Cosuppression of *I* Transposon Activity in Drosophila by *I*-Containing Sense and Antisense Transgenes

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ABSTRACT

We have previously shown that the activity of functional *I* elements introduced into Drosophila devoid of such elements can be repressed by transgenes containing an internal nontranslatable part of the *I* element itself and that this repressing effect presents features characteristic of homology-dependent gene silencing or cosuppression. Here we show that transgenes containing a fragment of the *I* element in antisense orientation induce *I*-element silencing with the same characteristic features as the corresponding sense construct: namely, repression takes several generations to be fully established, with similar rates for sense and antisense constructs, and it is only maternally transmitted, with reversal of the effect through paternal transmission. We also show that transcription of the transgenes is necessary to produce the silencing effect and that repression can be maintained for at least one generation following elimination of the transgenes, thus strongly suggesting that a transgene product and not the transgene *per se* is the essential intermediate in the silencing effect. The data presented strongly support models in which the repressing effect of antisense transcripts involves the same mechanisms as cosuppression by sense constructs and emphasize the role of symmetrically acting nucleic acid structures in mediating repression.

THE *I* element is a Drosophila LINE-like transposon, which transposes through reverse transcription of an RNA intermediate (Jensen and Heidmann 1991; Pélisson *et al.* 1991). It is present in most *Drosophila melanogaster* strains, but there still exist some strains lacking functional *I* elements, mainly as a result of their sequestration in laboratories after they had been caught in the wild several decades ago. Such strains, also called reactive strains, are essential tools to assay the effect of transposable elements on "virgin" genomes. Actually, introduction of *I* elements by crossing into Drosophila genomes devoid of such elements results in high frequency transposition of the incoming transposon, high mutation rate, chromosome nondisjunction, and female sterility, a syndrome referred to as hybrid dysgenesis (Picard and L'Héritier 1971; reviewed in Brégliano *et al.* 1980; Brégliano and Kidwell 1983; Finnegan 1989; Bucheton 1990). High frequency transposition is only transient, as the number of *I* elements reaches a finite value, and transposition ceases after about 10 generations (Pélisson and Brégliano 1987; Pritchard *et al.* 1988). We had previously shown that the activity of incoming *I* elements can be silenced by the prior introduction by transgenesis of a small internal region of the *I* element itself (Jensen *et al.* 1995, 1999). This autoregulation presents the characteristic features of homology-dependent gene silencing, a process also called cosuppression, first discovered in plants (reviewed in Vaucheret *et al.* 1998; Wassenegger and Pélissier 1998; Grant 1999; Selker 1999) and recently also demonstrated in Drosophila (Pal-Bhadra *et al.* 1997): repression of the *I* element does not require any translatable sequence, and its extent increases with transgene copy number. We further showed that repression develops in a generation-dependent manner, via the germline transmission, only by females, of a silencing effector. Altogether these results established that transposable elements are prone to homology-dependent gene silencing, and that the self-repression observed under the "natural" conditions of hybrid dysgenesis with *I* elements is mediated by cosuppression (Jensen *et al.* 1999).

Yet, the refined molecular mechanisms involved in cosuppression are still poorly understood, despite extensive studies carried out essentially in plants. In all cases, including the present one, where transcription of the transgene(s) was demonstrated to be absolutely required for the repressing effect, a paradoxical feature is that sense constructs, *i.e.*, transgenes producing RNA molecules of the same polarity as the endogenous gene to be repressed, are very effective for cosuppression, thus excluding simple models involving pairing between complementary RNAs from the endogenous gene and homologous transgenes. More complex models have therefore been proposed, among which are models involving either synthesis by the transgenes of double-stranded RNA (dsRNA) molecules generated by still unresolved mechanisms or direct effects of transgene

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transcripts on the structure of the homologous DNA chromosomal sequences, for instance, through methylation or changes in chromatin structure (reviewed in Montgomery and Fire 1998; Wassenegger and Pélissier 1998; Selker 1999; Sharp 1999). A common feature of these models is symmetry, in the first case of the effector molecule (dsRNA), and in the second of the target (genomic DNA). In both cases it can be predicted that sense and antisense transgenes should identically trigger cosuppression. In this study, we have therefore assayed both sense and antisense *I*-containing transgenes, tested under identical conditions, for cosuppression of *I* elements. We show that antisense constructs trigger cosuppression as well, with characteristic features closely related to those of the sense construct, including the maternal transmission of the repressing effect and the rate of the generation-dependent establishment of the repressed state.

