

# Transgene Cosuppression in Animals

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WHEN TRANSGENES WERE FIRST INTRODUCED INTO PLANT SPECIES, it was discovered that their expression was often compromised. Matzke et al. (1989) described a situation in which transgenes, with sequence homology between the promoter regions, were silenced when combined together into tobacco. Napoli et al. (1990) and van der Krol et al. (1990) found that introduction of transgenes for anthocyanin pigment synthesis into petunia plants silenced not only multiple transgenes, but the endogenous gene as well. This process is referred to as cosuppression (Jorgensen 1995) or homology-dependent gene silencing (HDGS) (see also Chapter 1).

The numerous examples of cosuppression subsequently described were classified into two categories. Many cases in which homology existed between the RNA-encoding portions of the transgenes were found to have normal rates in transcriptional run-on assays (e.g., see Blokland et al. 1994). These results led to the concept that the silencing occurred posttranscriptionally, presumably by affecting RNA turnover (e.g., see Depicker et al. 1996; Metzloff et al. 1997). The other class of silencing occurred between transgenes with homology in the promoter regions. This type was accompanied by increased methylation in the regions of homology and was correlated with reduced rates of transcription (e.g., see Matzke et al. 1989).

It was also known that transgenes expressing portions of plant viruses (most of which are of the double-stranded [ds] RNA type) would confer resistance to the homologous virus (e.g., see Goodwin et al. 1996). Subsequently, it was determined that the viral RNA was turned over in a manner similar to that found for posttranscriptional silencing (Baulcombe and English 1996). This led to the concept that one of the evolutionary pressures for cosuppression was as a defense against viruses (Baulcombe and English 1996). Another evolutionary rationale for silencing was described as a means of holding the expression of transposable elements at a low level to keep the mutation rate in check (Chabossier et al. 1998; Jensen et al. 1999; Ketting et al. 1999; Tabara et al. 1999).

Although good evidence exists that the process does function as a defense against foreign elements and transposons, the genes responsible for silencing in a variety of species also affect developmental processes. There is also an overlap of genes involved with the maturation of small temporal RNAs and posttranscriptional silencing (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001). A full appreciation of the cellular processes involved and all of their ramifications has yet to be realized.

For many years, transgene silencing was thought to be a phenomenon unique to plants. In the mid 1990s when the authors first attempted to publish an example of trans-

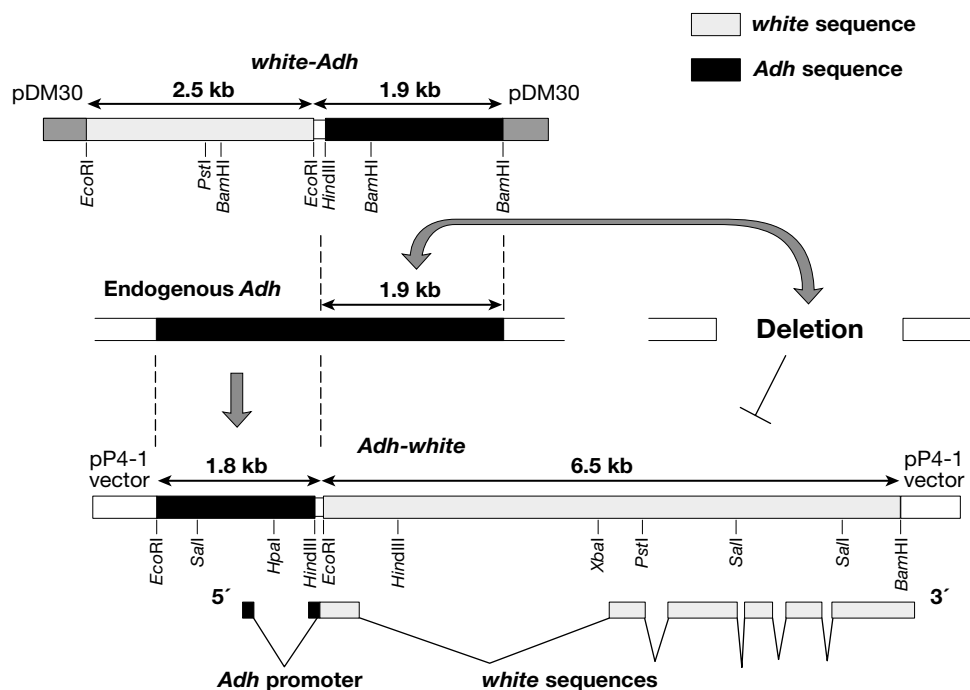
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gene silencing in *Drosophila*, a reviewer, while admitting no criticism of the data, likened the phenomenon to “cold fusion.” Subsequently, we have found that most transgenes examined will exhibit some level of silencing if the dosage is great enough. Transgene silencing in *Drosophila* differs from many cases in plants in that the degree of silencing is not as strong and seldom reaches complete null levels. This fact explains why transgene silencing was not recognized earlier in the animal kingdom.

## TRANSGENE SILENCING IN *DROSOPHILA*

In the course of studies on dosage-dependent gene regulation, our lab produced a promoter-reporter construct to test whether modifier effects operating on the *white* locus could be conferred onto another gene via the *white* promoter (Rabinow et al. 1991). The promoter region of *white* extending into the mRNA encoding sequences to the *Hph*I site (174 bp from the transcriptional initiation site) was fused with the structural part of the *Alcohol dehydrogenase* (*Adh*) gene beginning 36 bp 5′ to the initiation AUG (see Figure 2.1). This fusion (*w-Adh*) was transformed back into *Drosophila* carrying a null allele of the endogenous *Adh* gene (*Adh<sup>fin6</sup>*). The homozygous constructs produced no ADH activity. However, one hemizygous copy on the single X chromosome in males was active. With this result, it was thought perhaps the explanation resided in the phenomenon of transvection, a situation in *Drosophila* in which the pairing (or lack thereof) of alleles on homologous chromosomes affects their expression (Wu and Goldberg 1989; Geyer et al. 1990). It should be noted that homologous chromosomes are paired in somatic cells in *Drosophila*, allowing genes at homologous sites to be in close association.

To examine this possibility, single *w-Adh* constructs present on different chromosomes were examined each in an unpaired hemizygous condition. These flies all produced significant levels of ADH activity. This result would be consistent with transvection as the



**FIGURE 2.1.** Structure of the *w-Adh* and *Adh-w* transgenes illustrating their relationship to endogenous *Adh*. Deletion of the endogenous gene removes any homologous connection between the reciprocally formed transgenes.

explanation, were it not for the fact that a control of two copies, represented by one unpaired insertion in two places in the genome, produced no more ADH than either single copy alone.

When higher copy numbers of the transgene were assayed by northern blots and RNase protection assays, the level of *Adh* message declined in quantity as the number of *w-Adh* constructs was increased from one to six copies (Pal-Bhadra et al. 1997). Flies with six copies had only about 15% of the *Adh* message level as flies with one copy. This result raised the issue of an analogy with the process of cosuppression, defined in plant species, in which transgenes introduced into the genome are silenced.

