Interrelationship of RNA Interference and Transcriptional Gene Silencing in *Drosophila*

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Gene silencing processes that operate within the nucleus first came to light with studies of transgene silencing in plants (Matzke et al. 1989; Napoli et al. 1990; van der Krol et al. 1990). This body of work suggested the involvement of double-stranded RNA molecules as the catalyst for posttranscriptional silencing (Metzlaff et al. 1997). Studies of plant viruses, which are targets of posttranscriptional silencing (Goodwin et al. 1996), resulted in the finding of RNA-dependent DNA methylation in the nucleus (Wassenegger et al. 1994; Mette et al. 1999, 2000; Jones et al. 2001). When the finding was made that the experimental procedure of RNAi likely worked through a double-stranded RNA process (Fire et al. 1998), biochemical studies were inspired that led to the discovery of what is referred to as the “RNAi machinery” (Tuschl et al. 1999; Hammond et al. 2000, 2001; Zamore et al. 2000; Bernstein et al. 2001). This machinery not only works to destroy homologous messenger RNAs in a posttranscriptional manner (Montgomery et al. 1998), using siRNAs as a guide (Hamilton and Baulcombe 1999), but also is involved with processing of micro-RNAs that play a role in normal development of plants and animals (Grishok et al. 2001; Hutvagner et al. 2001) as well as with targeting sites on the chromosomes for chromatin modifications, which ultimately result in transcriptional silencing (Pal-Bhadra et al. 1997, 2002, 2004; Morel et al. 2000; Sijen et al. 2001; Hall et al. 2002; Volpe et al. 2002; Schramke and Allshire 2003; Verdel et al. 2004). In this paper we will focus on studies in our laboratory concerned with novel aspects of transcriptional gene silencing and its interrelationship with the RNAi machinery.

These studies began with the construction of a promoter–reporter fusion transformant designed to study regulatory factors effective on the *white* eye color gene in *Drosophila* (Rabinow et al. 1991). This plasmid consists of the *white* gene promoter fused to the *Alcohol dehydrogenase* structural gene in the respective 5′ leader sequences. It was transformed into a background carrying an *Adh* allele with very low expression. This *w-Adh* transgene was expressed less when homozygous at any location in the genome (Rabinow et al. 1991) than when only

a single copy was present. This behavior was reminiscent of the phenomenon of pairing-sensitive silencing described by Judy Kassis for *engrailed-white* transgenes (Kassis et al. 1991; Kassis 1994; Americo et al. 2002).

A further unusual property of the *w-Adh* transgenes was that they were not additive in expression when multiple copies were present in the cell at dispersed locations (Pal-Bhadra et al. 1997). This type of behavior was analogous to the phenomenon of cosuppression that had been described in plant species for numerous transgenes. This silencing was modulated by various mutations in the genes encoding the Polycomb complex of repressive chromatin proteins. Indeed, when silencing is operative, the silenced transgenes become ectopically associated with the Polycomb complex. This type of silencing occurs at the transcriptional level (Pal-Bhadra et al. 2002).

Other transgenes, such as a full-length *Adh* construct, exhibit silencing that operates at the posttranscriptional level (Pal-Bhadra et al. 2002). This type of silencing is associated with the production of siRNAs and thus fulfills all the hallmarks of operating via the RNA interference pathway. Mutations in this type of silencing are blocked by mutations in the *piwi* gene (Cox et al. 1998), which is a member of a gene family characterized by the PAZ domain. Members of this family are involved with RNAi functions in many species (Cerutti et al. 2000).

The transcriptional silencing characteristic of an interaction between *w-Adh* and a reciprocal *Adh-white* transgene is also blocked by mutation in the *piwi* gene (Pal-Bhadra et al. 1999, 2002) and to a lesser extent by mutations in another member of the same gene family, *aubergine* (Schupbach and Wieschaus 1991; Schmidt et al. 1999; Birchler et al. 2003). These findings indicated a connection between posttranscriptional and transcriptional silencing in *Drosophila*.

With the finding that heterochromatic silencing in fission yeast was suppressed by mutations in the RNAi machinery (Hall et al. 2002; Volpe et al. 2002), tests were conducted as to whether such mutations in *Drosophila* would suppress various types of heterochromatin-based silencing (Pal-Bhadra et al. 2004). Both *piwi* and *aubergine* suppress *mini-white* insertions in the fourth chromosome heterochromatin. Another gene, *homeless*, which encodes a double-stranded RNA helicase required for RNAi (Aravin et al. 2001; Stapleton et al. 2001; Kennerdell et al. 2002), suppresses these transgene insertions as well as repeat-induced silencing of a tandem array of

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miniwhite (Dorer and Henikoff 1994), which shows variegated silencing. These findings provide further evidence of an involvement of the RNAi machinery in the establishment of chromatin domains.