MATERIALS AND METHODS

DNA constructs: Hsp[i1-2Δ/S]pA and hsp[i1-2Δ/AS]pA were obtained by inserting the "i1-2Δ" fragment [nucleotides 167–2484 in Fawcett *et al.* (1986)] obtained by *Eco*RI-*Xba*I restriction from pAct[I] (Jensen *et al.* 1994), respectively, in sense or antisense orientation, into the pW8-hsp-pA vector (Jensen *et al.* 1999) restricted by *Hpa*I. For all but two transgenic strains with the sense construct, stop codons were introduced into [i1-2Δ] as described in Jensen *et al.* (1999). The control construct corresponds to pW8-hsp-pA, and the promoterless pA′[i1-2Δ]pA construct is described in Jensen *et al.* (1999).

Drosophila, *P*-mediated transformation and characterization of transgenic strains: Flies were raised at 22° ± 1 on standard medium, and strains were maintained by using only young flies, as described in Jensen *et al.* (1995, 1999). The w^{1118} (Hazelrigg *et al.* 1984) and the reactive w^{K} (Lüning 1981) strains were gifts from D. Coen and C. McLean. P-mediated germline transformation was performed essentially as described by Rubin and Spradling (1982). The transgene copy number was assessed by Southern blots probed with a *hsp70* promoter fragment, by counting the number of fragments containing flanking DNA and by quantitating the intensity of the internal, transgene specific fragment using PhosphorImager technology (Storm 840 scanner). Results were confirmed by quantitative PCR (TaqMan; Perkin Elmer, Norwalk, CT), using the following primers: 5′-GAATCATCCTTTA TAGCGCAAACC-3′ (within the *I* element) and 5′-CATGCAA CGTAGCTTGGCTG-3′ (downstream of the *I* fragment). PCR was performed using an ABI PRISM 7700 Sequence Detection System apparatus and a 5′FAM(6-carboxyfluorescein)-TCACCTAACACACATCCATACAGGGTTCCA-3′TAMRA (6-carboxy-tetramethylrhodamine) TaqMan probe. The same primers and method were used in some experiments to assay sense and antisense transcripts of the transgene, after reverse transcription of total RNA (300 ng) using the above primers and Moloney murine leukemia virus reverse transcriptase (PE Biosystems, Foster City, CA).

Measurements of the level of *I* element activity: The level of *I* element activity was assessed as described in Jensen *et al.* (1999). Groups of 15 females were mated with 20 w^{1118} males (containing functional *I* elements) when less than 4 days old. The first 20 females and 20 males born from each batch of test crosses were collected and allowed to mate. When less than 4 days old, these flies were transferred to an egg collector. Sixteen hours later, five batches of 40 eggs were deposited as 4 × 10 matrices, thus allowing unambiguous counting (a further 48 hr later) of hatched and nonhatched (dead) embryos. The temperature was kept at 22° ± 1 throughout the experiments, as the intensity of the hybrid dysgenesis syndrome is influenced by temperature changes. The transgenic strains were controlled (at generations >14 after transgenesis) for the absence of contamination by functional *I* elements as in Jensen *et al.* (1995) by crossing transgenic males with reactive w^{K} females.

RESULTS

Rationale of the assay: We had previously shown that the activity of incoming *I* elements could be silenced by the prior introduction through transgenesis of an internal part of the *I* element, either translatable or not, demonstrating that *I* elements are prone to homology-dependent gene silencing (Jensen *et al.* 1999). We have now assayed transgenes containing part of the *I* element inserted either in the sense or antisense orientation, under the control of the *hsp70* promoter and the *Actin 5C* polyadenylation signal. The transgenes and the rationale for this assay are shown in Figure 1. The *I* element sequence contained in the hsp[i1-2Δ/S]pA and hsp[i1-2Δ/AS]pA transgenes corresponds to a 2318-bp fragment containing ORF1 and the 5′ part of ORF2 inserted in the sense and antisense orientation, respectively, between the promoter and the polyadenylation signal. The same *I* fragment was also inserted in a promoterless construct with a polyadenylation signal-containing sequence inserted in place of the promoter. These constructs, as well as a control construct without any insert, were introduced into Drosophila of a reactive strain (the w^{K} strain, devoid of active *I* elements) by *P*-mediated transgenesis, and several independent transgenic strains were established for each construct. The integrity of the transgenes and the transgene copy number were assessed by Southern blots and quantitative PCR. The majority of the strains contain only one copy of the transgene per haploid genome, unless otherwise indicated. The ability of the transgenes to repress *I*-element activity was then determined, at different generations after transgenesis, by introducing functional *I* elements by crossing. The activity of the latter was measured according to standard procedures by quantifying their lethal effect in the progeny of the cross (percentage of dead embryos; Picard 1978; Jensen *et al.* 1995).