To test whether the analogy to cosuppression extended to an effect on the endogenous *Adh* gene, stocks carrying the *Adh* null allele and zero to four copies of the *w-Adh* transgene were crossed to the Canton S wild-type strain. The level of *Adh* endogenous mRNA was measured by RNase protection and northern blots. One copy of the transgene produced RNA levels that were additive with the endogenous *Adh*, but two to four copies lowered the total *Adh* message progressively to 16% of the level in flies with no transgenes. Thus, the *w-Adh* gene not only lowers its own expression with increasing doses, but also lowers that of the endogenous *Adh* gene held at one normal copy (Pal-Bhadra et al. 1997). In contrast, the copy number of *w-Adh* does not affect the endogenous *white* gene expression.

### Cosuppression with Other Constructs

To test whether silencing occurred with other constructs, three dosage series were produced: full-length *Adh*, full-length *white*, and the reciprocal construct to *w-Adh*—one carrying the *Adh* regulatory sequences fused to the *white* structural gene (see Figure 2.1). The *white-Adh* construct has 2.5 kb of *white* joined to 1.9 kb of *Adh* in vector DM30 (Rabinow et al. 1991). The *Adh-white* construct has 1.8 kb of *Adh* fused to 6.5 kb of *white* in vector P4-1 (Birchler et al. 1990). The *Adh* sequences begin 5' to both of the two *Adh* enhancers and promoters (Benyajati et al. 1983) and end at the distally promoted RNA splice site, leaving only 34 bp of the proximally promoted RNA and 87 bp from the distally promoted RNA joined to the *HphI* site 51 bp before the initiation AUG of *white*. Full-length *Adh* and *white* are present in the Carnegie 20 vector (Pal-Bhadra et al. 1997, 2002).

The expression of the full-length *white* construct is linear up to six copies—the highest tested. This result was consistent with the work of other investigators, which demonstrated that *mini-white*, a shortened version, exhibits a dosage effect. The *Adh* gene showed a dosage effect up to five copies, but beyond that level, the expression is reduced from the expected amount (Pal-Bhadra et al. 1997, 2002).

The *Adh-w* construct, however, shows silencing at lower dosage (M. Pal-Bhadra, U. Bhadra, and J.A. Birchler, unpubl.). This hybrid transgene can be scored phenotypically because it is present in a *white* deficiency background (*w*<sup>67c23</sup>). At any one insertion site, *Adh-w* exhibits greater pigment as a homozygote compared to the respective hemizygote, as one might expect, but this result is in contrast to that observed with *w-Adh*. However, two unpaired copies show less expression than the additive effect predicted. Introduction of three copies into the genome causes a further decline in pigment. When an endogenous *white* gene is crossed into a multiple *Adh-w* stock, it is also reduced in expression. Thus, in this case, the endogenous gene is also drawn into the silencing pool.

In addition, a single *w-Adh* transgene will reduce the expression of a single *Adh-w* copy, even though they have no homologous sequences in common (see Figure 2.1) (Pal-Bhadra et al. 1999). The basis for this interaction appears to be that the two nonhomologous transgenes interact via the endogenous *Adh*. This hypothesis was tested by producing flies heterozygous for two overlapping deficiencies, which delete the *Adh* gene

entirely, but produce a viable fly. Accordingly, a *w-Adh* insert on chromosome 3 and an *Adh-w* insert on the X chromosome were combined with both deficiencies on chromosome 2. When the two stocks with different *Adh* deficiencies were crossed together, the flies with both *Adh-w* and *w-Adh* but with the endogenous *Adh* deleted returned to the eye color characteristic of a single *Adh-w* insert alone. RNA analysis of *white* paralleled the phenotypic results. This led to the conclusion that the *Adh* structural region of *w-Adh* interacts with the part of the endogenous *Adh* in common (Pal-Bhadra et al. 1999). The signal might “spread” in the endogenous gene to the extreme 5′ leader region plus the promoter of *Adh*, followed by an interaction with the homologous sequences in the *Adh-w* insertion to cause the reduction in expression. Another possibility is that the three types of sequences might become associated in the nucleus to initiate silencing. Deletion of the endogenous *Adh* would fail to bring together *w-Adh* and *Adh-w*. Finally, the interaction might be mediated by ectopic transcription of the *Adh* promoter region, which triggers a chromatin change in *trans* via an interaction with the RNA or a doubled-stranded version of it. With the deletion of the endogenous copy, no homologous RNA could exist between the two types of transgenes.

Reintroduction of a full-length *Adh* transgene in the absence of endogenous *Adh* restores the interaction between *w-Adh* and *Adh-w*. Furthermore, a full-length *Adh* transgene alone was sufficient to silence the *Adh-w* construct in the absence of the endogenous *Adh*. This result allowed a test of a series of truncated *Adh* transgenes having deletions in the regulatory sequences of *Adh*. The smallest segment that is required for silencing surrounds the adult enhancer (Pal-Bhadra et al. 1999). This region of *Adh* is not known to be transcribed, leading to the suggestion that the silencing interaction is initiated by DNA-to-DNA association. However, some undetectable level of ectopic transcription of the regulatory sequences might foster an RNA-mediated mechanism, especially considering the impact of the *piwi* and *aubergine* mutations described below.

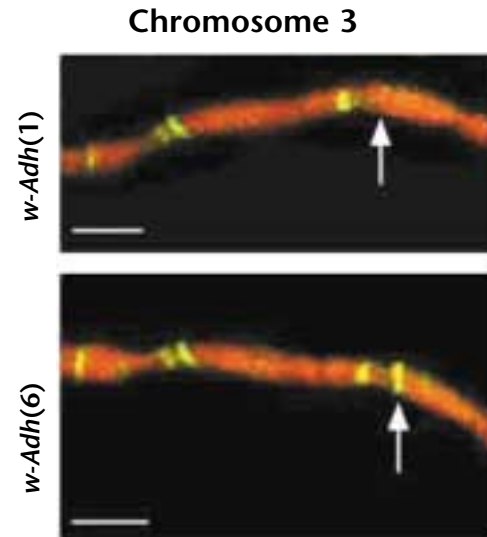
### Polycomb-Group Gene Effects

The setting of partially repressed states of gene expression suggested an involvement of the Polycomb-Group (Pc-G) genes (Kennison 1995). To test this, stocks carrying mutations of *Polycomb* (chromosome 3) and *Polycomblike* (chromosome 2) were crossed to the six-copy *w-Adh* stock and the *Adh<sup>fr16</sup> cn; ry<sup>506</sup>* transformation recipient. Neither *Pc* nor *Pcl* has an effect upon *Adh* RNA levels alone. However, when three *w-Adh* copies are included in the genotype, endogenous *Adh* is reduced, unless the *Pc* or *Pcl* mutations are also present, in which case the RNA levels return toward normal (Pal-Bhadra et al. 1997).

