PERSPECTIVE

RNAi – Is one suffix sufficient?

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RNA-directed translational gene silencing in many organisms appears to represent a later evolved function for this versatile molecule, and the observation that protein domains similar to those of RISC complex components in *Tetrahymena*, fission yeast, fungi and archaeal bacteria suggests that RNA interference as it is currently understood may have developed as a functional entity in the last common ancestor of eukaryotes (Cerutti and Casas-Mollano, 2006). Crystal structures indicate that the PIWI domain of ARGONAUTE proteins is a member of the RNase H superfamily, supporting the notion that RNAi evolved as a defense against genomic parasites (Parker et al, 2004; Cerutti and Casas-Mollano, 2006). A range of new studies are now beginning to reveal both a diversity of cellular small RNAs, and potential functions other than translational silencing in higher organisms. It is becoming increasingly apparent that the role of RNA in coordinating and facilitating a wide variety of cellular processes has been underestimated.

Traditional exogenous short RNA mediated gene silencing efforts have been designed to target an mRNA sequence. Most endogenous miRNA also appear also to target the transcribed RNA and so RNAi has generally been considered a post-transcriptional regulatory mechanism (Bernstein et al, 2001; Broderson and Voinnet, 2006). Experiments in fission yeast have showed that complexes containing siRNAs can be co-purified with proteins involved in transcriptional silencing, suggesting that small RNAs might have nuclear silencing roles also (Reinhart and Bartel, 2002; Volpe et al, 2002). Whilst transcriptional regulation by RNA has been implied since the observation of RNA-induced DNA methylation in plants over 10 years ago, (Wassenegger, 1994), the relevance of this observation to small RNAs and animal cells has only recently come into focus.

Small RNAs play a role in histone H3 methylation at centromeres in *S.pombe*, indicating that such a mechanism is not limited to plants (Volpe et al, 2002; Reinhart and Bartel, 2002; Buhler et al, 2006). Experiments in *C. elegans* and *Drosophila* identified potential transcriptional

regulatory roles for small RNAs that involve chromatin modification (Pal-Bhadra et al, 2004; Grishok et al, 2005). RNAi-induced Transcriptional Gene Silencing (RNAi-TGS) in *C. elegans* is dependent upon heterochromatin factors, reduced by histone deacetylase inhibitors and is associated with a decrease in acetylated histone H4 protein and less polymerase II recruitment to the initiation complex. Mutations in the RNAi pathway genes *dcr-1*, *rde-1*, *rde-4*, and *rrf-1* refract RNAi-TGS, strongly indicating that this is a nuclear mechanism of transcriptional regulation mediated by the RNAi machinery (Grishok et al, 2005).

The experimental application of RNA interference has become routine, but several new observations suggest that small RNAs might induce unappreciated cellular effects such as sequence specific transcriptional silencing or activation. Twenty-one nucleotide dsRNA targeted to the promoter regions of stably transfected CCR5 and RASSF1A or endogenous Huntingtin, Androgen receptor or Progesterone receptor loci represses transcription in cell culture, whilst at the promoters of E-Cadherin, p21 and VEGF genes, exogenous dsRNA enhances the steady state level of transcription (Janowski et al, 2006; Kim et al, 2006; Li et al, 2006) .

Answering the question of whether these observations result from currently documented silencing mechanisms, or represent novel (perhaps endogenous) pathways of small RNA regulation is of some significance as, if borne out, the findings might offer a means to specifically activate transcription with small RNAs in an analogous manner to gene silencing by post transcriptional RNAi.

'agRNAs'

Demonstration of transcriptional silencing in higher organisms remains controversial, despite the observation of transcriptional gene silencing on application of small RNAs in some human cell lines. Transfection of short double-stranded RNA corresponding to transcription start sites and promoter regions leads to gene silencing in some instances (Janowski et al, 2005; Suzuki et al, 2005). Pep-

tide nucleic acids (PNA) targeted to the same sequence as such 'antigene' (agRNAs) provide a similar level of transcriptional inhibition through a mechanism that may involve direct binding to either single or double stranded genomic DNA (Janowski et al, 2005). Given the strong indication that components of the RNAi pathway are involved in RNAi-TGS, the question of whether such agRNAs are acting through an endogenous mechanism or by steric interference remains to be addressed – as must be conflicting reports indicating that agRNAs may induce DNA methylation. It may be noteworthy that agRNAs appear slightly more effective at inhibiting transcription that their PNA counterparts, though activity varies significantly depending on the sequence chosen (Janowski et al, 2005). If an endogenous mechanism exists, selection of a 'natural' target site might provide a more obvious difference in activities between agRNAs and PNAs.

There are many difficulties in delineating the mechanisms behind the observed effects in immortalized cell lines. Precise definition of transcriptional start sites for many genes is required to eliminate an mRNA or pre-mRNA based effect. Reports employing genomic scanning arrays suggest low-level sense and antisense non-coding transcription across a large number of genomic loci, and so an in-depth characterization of transcription around a small RNA target site is especially important in understanding how the effector oligonucleotide interacts with the target (Kapranoc et al, 2005).