Silencing by *I*-element-derived sense and antisense transgenes: As previously reported for the sense hsp[i2Δ]pA transgene, which contains an internal *I* fragment only 969 bp long corresponding to the 5′ part of ORF2 in the transgenes in Figure 1 (Jensen *et al.* 1999), the new hsp[i1-2Δ/S]pA construct was also found to silence incoming *I* elements, with a resulting *I*-element activity, as measured at generations >10 after transgenesis, as low as 11% on average (activity values were <21%

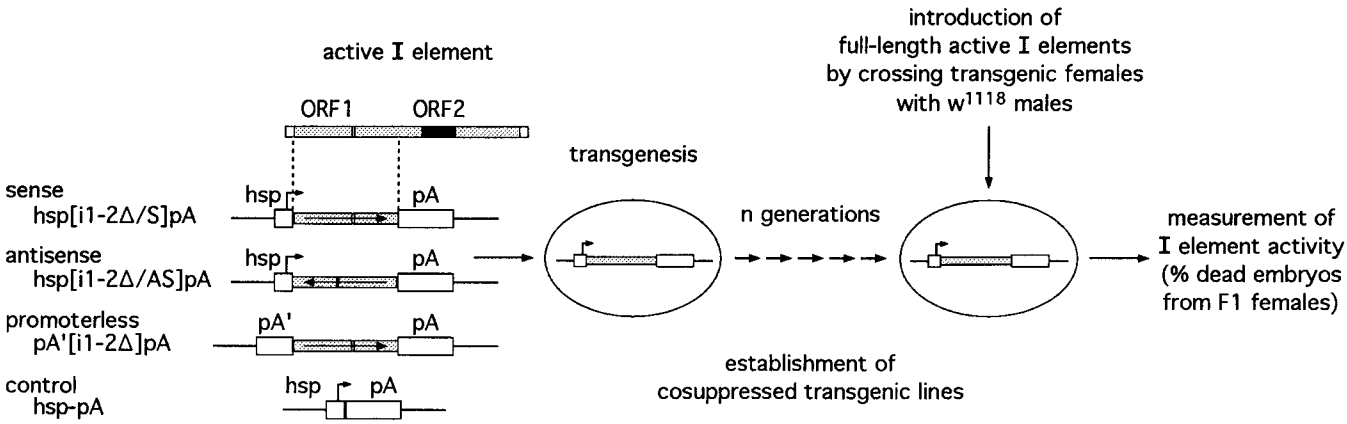

Figure 1.—Constructs and rationale of the assay for measurement of the level of *I*-element activity in transgenic strains. The structures of the full-length *I* element with its two open reading frames (ORFs), including the reverse transcriptase domain (in black), of the hsp[i1-2Δ/S]pA and hsp[i1-2Δ/AS]pA constructs containing part of the *I* element in either sense or antisense orientation under the control of the *hsp70* promoter (hsp) and the *Actin 5C* polyadenylation signal (pA), and of the promoterless construct with the *hsp70* polyadenylation sequence (pA′) in place of the promoter are shown on the left. The control construct contains the same components as hsp[i1-2Δ/S]pA and hsp[i1-2Δ/AS]pA except the *I* element sequence. The constructs were introduced into Drosophila of the reactive w^K strain (devoid of functional *I* elements) by *P*-mediated transgenesis, and several transgenic strains were established for each of the four constructs. *I*-element activity was assessed at different generations after transgenesis by introduction of full-length active *I* elements by crossing and subsequent measurement of the percentage of dead embryos laid by the F_1 progeny of the cross.