To examine this issue further, immunostaining with antibodies against *Pc*- and *polyhomeotic* (*ph*)-encoded proteins, both core components of the Pc-G complex, was conducted on salivary gland chromosome preparations from larvae with and without cosuppression. The Polycomb complex including these two proteins normally colocalizes to many sites on the chromosomes. Two of the three *w-Adh* insertions are not present at these sites and thus could be tested for accumulation of *Pc*- and *ph*-encoded proteins. The recipient strain and the single-copy genotypes do not show binding, but the six-copy *w-Adh* stock shows two additional bands of labeling that correspond to the testable sites (Figure 2.2). Thus, *Pc*- and *ph*-encoded proteins accumulate at the insertions under cosuppressing conditions but not otherwise. The mutational and binding studies provide evidence for an involvement of the Pc-G complex in cosuppression of this type. The association of the Pc-G on the transgene may act as a mechanism for maintenance of a set level of histone acetylation (Cavalli and Paro 1999; Cao et al. 2002; Czremin et al. 2002; Muller et al. 2002).

The cosuppressive effect of *w-Adh* transgenes on the *Adh-w* transposons also produces accumulation of Pc on the polytene chromosomes at the site of *Adh-w* insertion. Two dif-

**FIGURE 2.2.** Accumulation of the Polycomb complex on a silenced transgene. A portion of chromosome 3 of *Drosophila* is shown that includes the site of a *w-Adh* transgene (arrow). The chromosomes were stained with propidium iodide and the antibody labeling against Polycomb is green. The merged image shows the sites of binding in yellow. Many locations accumulate the Polycomb complex normally, but none of these sites overlap the location of this transgene. When only a single transgene is active, there is no detectable Polycomb binding (*top*). When there is strong silencing in the presence of six *w-Adh* transgenes in the nucleus, there is accumulation of the Polycomb complex over the transgene (*bottom*).



ferent *Adh-w* insertions have no Pc labeling when present in a single copy in the genome. However, with the introduction of *w-Adh* transposons, the expression of *Adh-w* is reduced and Pc labeling occurs on the respective *Adh-w* sites. Deletion of the endogenous *Adh* gene, the presumed intermediary of this interaction as described above, eliminates the Pc binding.

The *Adh-w* transgene alone shows strong expression, as evidenced by in situ RNA analysis, as early as 2.5 hours of development and continues throughout the embryonic stages (Pal-Bhadra et al. 1999). However, when two *w-Adh* transgenes are also present, *Adh-w* again shows strong expression at 2.5 hours, but there is an obvious decline observed by 4 hours, which continues until about 13 hours, at which point the expression plateaus. By comparison, it should be noted that pairing of homologs after fertilization begins near the end of syncytial blastoderm ( $\pm 3$  hours) (Hiraoka et al. 1993). The initiation of Pc binding begins by cellular blastoderm and then spreads from the core association sites by the time of germ-band extension (Orlando et al. 1998). The time course of silencing is consistent with the possibility that the homologous copies associate during the homology search that establishes the homolog pairing in *Drosophila* and that Pc-G accumulation maintains the reduced level of gene expression. The time course cannot rule out the alternative possibility that expression of *w-Adh* ultimately triggers the *Adh-w* silencing, especially considering that posttranscriptional silencing readily occurs at this stage of development (see below).

### Interactions of PRE-containing Constructs

Hagstrom et al. (1997), Sigrist and Pirrotta (1997), and Muller et al. (1999) reported the *trans*-interaction of transposons carrying a Polycomb response element (PRE) and *mini-white* (*m-w*). PREs are DNA sequence motifs that attract the Polycomb complex (Mihaly et al. 1998) and were originally defined by their role in maintaining repressed states of gene expression of segment identity genes. When present in a transposon together with an *m-w* eye color reporter, the latter has altered expression. Typically, these *m-w* transgenes are active when hemizygous, but many insertion sites show strong silencing when homozygous. In addition, two unpaired copies in the genome are not additive in their expression and increasing copy number causes a decline in the total expression. We suspect that the interaction of PRE-containing transgenes is related to the cosuppression of

the *w-Adh* and *Adh-w* examples described above. Cosuppression of *w-Adh* or *Adh-w* by *w-Adh* can involve *trans*-interactions of copies that do not label with Polycomb when active, but do label when the silencing becomes evident. However, when a bona fide PRE is present on the transposon, as was the case in the aforementioned studies, the silencing in *trans* appears to be much more severe and operates at a much lower dosage. Transgenes that carry a PRE together with *m-w* and *lacZ* (Muller 1995) or *m-w* and *yellow*<sup>+</sup> (Mallin et al. 1998) exhibit silencing of both genes. Chromatin insulators placed around *yellow*<sup>+</sup> can protect it from silencing by the PRE, even though the unprotected *m-w* is silenced (Mallin et al. 1998). By using a transformation cassette buffered from *y*<sup>+</sup> silencing by chromatin insulators, Sigrist and Pirrotta (1997) found that many hemizygous transformants (20%) are basically null for *white* and would not have been recognized in previous experiments. This example illustrates that unless one is vigilant to the possibility of silencing, such transformants would not be recognized.

### Cosuppression and Pairing-sensitive Silencing

Transvection is the phenomenon whereby the expression of alleles is affected by the degree of pairing of the homologs. The change can be either a reduction in expression or complementation between alleles. This phenomenon was first discovered by Ed Lewis, using the *bithorax* locus (Lewis 1954; Wu and Goldberg 1989). Several subsequent examples have been found including the loci *white* (Jack and Judd 1979; Babu and Bhat 1980; Pirrotta 1991), *decapentaplegic* (Gelbart and Wu 1982), and *yellow* (Geyer et al. 1990). Although the mechanism is still unknown, a popular explanation is that transcriptional enhancers are shared between paired homologs (Geyer et al. 1990; Goldsborough and Kornberg 1996). In the case of complementation, one homolog might carry an allele with an enhancer that is inactivated and the other homolog, an allele in which the structural gene is impaired, but a nearly normal phenotype will still result. This occurs because the normal enhancer will work with the normal structural gene in *trans* across homologous pairs, as long as they can physically associate. If chromosomal aberrations or mutations are introduced that disrupt this association, a mutant phenotype will result. A pairing phenomenon referred to as “pairing-sensitive silencing” in which paired transgenes become inactive may or may not be related to transvection, as discussed below. The *w-Adh* transgene, which exhibits cosuppression, exhibits pairing silencing, so it would be of interest to determine whether there is a relationship between the two phenomena.

