations suggest that more than one RNA mediated mechanisms can lead to gene silencing and CpG island methylation. Based on the research summarized in this review, alternative mechanisms through which *cis* AS-RNA transcripts can lead to CpG island methylation have been proposed. Future work will aim to determine whether any of these mechanisms are at work to regulate expression of CpG island associated genes during development and differentiation, and if they contribute to altered gene expression in disease.

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