for 13 out of the 14 transgenic strains and 30% for the remaining strain; see Figure 2). The silencing efficiency of the hsp[i1-2Δ/S]pA construct is actually stronger than that of the hsp[i2Δ]pA construct [mean activity value: 38% as measured at generation 20 after transgenesis; see Jensen *et al.* (1999)], most probably due to the fact that the *I* fragment in hsp[i1-2Δ/S]pA is more than twice as long (2318 bp) as that in hsp[i2Δ]pA (969 bp). The very high repressing effect of the hsp[i1-2Δ/S]pA construct is also consistent with the high rate of establishment of repression (data not shown, but see below), as the *I*-silencing efficiency of all of the hsp[i1-2Δ/S]pA strains reached a maximum within <10 generations, and, in most cases, even as soon as on the first measurement after transgenesis, at generation 3. Interestingly, the antisense hsp[i1-2Δ/AS]pA construct also regulates *I*-element activity, with a silencing efficiency, as measured under identical conditions, closely related to that of the sense construct: activity values for all the transgenic strains except one were <25%, with a mean value of 18% (Figure 2). Again, the rates of establishment of the repressed state were high, with maximum repression being reached within <10 generations (data not shown, but see below). Finally, the data in Figure 2 also show that transcription of the *I* sequences is required for the repressing effect, as the promoterless *I*-containing construct has no silencing effect on *I*-element activity, with values very close to those of the control without inserted *I* sequences (96.4 and 96.6%, respectively; see Figure 2).

To further characterize and compare the repressing effects triggered by the sense and antisense constructs, two essential properties that had been previously identified as characteristic features of the cosuppression effect mediated by the sense hsp[i2Δ]pA transgene, namely, transmission of the repressing effect through females

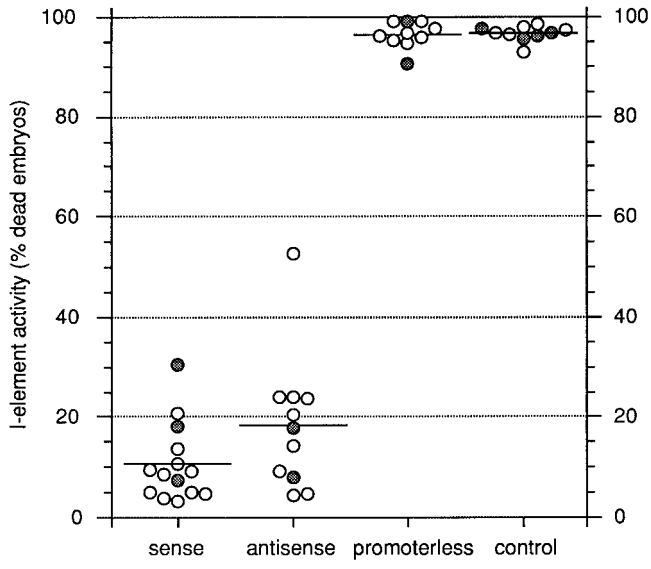

Figure 2.—Regulation of *I*-element activity by the transgenic strains is not dependent on *I* sequence orientation, but requires their transcription. *I*-element activity was quantitated by measuring the percentage of dead embryos laid by the F_1 progeny (transgene-containing) resulting from the cross of transgenic females with w^{1118} males containing full-length functional *I* elements. The data in the figure correspond to plateau values, attained within <10 generations, and are the mean of at least four measurements along the subsequent generations. Open and shaded circles refer to the progeny of transgenic females carrying one or two copies of the transgene per haploid genome, respectively.