One mutation that will disrupt transvection at many loci is a “loss-of-function” allele at the X-linked *zeste* locus (Babu and Bhat 1980, 1981; Wu and Goldberg 1989). This allele of *zeste* is called *z-a* and disrupts transvection at *white* (Babu and Bhat 1980), *decapentaplegic* (Gelbart et al. 1985), and *bithorax* (Goldsborough and Kornberg 1996). Thus, a relationship between transvection and cosuppression was tested by examining whether cosuppression is eliminated in flies carrying the *z-a* allele. To do this, a stock homozygous for *z-a* and the *Adh* null allele was produced. Second, a stock was constructed that was homozygous for *z-a* and four copies of the *w-Adh* transgene. Once these were generated, the two lines were crossed together to produce *z-a* flies with one transgene on chromosome 2 and a second on chromosome 3. Then, comparisons for the level of *Adh* mRNA were made of the three types of flies, namely, (1) *z-a*, *Adh* null; (2) *z-a*, *Adh* null + hemizygous transgenes on two chromosomes; and (3) *z-a*, *Adh* null + two homozygous copies (= 4 copies). If *z-a* were to eliminate cosuppression, then the four copies of the transgene would have twice the level as two copies. If *z-a* does not eliminate cosuppression, the four copies would produce about one-third the level of mRNA as the two copies, based on our previous results. The latter result was found (Pal-Bhadra et al. 1997).

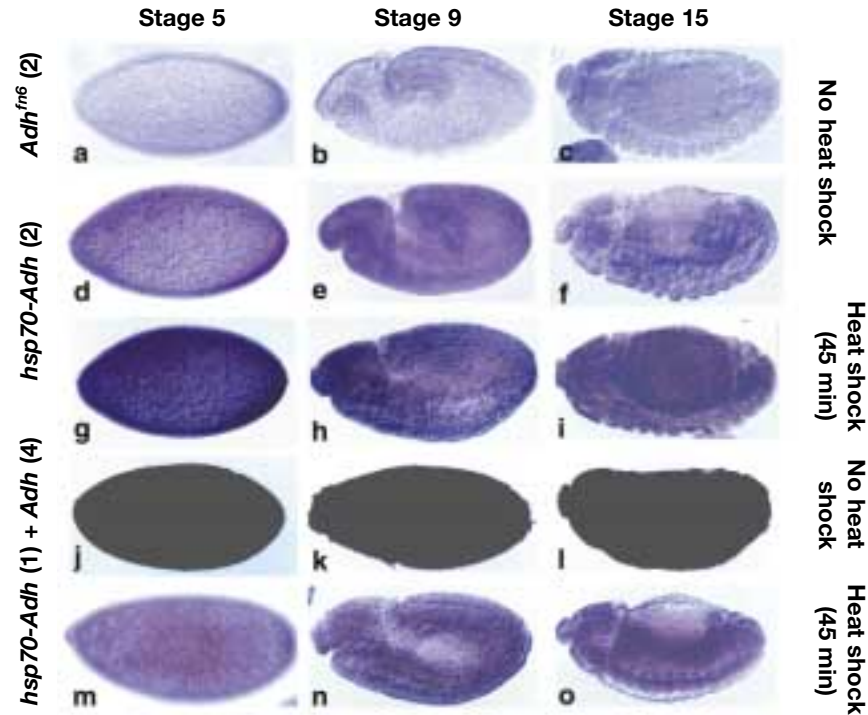
Despite the failure to find a relationship with *zeste*-mediated transvection, it should be noted that the *w-Adh* construct shows strong pairing-sensitive silencing. Its behavior is similar to that of the *engrailed-white* (*en-w*) transgenes that defined the phenomenon of pairing-sensitive silencing (Kassis et al. 1991, 1994). This transgene carries a portion of the nontranscribed regulatory region of the *engrailed* locus adjacent to *m-w*. The *en-w* transgenes express well as hemizygotes but are silenced as homozygotes. Interestingly, the original studies noted that two unpaired copies of *en-w* on different chromosomes very often were not expressed at greater levels than either single copy, just as with *w-Adh*. Furthermore, *z-a* had no effect upon the action of *en-w*. We have examined a dosage series of *en-w* for the presence of dispersed silencing (M. Pal-Bhadra, U. Bhadra, and J. Birchler, unpubl.). Indeed, double hemizygotes are not additive, and flies containing three and four copies of *en-w* progressively decline in eye color below the level seen in homozygous insertions. The flies with four copies of *en-w* show the most extreme reduction. Thus, the same type of silencing response occurs with *en-w* as was found with *w-Adh*. Further work is needed to understand the relationship of pairing-sensitive silencing between transgenes in identical positions on homologs versus transgene silencing among disperse locations in the genome (cosuppression). It is possible that the ability of the genes to associate enhances the silencing mechanism.

The question arises as to whether gene-to-gene encounters are involved in triggering silencing. There is very little known about homology searching in the nucleus. We reason that it occurs at meiosis, at the establishment of somatic pairing at some point following fertilization (Hiraoka et al. 1993), and in repair of P element excisions (Gloor et al. 1991). In addition, transposons containing PRE have a tendency to “home,” in that they preferentially insert in chromosomal sites bound with the Polycomb complex (Kassis et al. 1992). Thus, it is possible that Pc-G-bound genes might foster gene-to-gene searches.

## POSTTRANSCRIPTIONAL TRANSGENE SILENCING IN *DROSOPHILA*

Work in *Caenorhabditis elegans* several years ago demonstrated that the injection of sense RNA into embryos would result in the extinction of homologous endogenous gene expression (Guo and Kemphues 1995). Subsequently, it was discovered that dsRNA was much more potent at silencing (Fire et al. 1998). This “RNA interference” or RNAi also occurs in *Paramecium* (Ruiz et al. 1998), trypanosomes (Ngo et al. 1998), *Drosophila* (Kennerdell and Carthew 1998; Misquitta and Paterson 1999), and mammals (Wianny and Zernicka-Goetz 2000; Elbashir et al. 2001b), as well as many other species. Extremely low levels of dsRNA can trigger interference, suggesting that there is an amplification of the signal. The silencing signal can act systemically, and, in some cases, the silenced state is transmitted to the subsequent generation (Tabara et al. 1998). Intron sequences are not effective, suggesting that RNA rather than DNA is the molecule targeted (Montgomery et al. 1998).

The 1–10-dosage series of full-length *Adh* described above shows a linear expression correlated with dosage up to five copies. After that level, the expression falls off and in some cases is quite low (Pal-Bhadra et al. 1997, 2002). In comparison, when a *heat-shock* (*hs*)-*Adh* construct is induced, an increase in the level of *Adh* RNA follows. However, when four copies of full-length *Adh* and the *hs-Adh* construct are combined, heat shock induction produces a collapse of the RNA levels as assayed by northern blots of adult RNA (Pal-Bhadra et al. 2002) or RNA in situ hybridizations of early embryos (Figure 2.3). The 45-minute heat shock has no effect on the levels in the five-*Adh*-copy flies and causes an increase with *hs-Adh* alone; but with the combination, the RNA levels are virtually extinguished. The rapid decline in *Adh* RNA under these circumstances suggests that an active degradation is occurring within a matter of minutes (Figure 2.4). The silenced con-