IS THERE A RELATIONSHIP BETWEEN COSUPPRESSION AND PAIRING-SENSITIVE SILENCING?

The *trans* silencing of *w-Adh* and involvement of the Polycomb complex raises the issue of whether an analogous mechanism operates on the silencing observed with transgenes that carry a Polycomb Response Element (PRE). These transgenes typically exhibit pairing-sensitive silencing, as does *w-Adh*. In those cases examined, increased copy number in the nucleus results in further decline in total expression of the collective homologous transgenes (Hagstrom et al. 1997; Sigrist and Pirrotta 1997; Muller et al. 1999), again in analogy with *w-Adh*.

The *engrailed-white* transgenes carry a PRE and were the prototypical case of pairing-sensitive silencing (Kassis et al. 1991; Kassis 1994; Brown et al. 1998; Americo et al. 2002). We used multiple dispersed copies to test whether dispersed silencing was also operative with this transgene. When a large fragment of *engrailed* (2595 bp) was carried on the transgene adjacent to the *mini-white* reporter insertions at various locations in the genome, they exhibited the normal pairing-sensitive silencing. The general assay for pairing-sensitive silencing is a test of whether two paired copies are expressed less than two copies unpaired in the genome. This is indeed the case for insertions of this class (the G series). An example is shown in Figure 1. However, two unpaired copies are not additive in their expression, and further increase in copy

Figure 2. One to four doses of the *en-w* (series G) transgene shows declining total expression. At the *bottom* is one copy of *en-w*. *Counterclockwise* are two copies, each unpaired; three copies consisting of two copies paired with each other and a third copy unpaired; four copies in which two insert sites are both homozygous. Although not shown, the four copies (two insertion sites homozygous) are expressed less than either paired insert alone.

number causes less total expression (Fig. 2). The pairing effects and *trans*-acting silencing are also reflected in the RNA level of *white* expression (Fig. 3). This departure from additivity illustrates that this class of *en-w* transgene participates in dispersed transgene silencing as well, a phenomenon referred to as cosuppression.

A second class of *en-w* transgenes (the H series) has only 400 bp of *engrailed* adjacent to the *mini-white* reporter. In this case the sequences of *engrailed* thought to be the major determinant of pairing-sensitive silencing are missing. When the transgenes are paired at homologous sites, the expression of the *mini-white* reporter is additive in the homozygotes for the respective insertion sites (Figs. 1 and 4). With respect to dispersed silencing, there is no cosuppression observed with this particular transgene (Figs. 1 and 4).

Last, a transgene containing only the portion of *engrailed* thought to play a major role in attracting the Polycomb complex was tested. This transgene series (TVup) contained only 181 base pairs of the *engrailed* gene. This small segment is sufficient to condition pairing-sensitive silencing (Figs. 1 and 5). Interestingly, however, this transgene construct does not exhibit dispersed transgene silencing. Therefore, although there may be determinants in common between pairing-sensitive silencing and cosuppression, the results with this series suggest that those sequences that can confer pairing-sensitive silencing are insufficient to produce cosuppression or that some sequence in this fragment can interfere with cosuppression.

MUTATIONS IN THE RNAi MACHINERY AFFECT PAIRING-SENSITIVE SILENCING

Given that the Polycomb complex has been implicated in both cosuppression and pairing-sensitive silencing, albeit with mechanistic distinctions, it was of interest to test

Figure 1. The eye color of multiple *engrailed-white* (*en-w*) transgenes showing a comparison of pairing-sensitive silencing and cosuppression. The length of *engrailed* sequences, chromosomal location, and allelic combinations are noted in the panel. Each *en-w* construct was tested in at least two genomic locations. The *engrailed-white* gene carrying approximately 2595 bp of *engrailed* sequences shows pairing-sensitive silencing. The presence of 400 bp of *engrailed* sequences next to *mini-white* shows a gene dosage effect in paired copies at each location. When only 181 bp upstream to the 400-bp sequence are present, pairing-sensitive silencing occurs, but cosuppression of dispersed copies is not observed.