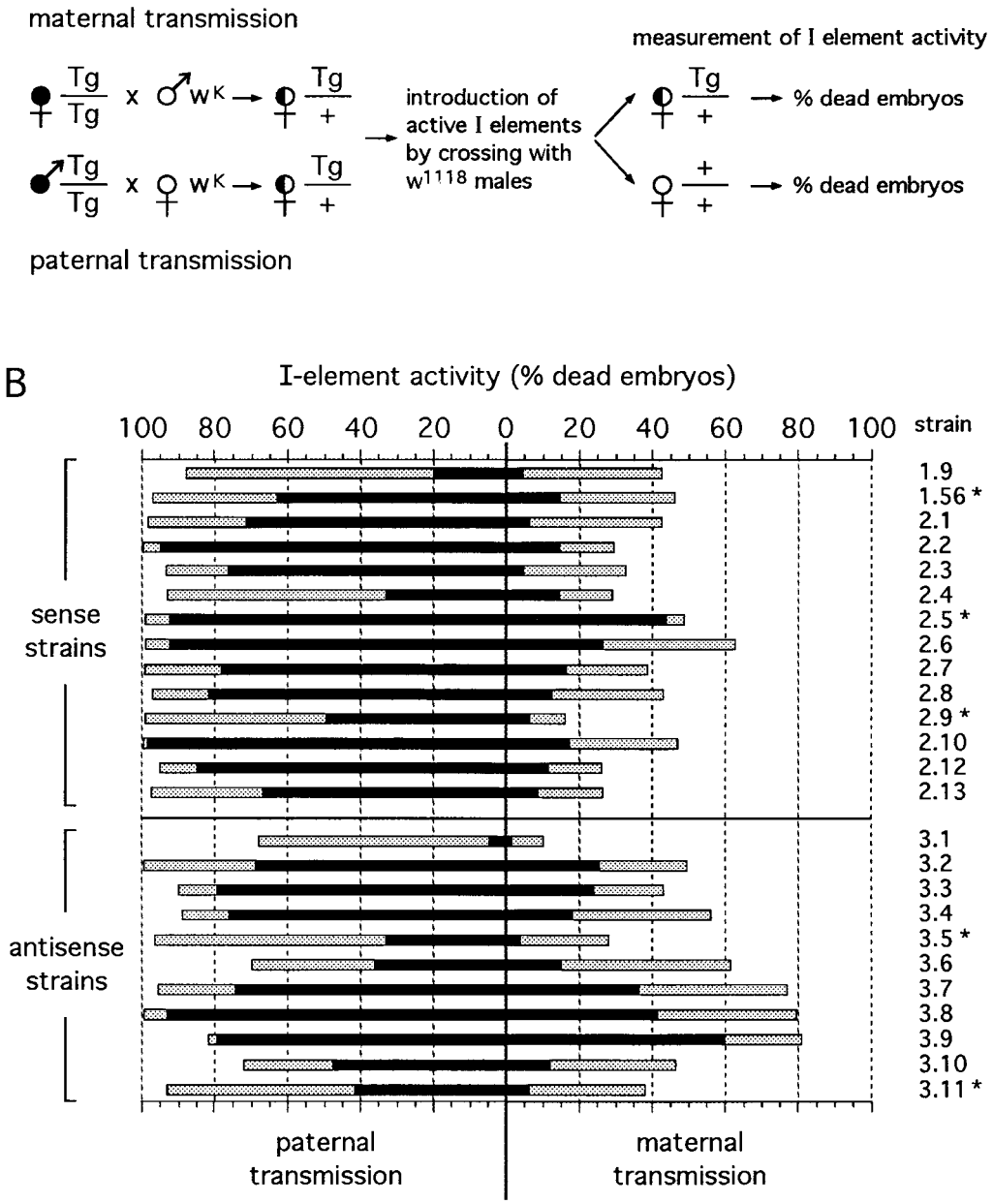

Figure 3.—The repressed phenotype is maternally transmitted in both the sense and antisense construct-containing strains. (A) Mating schemes for the maternal and paternal transmission of the transgene and rationale of the assay. Female or male transgenic Drosophila (solid symbols) were crossed, >10 generations after transgenesis, with w^K flies (open symbols); the resulting heterozygous females were crossed with w^{1118} males to introduce active *I* elements. *I*-element activity was quantitated by measuring the percentage of dead embryos laid by the transgene-containing (half-solid symbol) or transgene-free (open symbol) female progeny. (B) Results (means of five batches of 40 embryos) for maternal (right) and paternal (left) transmission of the transgenes; top, sense strains; bottom, antisense strains. Activity values for transgene-free F_1 females are indicated with shaded bars, those for transgene-containing F_1 females with solid bars (superimposed on shaded bars for the sake of clarity). Strains containing two copies of the transgene per haploid genome are marked by an asterisk.

only and cumulative repressing effects along generations (Jensen *et al.* 1999), were analyzed for both constructs, in a detailed and quantitative manner.

Maternal transmission of cosuppression in both the sense and antisense construct-containing strains: We had previously shown that the repressing effect induced by hsp[i2Δ]pA was only maternally transmitted (Jensen *et al.* 1999). We have therefore tested, for all the transgenic strains in Figure 2, whether transmission of repression followed the same rule for both the sense and antisense constructs. The scheme in Figure 3 illustrates the nature of the crosses that were used to carry out maternal and paternal transmission of the transgene and also shows how the extent of repression was measured in the female progeny of heterozygous flies, both for transgene-containing and transgene-free females. The first important result from this series of measurements is the observation that, for both the sense and antisense transgenes, silencing of *I*-element activity is maintained in the maternal transmission assays (mean activity values of 14 and 22% for the sense and antisense constructs, respectively; see solid bars in Figure 3, right), while paternal transmission of the transgenes results, in one generation, in a substantial or even full reversal of repression (mean activity values of 72 and 59% for the sense and antisense constructs, respectively; see solid bars in Figure 3, left). Thus, for both the sense and antisense constructs, repression is essentially transmitted via females.