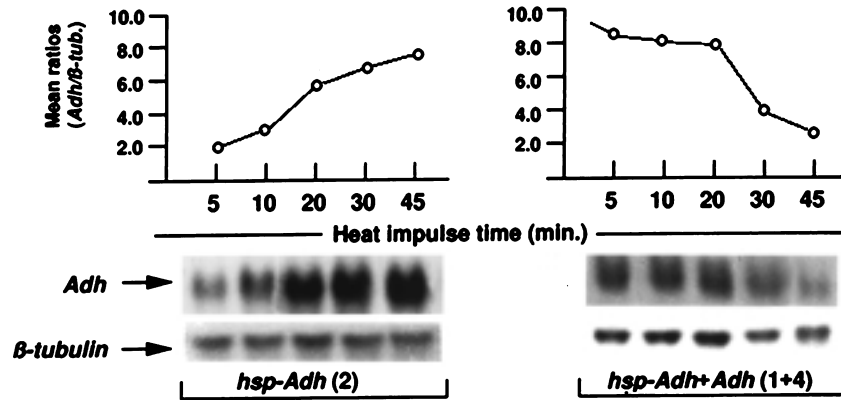
**FIGURE 2.3.** Threshold posttranscriptional gene silencing (PTGS) of *Adh* transgenes in early embryos. Silencing of five copies of *Adh* by the induction of *hsp70-Adh*. In situ analysis of RNA, using an *Adh* probe, was performed on mixed ages of embryos of different genotypes and treatments. (a–c) *Adh* null (*Adh<sup>ins6</sup>*); (d–f) two copies of *hs-Adh* uninduced; (g–i) two copies of *hs-Adh* induced; (j–l) four copies of *Adh* + one *hs-Adh* uninduced; (m–o) four copies of *Adh* + one *hs-Adh* induced. All embryos were fixed at the end of the 45-minute heat shock treatment.

dition persists at a reasonably low level for about 20 hours in adults, but will eventually recover to normal by 120 hours. This suggests that in *Drosophila*, a continuous overexpression of RNA is needed for silencing of this type. There is no influence of the *Polycomblike* mutation on the level of silencing as assayed immediately following heat shock or at 20 hours (Pal-Bhadra et al. 2002).

The dosage series of *Adh* and the heat-shock-induced silencing were examined for the presence of small 21–25-bp dsRNAs that are characteristic of RNAi and posttranscriptional gene silencing (Hamilton and Baulcombe 1999; Tuschl et al. 1999; Hammond et al. 2000; Yang et al. 2000; Zamore et al. 2000; Elbashir et al. 2001a) (see also Chapter 13). In the silencing doses, the quantity of such RNAs is quite abundant (Pal-Bhadra et al. 2002); in addition, with the threshold-induced silencing, they correlate with the collapse of *Adh* mRNA levels. Therefore, it appears that at a certain threshold of *Adh* mRNA, a double-stranded molecule is formed, presumably by an RNA-dependent RNA polymerase activity (Bass 2000; Lipardi et al. 2001) (see also Chapter 9). Such dsRNA structures would be cleaved to 21–25-bp units by an RNase type III nuclease (Bernstein et al. 2001) and subsequently incorporated into a larger RNase complex to target the homologous mRNA for digestion (Hammond et al. 2000).

## RUN-ON TRANSCRIPTION ANALYSIS

The ability to induce silencing of full-length *Adh* constructs suggested a posttranscriptional mechanism. To test this, run-on transcription experiments (So and Rosbash 1997)



**FIGURE 2.4.** Time course for threshold-induced posttranscriptional silencing of *Adh*. Northern blot autoradiograms from two classes of adult RNAs carrying two copies of *hsp70-Adh* or one copy of *hsp70-Adh* and four copies of *Adh* were probed with radiolabeled *Adh* antisense RNA. The flies were heat-incubated for 45 minutes at 37°C. The total cellular RNA was isolated from each genotype at several intervals throughout the incubation period. The  $\beta$ -*tubulin* level acts as a gel-loading control. The mean *Adh*/ $\beta$ -*tubulin* ratios from triplicate measurements were plotted at time intervals during the heat incubation. At the time point that the *hsp-Adh* construct has significantly contributed *Adh* to the pool, the degradation of total RNA in the combined genotype begins to decline. Once the threshold for silencing is achieved, destruction of the RNA follows rapidly.

were performed with 0–10 copies of the *Adh* transgene, with 0–6 copies of *w-Adh*, and with the *Adh-w/w-Adh* combination (Pal-Bhadra et al. 2002). The amount of run-on transcription in the former case showed a linear relationship with transgene dosage, in contrast to the decline of the steady-state RNA level. The lack of linearity in the northern analysis indicates that the silencing in that case is indeed posttranscriptional. This result is consistent with the production of short interfering RNAs (siRNAs) in silenced doses. With *w-Adh* and *Adh-w/w-Adh*, the transcription followed a similar pattern with the northern data, indicating a transcriptional silencing (Pal-Bhadra et al. 2002). This result was foreshadowed by the finding of an association of the Pc-G complex with the silenced *w-Adh* transgenes.

## INFLUENCE OF *PIWI* AND *AUBERGINE* ON POSTTRANSCRIPTIONAL AND TRANSCRIPTIONAL SILENCING

Although transcriptional and posttranscriptional silencing might appear to be unrelated, some data demonstrate that RNA-mediated silencing can cause epigenetic changes in the homologous nuclear genes. The first indication that this might be the case was the finding that a transgene of a plant virus became methylated upon infection by the corresponding virus (Wassenegger et al. 1994; Jones et al. 1998, 1999; Pelissier and Wassenegger 2000). Second, the introduction into cells of DNA constructs with no means of expression can trigger the RNA degradation reaction (Voinnet et al. 1998). Third, mutations in plants that exhibit reduced DNA methylation will relieve gene silencing (Jeddeloh et al. 1998; Mittelsten-Scheid et al. 1998), and there is a relief of posttranscriptional silencing in some individuals of this genotype (Morel et al. 2000). Furthermore, Mette et al. (1999, 2000) showed that transcription of promoter regions to produce an aberrant RNA would cause methylation of the homologous sequences on a different transgene in the nucleus and silencing of the reporter gene being promoted. In