Figure 3. Autoradiograms of Northern blot hybridization from adult female flies carrying 1 and 2 copies of *en-w* constructs demonstrate the trends of interaction at the transcript level. Although only blots using female RNA are shown, males and females follow a similar pattern. The genotypes depicted in the autoradiogram are as follows: (1) *en-w#2/+* (chromosome 2), (2) *+en-w#3* (chromosome 3), (3) *en-w#2/enw#2*, (4) *en-w#3/en-w#3*, and (5) *en-w#2/en-w#3*. The bottom panel represents β -tubulin transcript level after reprobing the same blot, which acts as a gel loading control. The abundance of *white* transcript relative to β -tubulin loading from three independent blots is presented by a bar diagram above the panels. The error bars delimit the 95% confidence intervals. Those values significantly different between the two inserts are designated with an open circle. Those significantly different between the paired and unpaired condition are designated with an asterisk. All flies are in a *y w[67c23]* background. The autoradiogram shows a reduction of *white* transcripts for the paired copies relative to the single insert at each location. Note that both pairing silencing and cosuppression are reflected in the level of the *white* transcripts.

whether pairing-sensitive silencing would be modulated by mutations in the RNAi machinery as is cosuppression. Thus, various insertions of the *engrailed-white* transgene were combined with different alleles of *piwi* and *homeless*.

The insertions A2 and G1A, both of which exhibit pairing-sensitive silencing, were genetically combined with *piwi*¹, *piwi*², and the heteroallelic combination. The homozygous *piwi* mutations do not have any discernible phenotypic effect on a single unpaired copy of either insertion. However, the paired copies have reduced expression, which is diminished even further in the mutant *piwi* homozygotes. In other words, the silencing is greater in the absence of the functional *piwi* gene product.

Similarly, the combination of *en-w* 1-1B construct, which resides on the X chromosomes and exhibits pair-

Figure 4. The *white* transcripts of truncated (400-bp) *en-w* inserts are proportionately increased with the copy number, suggesting a gene dosage effect rather than pairing-sensitive silencing and cosuppression. Copy number of the representative transgenes is arranged as described in Fig. 3.

ing-sensitive silencing, with *homeless* alleles produced a related result. The alleles of *homeless* tested were *DE8* and *delta125*, as well as their heteroallelic combination. Again, the *homeless* alleles had no apparent phenotypic effect on an unpaired *en-w* transgene when homozygous, but cause a greater silencing of the paired situation. The magnitude of enhancement of pairing-sensitive silencing

Figure 5. The TVup series exhibits pairing-sensitive silencing, but not cosuppression. Copy number of the representative transgenes is arranged as described in Fig. 3.

Figure 6. Pairing-sensitive silencing is enhanced by *homeless* mutations. The 1-IB *engrailed-white* construct on the X chromosome was combined with the *homeless* alleles, *delta125* and *DE8*, as well as their heteroallelic combination. Similar results were found in all cases. Depicted are flies segregating for the *delta125* allele. *Bottom right*, a male carrying a single copy of the *en-w* transgene. *Top right*, a female homozygous for this construct, which exhibits pairing-sensitive silencing. *Bottom left*, a male with an unpaired *en-w* insertion and with the *homeless* mutation homozygous. *Top left*, a female homozygous for the transgene and for *homeless*. Note that *homeless* has little effect on the unpaired transgene, but conditions a stronger silencing of *en-w* when it is paired.

by *homeless* was greater than with *piwi*. The effect of *homeless* on pairing-sensitive silencing is shown in Figure 6.

CONCLUSIONS

Several lines of investigations indicate that the RNAi machinery operates via a posttranscriptional mechanism to destroy specific messenger RNAs or viruses. The trigger for these processes appears to be the production of a double-stranded RNA moiety. One natural function is likely to be a defense mechanism against the overexpression of transposable elements (Chabossier et al. 1998; Jensen et al. 1999; Ketting et al. 1999; Tabara et al. 1999) and against viruses. Transposable elements are present at many sites in the chromosomes and can therefore be transcribed in various orientations in such a manner as to contribute to a pool of aberrant RNAs that will trigger such a response. The double-stranded RNAs are cleaved to siRNAs, which are incorporated into the RISC complex to serve as a guide for the enzymatic destruction of the homologous RNAs.