Figure 3 also shows that maternal transmission of the

silencing effect takes place even in the absence of any zygotic expression of the transgene. This is evidenced by the fact that repression can persist, for at least one generation, in the absence of DNA copies of the transgene: nontransgenic offspring from silenced heterozygous transgenic mothers in the maternal transmission assay actually still repress *I*-element activity, again with no significant difference between the effect induced by the sense and antisense constructs (mean activity values for *I*-element activity of 38 and 52%, for the sense and antisense strains, respectively; see shaded bars in Figure 3).

Compared rates of establishment of repression by the sense and antisense constructs: Taking into consideration the high rate of establishment of repression after transgenesis that precluded any quantitative analysis, we derived heterozygous flies from arbitrarily chosen single-copy strains, in an attempt to slow this rate (based upon a reduction in transgene dosage) and thereafter be able to compare the effects of the sense and antisense constructs. The scheme for the establishment of such heterozygous strains is presented in Figure 4. It comprises a first step in which homozygous males were crossed with w^K females to reset the resulting flies in a nonrepressed state via paternal transmission of the transgenes (see above and Figure 3). Thereafter, heterozygous flies were selected according to their eye color, since the transgenes are marked by the mini-*white* gene. As can be observed in Figure 4, under these conditions the kinetics of establishment of repression along generations can be resolved, with maximal effects still taking place within <10 generations for all the strains tested. Interestingly, small variations in the rates can be observed depending on the strain, most probably resulting from differences in the position of the transgene. Yet, no clear-cut difference can be detected between the groups of the sense and the antisense strains. Figure 4 also shows that the level of repression reached at equilibrium by the heterozygotes remains lower than that of the corresponding homozygotes (indicated with a dotted line in the figure), most probably as a result of a transgene dosage effect. As already observed in Figure 3 for the homozygous strains, the kinetic analysis in Figure 4 also shows that nontransgenic flies derived from heterozygous transgenic mothers still disclose a silencing effect (see the upper curves in Figure 4), with a decrease of *I*-element activity along generations correlated to that of their transgenic sisters and a difference at equilibrium ranging from 18 to 30%.

DISCUSSION

In this article, we show that transgenes containing a fragment of the *I* element in either the sense or antisense orientation, under the control of the *hsp70* promoter, both repress the activity of incoming functional *I* elements, with similar characteristic features. As previously demonstrated for sense constructs (Jensen *et al.* 1995, 1999), for the present sense and antisense constructs the repressing effect is only maternally transmitted and increases with increasing number of generations, without significant difference between the rates of establishment of repression between both types of constructs. Altogether, these data strongly suggest that the modes of repression by either transgene involve similar molecular processes. They also confirm that repression is not due to any protein that would be produced by the *I* element as repression is similarly observed with the antisense, noncoding strand, construct. Interestingly, previous studies in plants have also shown closely related cosuppression-associated effects for single-copy genes repressed by sense and antisense transgenes (see Sijen *et al.* 1996; Waterhouse *et al.* 1998 and references therein; but see Que *et al.* 1998). At the molecular level, among the possible models for homology-dependent gene silencing that would not distinguish between sense and antisense constructs (reviewed in Montgomery and Fire 1998; Vaucheret *et al.* 1998; Wassenegger and Pélissier 1998; Grant 1999; Sharp 1999), only those involving symmetrically interacting nucleic acid structures as initiators, effectors, or targets for repression therefore seem plausible. In some cases, homology-dependent gene silencing has been reported that did not require transcription of the transgene, but then occurred under rather specific conditions where the silencing sequences were introduced as tandem repeats or concatamers (Assaad *et al.* 1993; Dorer and Henikoff 1994; Chaboissier *et al.* 1998; Garrick *et al.* 1998). Under these conditions, repression seems to be a consequence of DNA-DNA interactions, possibly resulting in local changes in chromatin structure, not excluding long-range ectopic effects through a "scanning" process. With dispersed transgenes, *i.e.*, under conditions more closely related to that of transposable elements within the genome, we have shown that transcription of the transgenes is necessary to trigger repression, thus eliminating models where repression would be mediated by direct recognition of homologous *I* DNA sequences. Accordingly, in this case the transgene DNA sequences alone cannot be responsible for cosuppression. This would be consistent with our observation that repression can persist for at least one generation in the absence of the transgene in females (Jensen *et al.* 1995, 1999, and present article). It has been proposed that transgenes mediating transcription-dependent cosuppression might in fact produce both sense and antisense transcripts, due to the presence of either cryptic promoters on the antisense strand of transgenes, or of cellular RNA-dependent RNA polymerases (Schiebel *et al.* 1998) that could make an antisense RNA molecule using the transgene RNA transcript as a template (Waterhouse *et al.* 1998; reviewed in Grant 1999): according to such models, similar amounts of dsRNA molecules could be produced, whatever the orientation of the silencing *I* sequences in the transgenes. This possibility has been recently substantiated by the following: (1)