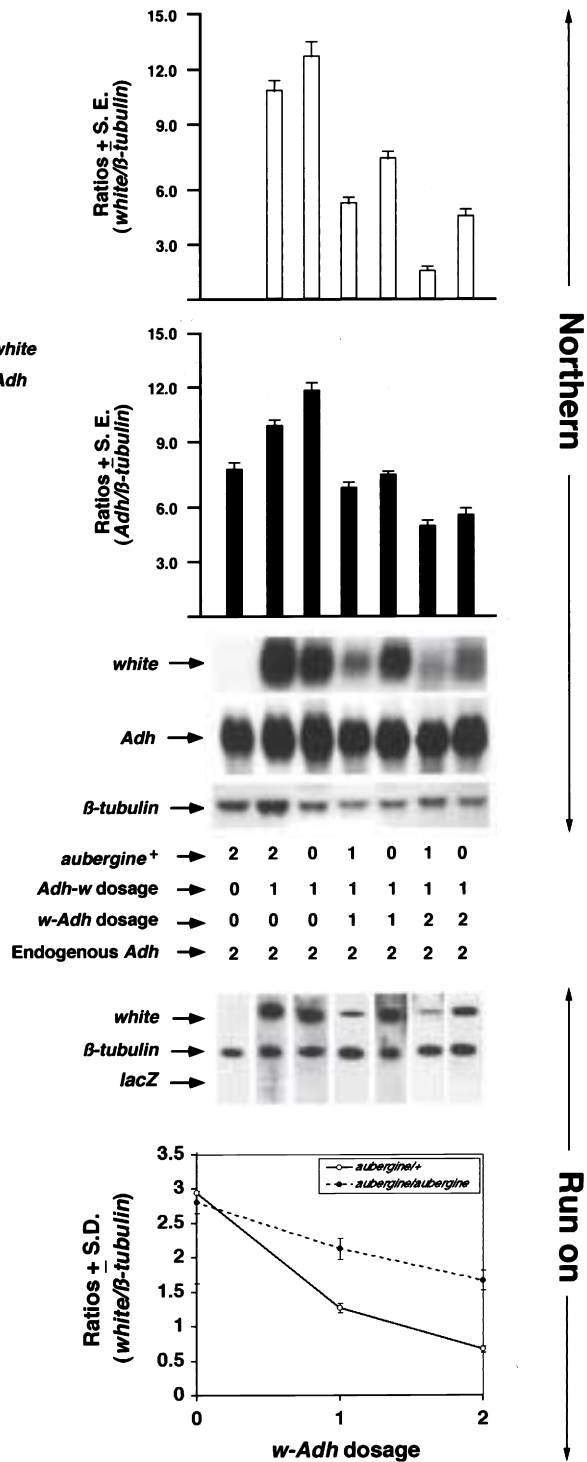
addition, the introduction of a promoter into the plant cell on a virus will trigger transcriptional silencing of another reporter construct in the same cell with a homologous promoter (Jones et al. 2001). In animal species, silenced transgene arrays in the *C. elegans* germ line are activated (Tabara et al. 1999; Ketting and Plasterk 2000) and appear to be less condensed (Dernburg et al. 2000) in some mutant backgrounds defective for RNAi. Such mutations were initially recovered on the basis of their inability to support RNAi. These results raise the issue of whether a relationship exists between the two types of silencing (Jones et al. 1998, 1999; Vionnet et al. 1998; Mette et al. 1999, 2000; Birchler et al. 2000; Marathe et al. 2000; Sijen et al. 2001).

Genes have been identified in plants, *C. elegans*, and *Neurospora* that are defective in RNA silencing processes (Elmayan et al. 1998; Cogoni and Macino 1999a,b; Ketting et al. 1999, 2001; Tabara et al. 1999; Grishok et al. 2000; Smardon et al. 2000). The predicted functions of some of these genes include RNA-dependent RNA polymerase (Cogoni and Macino 1999a; Dalmay et al. 2000), RNA or DNA helicases (Cogoni and Macino 1999b; Ketting et al. 1999; Tabara et al. 1999; Wu-Scharf et al. 2000; Dalmay et al. 2001), and RNases (Ketting et al. 1999; Hammond et al. 2000; Bernstein et al. 2001). The thinking is that these gene products generate or amplify the dsRNA, which is cleaved to siRNA. They, in turn, act as a guide for targeting the homologous mRNA for destruction via an RNase complex (Bass 2000; Hammond et al. 2000; Bernstein et al. 2001). The mutations that are defective for RNAi (*rde*) in *C. elegans* are all viable, so there may be vital genes involved that have yet to be identified. Several *Drosophila* genes are homologs or gene family members of the *C. elegans rde* genes, including *sting* (= *aubergine*) (Schupbach and Wieschaus 1991; Wilson et al. 1996; Schmidt et al. 1999; Aravin et al. 2001), *piwi* (Cox et al. 1998, 2000; Tabara et al. 1999; Cerutti et al. 2000), *argonaute* (Katoaka et al. 2001), *argonaute2* (Hammond et al. 2001), and *homeless* (Aravin et al. 2001; Stapleton et al. 2001).

Given the parallels between posttranscriptional silencing of *Adh* and RNAi, *Drosophila* mutations in gene family members related to RNAi-defective mutations in *C. elegans* were tested for an effect. The *piwi*, but not the *aubergine*, mutation will block threshold-induced silencing and eliminate the production of 21–25-bp dsRNAs. It should be noted, however, that the *aubergine* mutation will block the germ-line silencing of the *Stellate* gene by *Suppressor of Stellate*, which operates via an RNAi-like mechanism (Avarin et al. 2001). Furthermore, given the potential relationship between RNAi-like and transcriptional silencing noted above, these two mutations were tested for an effect on *Adh-w/w-Adh* transcriptional silencing. Neither has a significant effect on *w-Adh* silencing of endogenous *Adh*, but will prevent rather strongly the extension of this silencing to *Adh-w* (Figure 2.5) (Pal-Bhadra et al. 2002). Therefore, it appears that in *Drosophila*, as in plants, RNA silencing in the cytoplasm can have an impact on transcriptional silencing. The basis of this connection is unknown, but it is presumed to involve siRNAs as the homology guide to the sequences in the nucleus. The recent finding that chromodomain proteins contain RNA interaction motifs (Akhtar et al. 2000; Muchardt et al. 2002) provides the interesting possibility that siRNAs direct the Polycomb complex (whose members possess chromodomains) to the transgenes for establishment of the transcriptionally silenced state.

## TRANSGENE EFFECTS ON TRANSPOSABLE ELEMENTS

One function of silencing mechanisms appears to be as a means to keep transposable elements from exhibiting extensive transpositions, which would increase the mutation rate. Several groups have reported silencing of the I transposable element in *Drosophila*. The I element is a type of retroposon that is responsible for one type of hybrid dysgenesis. When females devoid of functional elements are crossed by male inducer strains carry-



**FIGURE 2.5.** Effect of the *aubergine* mutation on transcriptional silencing of *Adh-w*. Northern and transcriptional run-on analyses showing the effect of a heteroallelic *aubergine* mutant combination ( $\Delta P-3a/QC42$ ) (Schmidt et al. 1999; Schupbach and Wieschaus 1999) on *white* transcripts of the reporter *Adh-w* transgene in the presence of 0–2 copies of the reciprocal *w-Adh* construct. To determine the effect of *aubergine* on *w-Adh/Adh* silencing, each northern blot was probed with a radiolabeled *Adh* anti-sense RNA. The copy number of each construct or allele is noted below. The relative amount of *white* and *Adh* RNA (*white*/ $\beta$ -*tubulin*  $\pm$ s.e. and *Adh*/ $\beta$ -*tubulin*  $\pm$ s.e.) from triplicate measurements of each genotype is represented by the bar diagrams. The  $\beta$ -*tubulin* probe acts as a gel-loading control. Run-on transcriptional analysis of the same genotypes is shown below. A graphical representation of the means ( $\pm$ s.d.) of triplicate experiments illustrates the relief of silencing in the *aubergine* mutant flies.

ing full-length elements in their genomes, sterility is observed as well as high rates of mutation due to new insertions of I. Transgenes carrying different forms of the I element are capable of reducing the reactivity of these elements over the course of several generations (Pritchard et al. 1988; Jensen et al. 1995).