Another issue of interest in these reports is the discrepancy in the ability to immunoprecipitate AGO2 at target promoters - Janaowski et al detect AGO2 at their target promoters, whilst and Kim et al find no enrichment of AGO2 by ChIP 18 hrs after dsRNA transfection. If RNA directed protein recruitment drives histone modification, components of the RNAi silencing machinery may be recruited to, and then dissociate from, a locus prior to histone modification – if indeed chromatin modification is dependent upon dsRNA at all. Assaying 24-48 hours after dsRNA application might fail to recognize short term initial recruitment of AGO proteins to a target promoter, and so lead to the discrepancies observed.

The association of small RNAs with RITS and chromatin modifying factors indicates that RNA can direct recruitment of such proteins to sites in genomic DNA (Volpe et al 2002). The prospect of designing nucleic acids, or nucleic acid mimics that might target genomic DNA and modulate transcription offers further scope for developing efficient nucleic acid based therapies, and also highlights yet another potential role for small RNAs in dampening gene transcription. A far more intriguing idea is that small RNAs might be involved not just in silencing, but also transcriptional activation.

RNA activation ('RNAa')

An interesting and potentially exciting recent observation has been the ability of small RNAs targeted to promoter regions to activate transcription (Li et al, 2006). More than three decades ago, Britten and Davidson proposed the idea that "activator" RNAs, derived from non-coding genomic regions, might activate transcription of a number of protein coding genes (Britten and Davidson, 1969). Li et al (2006) now describe activation of transcription at the *E-Cadherin*, *p21* and *VEGF* loci in mammalian cells through transfection of small dsRNAs. Although the authors were not able to predict the gene activating ability of these promoter targeted dsRNAs, they did observe an apparent bias in activity based upon the strand loading characteristics of AGO proteins, implying that these dsRNAs might interact with some components of the RNAi machinery.

A commonality in current reports of transcriptional activation is the presence of retrotransposon or CpG repeat sequences in the promoter regions of such genes. As with transcriptional silencing, an understanding of the transcriptional activity in the region of the siRNA target sites is crucial in understanding by what means the observed effect is mediated. Many reports of transcriptional modulation by small RNAs concern promoters in CpG or transposon rich regions, and the inherent transcriptional silencing properties of these elements is poorly described - with some evidence linking the processing of such repeat sequences to the RNAi machinery (Yang and Kazazian, 2006). The binding of small RNAs nearby these chromatin silencing elements might disrupt their repressive activity and in itself lead to gene activation - without requiring a novel RNAi effect. Such silencing of silencers may still prove important, however, as CpG repeats have been estimated present at greater than 50% of human gene promoter regions.

An increasingly relevant observation in many immortal cell lines is the broad upregulation of transcription across many loci. It is not clear whether such loss of transcriptional dampening is a consequence of immortalisation, necessary to enhance the likelihood of a cell becoming immortalised, or due to experimental selection for rapidly dividing cells - but it has important implications for studies of transcriptional silencing and activation. Selection of cells in culture must be avoided; it must be carefully demonstrated that cells expressing higher levels of specific or global transcripts are not selected for due to toxicity of a specific siRNA transfection, as this may lead to apparent changes in transcription at assayed loci. Rescue experiments that alter the target, and then compensate with changes in the small RNA effector, provide more convincing proof, as the unpredictable off-target effects of small RNA sequences that might alter the translation of many proteins still complicate attribution of observed effects to a specific interaction.

The binding of small RNAs to promoters also has the ability to displace transcriptional activators or repressors in a manner that is not limited specifically to the target site, and may be translated some distance up or downstream through the effect of target site binding on adjacent sequences. Current reports have not been able to correlate activity of promoter targeted siRNAs to inhibition of transcription factor binding or polymerase seating. Li et al (2006) also pertinently note that the apparent involvement of AGO proteins in transcriptional regulation suggests new complications in designing effector RNAs, as ~7-8 nt seed sequence recognition may be sufficient to target the pro-

moters of many other genes, leading to unwanted off-target effects.

Sequence and temporally specific transcriptional activation by small RNAs indicates the potential for a nuclear transcriptional regulatory network perhaps as complex as that mediated by protein factors. The complementary base specificity of nucleic acids might enhance the fidelity of such regulation, and also provides researchers with highly discriminative targets for manipulating levels of transcription. The application of small dsRNAs to silence or activate gene transcription may also, however, face inherent obstacles relative to cytoplasmic post-transcriptional RNAi – such as achieving efficient nuclear import and identifying highly inhibitory target sites - yet if a mechanism of RNAi-based transcriptional control proves compatible with small RNAs then it will provide a new and powerful tool for manipulating gene expression. Unambiguous proof of a small RNA transcriptional regulatory system in mammalian cells is eagerly awaited.

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