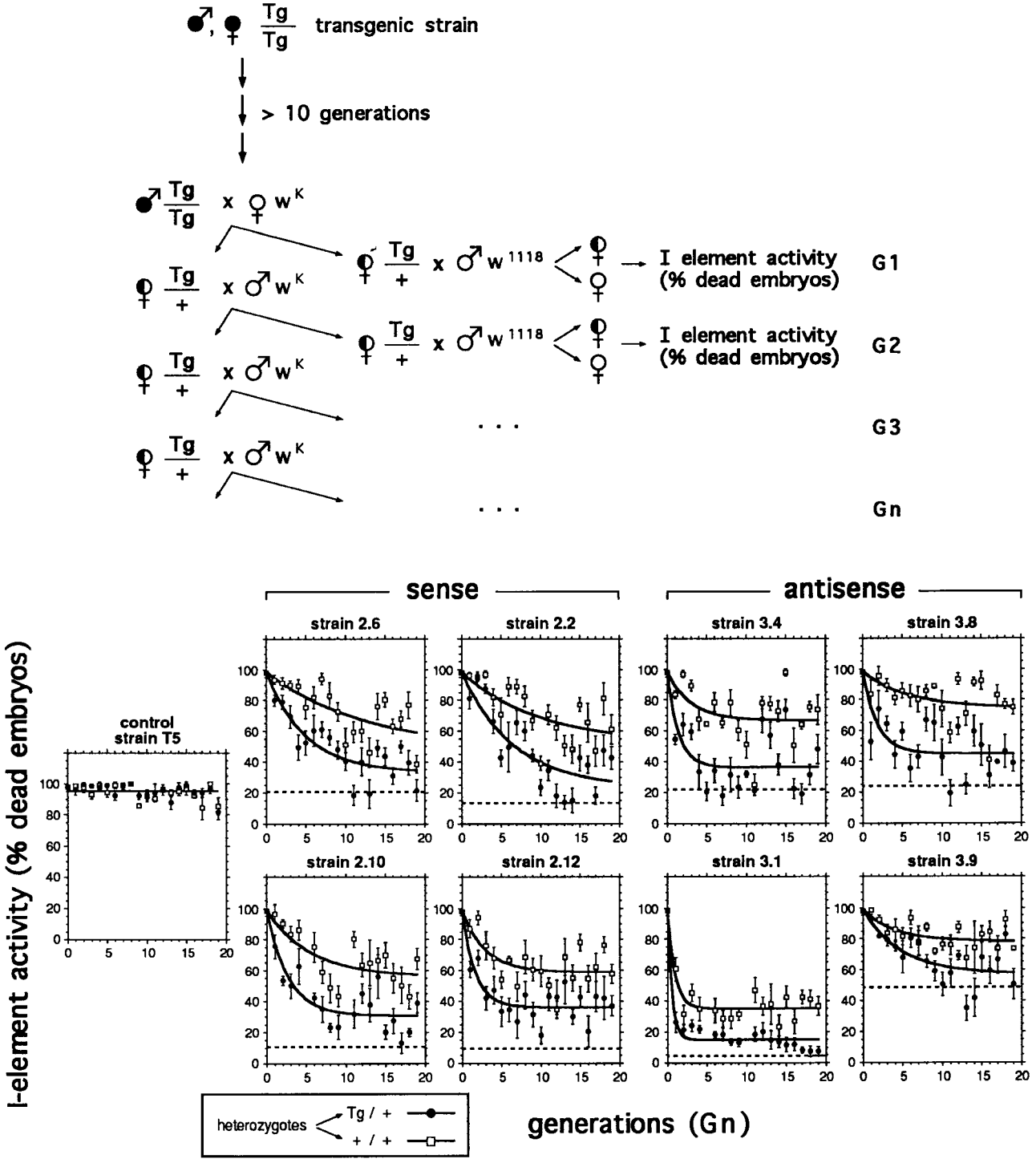

Figure 4.—Closely related kinetics of the generation-dependent repression process are observed for different heterozygous single-copy transgenic strains with the sense or antisense construct. *I*-element activity was assessed at different generations (Gn) following an initial resetting of the flies in a nonrepressed state by paternal transmission of the transgenes (see scheme at the top). The results are presented for four independent single-copy sense strains and four independent single-copy antisense strains, maintained in a heterozygous state to slow the rate of establishment of the cosuppressed state, for both the transgene-containing (solid symbols) and the transgene-free (open symbols) female progeny. The level of repression of the corresponding homozygous strains are indicated in dotted line. The data shown are the mean values (±SD) for five batches of 40 embryos.

direct evidence for the production of antisense transcripts produced at a low level in some transgene-mediated cosuppression experiments in plants (*e.g.*, Hamada and Spanu 1998), (2) the demonstration that the extent of cosuppression was much higher when both sense and antisense transcripts were simultaneously produced

by corresponding transgenes (Montgomery and Fire 1998; Waterhouse *et al.* 1998), and (3) recent evidence for repression of endogenous genes by direct injection of dsRNA molecules in both *Caenorhabditis elegans* (Fire *et al.* 1998) and Drosophila (Kennerdell and Carthew 1998) or by dsRNA transfection in *Trypanosoma brucei* (Ngo *et al.* 1998). This model would also be consistent with our observation of antisense transcripts in transgenic Drosophila containing *I*-derived sense constructs: actually, in five out of five tested hsp[i2Δ]pA transgenic strains, the quantitative RT-PCR TaqMan method using primers specific for sense or antisense transcripts (same primers and fluorescent probe used as for the TaqMan quantitation of transgene copy number; see materials and methods) provided evidence for low but significant amounts of antisense RNA, at levels ranging from 1.3 to 6.4% that of the related sense transcripts (S. Jensen, unpublished data). The second, and not exclusive, model, which would account for the symmetrical effects of sense and antisense constructs, relies on the possible involvement of DNA molecules as the symmetrical target for RNAs mediating the repressing effect. Yet, there is still no evidence for a direct, necessary targeting of DNA in cosuppression. Rather, recent experiments mentioned above have shown that repression of endogenous genes by injected dsRNAs had no effect when the dsRNAs corresponded to intronic domains of the gene to be regulated (Fire *et al.