To examine the relationship of this phenomenon to cosuppression, 186 bp of the 5'-untranslated region (5'UTR) of the I element were introduced into reactive flies

(Chaboissier et al. 1998). Strains with various numbers of this sequence ranging from 1 to 11 copies were produced, and the effect on an I element promoter-CAT (chloramphenicol acetyltransferase) reporter was determined. With increasing dosage of the UTR fragment, there was decreasing expression of the reporter. These transgenes were then tested for the fertility of dysgenic females. With increasing dosage of this segment, the fertility increased, suggesting a silencing of the elements in the genome. A regulatory mutation that decreases the expression of the I element transgene did not alter the silencing of the I element promoter-CAT construct significantly nor the effect on fertility.

In contrast, introduction of a different type of transgene transcribing internal portions of the I element had an effect on fertility, but when the transgene was not expressed, the response was absent (Jensen et al. 1999a). These constructs also showed a dosage effect with greater numbers of transgenes conditioning better fertility. Similar versions of the transgene that could or could not produce a protein were equally effective. The impact on fertility accumulates over several generations. The greater the number of transgenes, the fewer generations required to achieve the same level of effect. This accumulation only occurs through the female lineage and is reversed by one generation of passage through the male line. Different fragments of the I element were effective and the combined length was additive. Transgenes engineered not to express any RNA product were ineffective. Further analysis (Jensen et al. 1999b) demonstrated that the expression of sense or antisense fragments of the I element was equally effective in repressing incoming I elements. Both produced an accumulating silencing through the female germ line.

Gauthier et al. (2000) examined these issues further using antisense I element transgenes. With these constructs, the accumulation effect through the female lineage was also observed, although in this case, an effect could still be measured for two generations following the removal of the transgene. The strength of the effect of the transgene was dosage-dependent. Gauthier et al. also described a weaker paternal effect.

Malinsky et al. (2000) found that I element transgenes expressed by a heat shock promoter were quite effective at decreasing sterility. Sense or antisense constructs produced the same level of response. However, a transgene without the promoter was ineffective. Interestingly, when the same fragment of the I element was expressed by the I element promoter itself, there was no effect.

Jensen et al. (2002) reported that different nonhomologous portions of the I element were capable of producing the silencing interaction. The case is formally analogous to the *w-Adh/Adh-w* case described above. These authors suggested that pericentromeric copies of the I element that are normally present in the genome were capable of mediating the interaction of the two types of transgenes.

A fascinating aspect of the transgene silencing of the I element is the accumulating nature of the silencing. The transposition rate of the I factor progressively decreases over several generations as long as the transgene is inherited through the female germ line. It remains an open question whether the silencing operates at the posttranscriptional or transcriptional level. Determining the nature of this accumulating effect will reveal a process unique among cases of transgene silencing.

Data also exist which illustrate a *trans*-sensing mechanism for the P transposable element when present in telomeric-associated sequences (Ronsseray et al. 1996; 1998). At present, the relationship of this *trans*-interaction with the phenomenon of cosuppression is not known, although several parallels exist.

## STELLATE/SUPPRESSOR OF STELLATE INTERACTION IN *DROSOPHILA*

Although technically not an example of cosuppression, the *Stellate/Suppressor of Stellate* (*Su[Ste]*) interaction in *Drosophila* (Schmidt et al. 1999) is an example of homology-dependent

silencing (Avarin et al. 2001; Stapleton et al. 2001) that appears to be related to cases of cosuppression. The *Stellate* gene is present on the X chromosome and the *Suppressor of Stellate* is present on the Y. In males without a Y chromosome, the *Stellate* gene (a casein kinase II) is overexpressed in the testis and conditions male sterility. The respective genes on the two chromosomes share a region of homology. The *Su(Ste)* gene cluster generates sense and antisense RNAs that have homology with *Stellate*. These enter the pool for the formation of siRNAs that can silence both *Stellate* and *Su(Ste)*. Thus, the system represents a case in which RNA silencing acts as an autogenous control mechanism.

## COSUPPRESSION IN *C. ELEGANS*

When transgenes are introduced into *C. elegans*, they typically form highly repeated arrays without integration into the chromosomes. In the soma, most such arrays are expressed as expected and are capable of complementing mutations in the corresponding genes. In contrast, these arrays in the germ line are not active and, in many cases, are capable of silencing the endogenous gene. Arrays of a transgene lacking a promoter are not effective. This fact has led some investigators to postulate that the silencing is post-transcriptional (Dernburg et al. 2000; Ketting and Plasterk 2000), and the term “cosuppression” has been applied. However, some transgene array silencing is affected by mutations in the *mes* genes, which are homologs to a class of Pc-G genes (see Kelly and Fire 1998). This fact would suggest that their silencing is chromatin-based, although an indirect effect of the *mes* genes is possible. Therefore, it is not presently clear whether the silencing of transgene arrays in worms is analogous to the dispersed gene-silencing type of cosuppression or to repeat-induced silencing of tandem arrays that resembles heterochromatin (see Dorer and Henikoff 1994). Of course, these two types of silencing may be related.

As noted above, mutations were recovered in *C. elegans* that would not support RNAi (RNAi-defective or *rde*). Some of these mutations overlap with mutator (*mut*) genes that derepress transposable elements. Tabara et al. (1999) noted that transgene arrays were desilenced in a background of some of these mutations. Ketting and Plasterk (2000) tested a collection of these mutations on the ability to desilence transgene arrays in the germ line. The mutations, *mut-2*, *mut-7*, *mut-8*, and *mut-9*, which are defective in transposon silencing, also released the silencing of the transgene arrays. In contrast, *mut-6* did not affect RNAi or eliminate cosuppression. Furthermore, the *rde1* mutation does not support RNAi but has no effect on cosuppression, thus documenting some differences in the two phenomena.

Dernburg et al. (2000) also observed that *rde1* had no effect on silencing of transgene arrays. They too found that *rde2* and *mut-7* would release the silencing of the array tested. Arrays generated in these backgrounds were not silenced, but they could become so when outcrossed to normal. These two mutations also made the transgene arrays appear less condensed than in a wild-type background. No evidence of association of the transgene array with the normal chromosomes was observed, although transient associations could not be ruled out.

The tissue-specific dichotomy of silencing of transgene arrays in worms raises an interesting question concerning its basis. RNAi is effective in somatic tissue and therefore must have distinctions with the phenomenon of cosuppression, which is restricted to the germ line. The mutational analysis is consistent with a relationship between the two phenomena, but they are not equivalent. It is possible that the initiation of cosuppression of transgene arrays shares mechanistic steps with RNAi, but thereafter a divergence in maintenance functions occurs.