Transcriptional silencing is less well understood but requires functional gene products of many of the same components as posttranscriptional RNA interference (Pal-Bhadra et al. 2002, 2004). Transgene silencing accompanied by the accumulation of the Polycomb complex must involve homology recognition of the related sequences throughout the nucleus for the establishment of

this silencing complex. The most likely candidate for this *trans*-acting function are small RNAs, although how they are generated and function at the chromosomal level is still unknown.

Transcriptional silencing of heterochromatic repeats in *Schizosaccharomyces pombe* (Hall et al. 2002; Volpe et al. 2002) and *Drosophila* (Pal-Bhadra et al. 2004) also require the functional gene products of the RNAi machinery. In this case, it is postulated that the repeats generate small RNAs that in turn target chromatin modifications that foster silencing, namely, histone H3 methylated at lysine 9 (H3-mK9). This modified form of histone H3 is bound by Swi6 in fission yeast and by Heterochromatin Protein 1 (HP1) in *Drosophila*. Thus, in this type of transcriptional silencing as well, siRNAs are implicated in guiding the chromatin modification machinery to homologous sequences in the nucleus to silence the various copies of repeated sequences.

An additional type of transcriptional silencing involves the contact or pairing of gene sequences. Transfer of a silenced state from one allele to another was documented during meiosis in *Ascomobolus* (Colot et al. 1996). The silencing followed the same parameters as gene conversion, suggesting similarities between homology recognition for silencing and recombination. In *Neurospora*, also during meiosis, silencing of repeated sequences within the nucleus is triggered by the presence of an unpaired sequence (Shiu et al. 2001; Shiu and Metzenberg 2002). Such unpaired DNA will cause the silencing of all homologous copies whether paired or not. This process is referred to as meiotic silencing by unpaired DNA (MSUD). Interestingly, it is suppressed by mutations in the RNAi machinery (Shiu et al. 2001; Shiu and Metzenberg 2002; Lee et al. 2003).

As noted above, a pairing-dependent silencing phenomenon occurs in *Drosophila* and is referred to as pairing-sensitive silencing. It is typically manifested in the behavior of transgenes that express well as one copy, but less well when the transgene is homozygous. In this case, pairing of the transgenes can occur somatically given the property of *Drosophila* homologs to associate in all tissues.

We investigated the relationship of pairing-sensitive silencing to cosuppression and the RNAi machinery in the experiments described above. The larger *engrailed-white* transgenes participate in cosuppression in that they exhibit nonlinearity of expression relative to the copy number present in the nucleus.

Mutations in the RNAi machinery genes, *piwi* and *homeless*, were tested for an impact on pairing-sensitive silencing. Interestingly, they both increase the degree of silencing rather than suppress the loss of expression, raising interesting issues about how the RNAi machinery is involved in a process that appears to involve gene-to-gene contact.

One possibility might be that the paired versus unpaired configuration impacts the nature of gene transcription and modifies the production of messenger RNAs that are abnormal. Abnormal RNAs are thought to enter into the RNAi pathway to eventually generate siRNAs, which

may in turn feed back to the gene to modify the local chromatin environment.

A second possibility is simply that the RNAi machinery is intimately associated with the transgenes and acts to inhibit the silencing that might otherwise occur as a result of gene-to-gene contact. When the machinery is lost in the mutants, the silencing can be more severe.

A third possibility is that the gene products of the RNAi machinery are shared with other processes. The RNAi machinery must use the siRNAs as a homology guide for targeting the destruction of mRNAs in the cytoplasm as well as for guiding the sites of chromatin modifications in the nucleus. Pairing-sensitive silencing likely involves homology recognition in some form, followed by a change of chromatin state. It is possible, but not known at present, whether protein components of the RNAi machinery are utilized in other molecular complexes that perform related functions, such as DNA homology recognition.

A fourth possibility is that siRNAs might also be used as a guide to establish chromatin modifications in domains that foster gene activation, as well as those that foster gene silencing. Most research to date implicates siRNAs and the RNAi machinery as the responsible agent for determining the sites of chromatin modifications in the nucleus that result in silencing of all homologous sequences. If, however, the RNAi machinery helps maintain the activity of genes by related means, then the mutational inactivation of the machinery would allow greater silencing to occur.

Polycomb-dependent silencing and heterochromatic silencing are suppressed by mutations in the RNAi machinery. Here we provide evidence that components of this cellular process also affect gene expression as impacted by gene associations. Thus, there is increasing evidence that the RNAi machinery plays an important role in establishing the chromatin configurations along the length of the chromosome.

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