* 1998), thus favoring a direct effect—via a still-unresolved mechanism—at the level of the gene mRNA resulting in its degradation (reviewed in Grant 1999; Sharp 1999). It has also been shown that RNA is a target for dsRNA-mediated genetic interference in *C. elegans* (Montgomery *et al.* 1998). Similarly, repressing effects have been described for RNA viruses in plants, which clearly do not have replicative DNA intermediates (reviewed in Bruening 1998; Carrington and Whitham 1998; Vaucheret *et al.* 1998). Conversely, a series of experiments has unambigously demonstrated that RNAs from some transgenes may have a direct effect on endogenous genes by inducing DNA methylation and gene inactivation (Wassenegger *et al.* 1994; Jones *et al.* 1998; Mette *et al.* 1999; reviewed in Wassenegger and Pélissier 1998) and thus are possibly involved in long-term heritable silencing effects through changes in the chromatin state as manifested in parental imprinting in mammals (reviewed in Brannan and Bartolomei 1999) or paramutation in plants (reviewed in Hollick *et al.* 1997).

In conclusion, models involving dsRNA molecules as an effector for RNA degradation and an effect of these dsRNA molecules on DNA sequences will most probably turn out to be necessary to account for cosuppression. Accordingly, the presently observed cumulative, generation-dependent, germline transmission of *I* repression could be mediated by the transmission of dsRNA molecules via the oocyte, which would then act to generate new dsRNA molecules, either by direct synthesis from the transmitted dsRNA itself involving yet unknown RNA polymerases (Dougherty and Parks 1995; Wassenegger and Pélissier 1998), or by aberrant transcription from a homologous DNA sequence "modified" or "imprinted" by the transmitted dsRNA signal itself (reviewed in Grant 1999). Further studies will be necessary to unambigously determine how such dsRNA effectors are perpetuated and amplified through the successive generations and whether some DNA sequences—possibly the endogenous *I*-related ancestral elements acting as a relay—are involved in this process.

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