## COSUPPRESSION IN MAMMALS

Transgenes introduced into mammalian cells are often integrated as tandem arrays and exhibit silencing in a mosaic fashion in a field of cells (Garrick et al. 1998). Whether this type of silencing is related to that of dispersed single copies is unknown. The ability to conduct gene replacement in mammalian cells eliminates the need for experiments using individual transgenes. Thus, little data are available about dispersed gene silencing in mammals. Given the presence of RNAi in mammals (Elbashir et al. 2001b) and a large number of antisense transcripts (Lehner et al. 2002), it is likely that similar processes are present. In addition, silencing by sense RNAs has been documented in mammalian cells (Cameron and Jennings 1991; zu Putlitz and Wands 1999).

Indeed, an example of transgene silencing in rodent cells has been reported (Bahramian and Zarbl 1999). Electroporation of rat fibroblast cells with a collagen transgene causes a rapid reduction of the endogenous RNA. The stable integration of the same plasmid would exhibit normal expression approximately equal to the endogenous copy. The thousands of transgenes in the transient introduction to the cells did not express an RNA, leading to the interpretation that they were transcriptionally silenced. The transgene RNA could have been distinguished from the endogenous RNA by RNase protection, but it was not detected. Because the endogenous RNA was present, although reduced, the transgene RNA should have been found if it had been expressed to any degree. Various methods of introducing the transgene copies all produced the silencing. These observations were interpreted as a reflection of transcriptional silencing of the transgenes.

A collagen promoter-CAT construct also caused the rapid depletion of the endogenous RNA, but in this case, there was expression of the reporter gene. Such constructs with different promoter strengths were equally effective at silencing the endogenous RNA. Another plasmid with the promoter region and a portion of the protein-encoding part of the gene extending through the first exon and intron showed greater effectiveness at reducing the level of endogenous RNA. Sequences in the protein-encoding portion of the transgene were postulated to be involved with posttranscriptional silencing.

Transgenes delivered to mice in lentiviral vectors apparently have a low rate of silencing (Lois et al. 2002). A green fluorescent protein (GFP) construct was expressed from most insertions and, when judged qualitatively, was correlated with gene copy number. The emphasis of this study, as is often the case, was on recovery of expressing transgenes. Therefore, the basis of the silenced cases was not investigated.

## SUMMARY

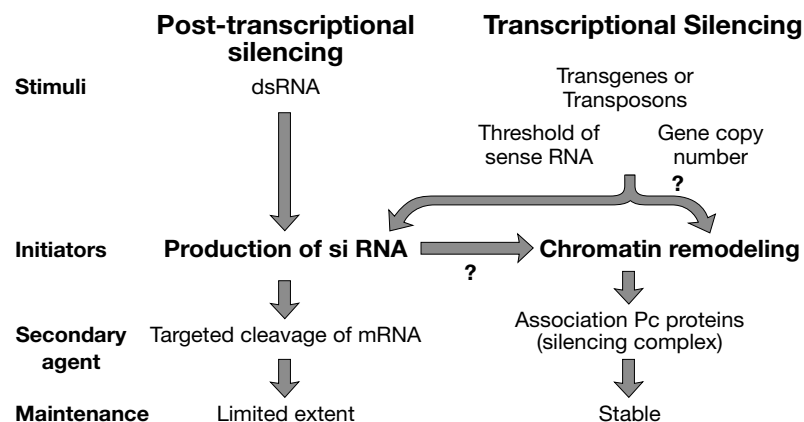
Gene silencing studies have led to the discovery of cellular mechanisms that were unknown until quite recently. The gene products identified to date indicate a group with implicated functions in RNA metabolism and another with chromatin functions. Many of these genes were previously identified for their role in developmental control. Further identification of the full spectrum of the participating gene products and their interactions is the challenge for understanding all of the processes involved and the evolution of their complex array of interactions.

Some transgenes show silencing at only a few copies, whereas others require much higher numbers, if they silence at all. The parameters that determine whether a particular transgene will silence and at what level are not known. In our experience, hybrid transgenes appear to be more susceptible, but the sample size is too small to make a generalization. It has long been recognized that transgenes vary considerably in expression, depending on the insertion site. Typically, these cases have been interpreted as reflecting

the chromatin environment. Clearly, this would influence expression, but it is also possible that some cases may involve RNA metabolism steps that have yet to be recognized.

Moreover, it is not obvious what the determinants are that condition transcriptional silencing versus posttranscriptional silencing. The *Adh* gene described above exhibits either type in different transgenes that have only subtle differences in structure. The involvement of pairing-sensitive silencing is a related issue. This type of silencing seems to involve gene-to-gene associations that are recognized by the cell, which modify the chromatin configuration of the targeted gene. Whether dispersed transgene silencing also involves gene-to-gene associations and what mediates such interactions, if they occur, are more difficult questions to address experimentally.

Another important issue in the field is whether there is a connection between post-transcriptional and transcriptional silencing. Clearly, posttranscriptional effects can occur in the absence of transcriptional silencing. However, numerous studies in plant species have indicated methylation of the nuclear gene when posttranscriptional silencing is induced. Nevertheless, mutations defective for posttranscriptional silencing in plants appear to have little impact on transcriptional silencing, and viral inhibitors of RNA silencing do not affect transcriptional silencing (Marathe et al. 2000). In animal species, *piwi* and *aubergine* will diminish the Polycomb-dependent silencing of *Adh-w* in the *Adh-w/w-Adh* interaction, suggesting some shared function. In *C. elegans*, Tabara et al. (1999) noted that *rde* mutants would desilence tandem arrays of transgenes in the germ line, a phenomenon that could involve transcriptional silencing. The silencing of the tandem arrays is suppressed by mutations in the *mes* genes, which are homologs of some members of the Polycomb-Group (Kelly and Fire 1998). It is conceivable that two independent mechanisms are operative, but under some circumstances, they have a connection (Figure 2.6). Alternatively, it is possible that all dispersed gene silencing has a basis in dsRNA mechanisms. It is important to establish whether such a connection can ever occur because this impacts the proper conduct of reverse genetic procedures and gene therapy, as well as expanding our basic understanding of the cellular processes involved.



**FIGURE 2.6.** Silencing pathways in *Drosophila* for transgene cosuppression. Posttranscriptional silencing of transgenes in *Drosophila* exhibits characteristics typical of RNA-silencing processes including the production of siRNAs. The silencing can be strong if induced by rapid threshold induction, but it is not stable. Full recovery in the case of *Adh* silencing occurs within 120 hours. Transcriptional silencing is associated with chromatin modification involving the Polycomb-Group complex. Some evidence suggests connections with pairing-sensitive silencing and RNA-silencing processes but the nature of these potential interactions is unknown. Once transcriptional silencing is established early in embryogenesis, it is stable throughout development.

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