Mutations at the *Darkener of apricot* Locus Modulate Transcript Levels of *copia* and *copia*-Induced Mutations in *Drosophila melanogaster*

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ABSTRACT

Mutations of the *Doa* locus of *Drosophila melanogaster* darken the eye color of the *copia*-induced *white^{apricot}* (*w^a*) allele and increase the accumulation of *white* promoter-initiated transcripts encoding functional mRNA. We show here that quantities of transcripts initiated in both long terminal repeats (LTRs) of the specific *w^a-copia* element are increased, and those initiating in the 5' LTR of the element are structurally altered, yielding a slightly shortened transcript. Accumulation of host-initiated transcripts of a *copia*-induced mutation within the *achaete-scute* complex, *Hairy-wing^{Ua}* (*Hw^{Ua}*), are reduced by *Doa* mutations. Finally, we show that homozygosity for *Doa* mutations increases the accumulation of *copia* transcripts from the population of elements in the genome. These results suggest that *Doa* modulates the severity of *copia*-induced mutations while functioning as a dosage-sensitive modulator of *copia* transcription.

THE *apricot* allele of the *white* locus (*w^a*), of *Drosophila melanogaster* is caused by the insertion of a *copia* retrotransposon in the second intron (BINGHAM and JUDD 1981; O'HARE *et al.* 1984; PIRROTTA and BROCKL 1984). This insertion reduces *white* function primarily due to abnormal termination of transcription in the long terminal repeats (LTRs) of the *copia* element (LEVIS, O'HARE and RUBIN 1984; PIRROTTA and BROCKL 1984; ZACHAR *et al.* 1985), yielding the mutant phenotype of orange (instead of red) eyes, and coordinate reduction of pigment in the Malpighian tubules and testes. *Copia* is arguably the best characterized of the retrotransposons known in *Drosophila* (FLAVELL 1984; FLAVELL *et al.* 1980, 1981; KIKUCHI, ANDO and SHIBA 1986; KUGIYAMA, IKENAGA and SAIGO 1983; MOUNT and RUBIN 1985; SCHWARTZ, LOCKETT and YOUNG 1982; SHIBA and SAIGO 1983; SINCLAIR *et al.* 1986; SNEDDON and FLAVELL 1989; YOSHIOKA *et al.* 1990), and its insertions have caused mutations in several other genes in addition to *w^a* (COTE *et al.* 1986; JACK 1985; MATTOX and DAVIDSON 1984; RUBIN, KIDWELL and BINGHAM 1982). Among these is a gain-of-function allele, *Hairy-wing^{Ua}* (*Hw^{Ua}*), of the *achaete-scute* (AS-C) complex (CAMPUZANO *et al.* 1986). This complex of several transcription units (ALONSO and CABRERA 1988; CAMPUZANO *et al.* 1985), is involved in the development of sensory chaetae (bristles and hairs) and their associated neurons (GARCIA-BELLIDO 1979). Under-

expression of the AS-C gives rise to the *achaete* (*ac*), and *scute* (*sc*), mutations, in which bristle number is reduced (GARCIA-BELLIDO 1979). Overexpression of the AS-C leads to the *Hairy-wing* (*Hw*) phenotype, where mutants have excess chaetae, due to overaccumulation of AS-C transcripts (BALCELLS, MODOLELL and RUIZ-GOMEZ 1988; CAMPUZANO *et al.* 1986). The transcriptionally anti-parallel *copia* insertion in *Hw^{Ua}* causes both overaccumulation and premature termination of transcription of the *sc* (T4) transcript.

Mutations in second-site modifier loci in *Drosophila* alter the phenotypes of many retrotransposon-induced mutations, with differing specificities for affected transposons and mutant alleles (CHANG *et al.* 1986; GREEN 1959; MODOLELL, BENDER and MESESON 1983; RUTLEDGE *et al.* 1988). Second-site modifier mutations enhance (increase) or suppress (decrease) the severity of affected alleles, and some have both properties, with opposite effects on different mutations. Single mutant alleles can both be enhanced and suppressed by different modifier mutations. Genetic criteria demonstrate that the mechanisms of phenotypic modification by second-site modifiers are diverse (MOUNT, GREEN and RUBIN 1988; RABINOW and BIRCHLER 1990; RUTLEDGE *et al.* 1988), and molecular analyses have implicated their gene products in functions such as DNA-binding, transcription (GEYER, GREEN and CORCES 1988; PARKHURST and CORCES 1986; PARKHURST *et al.* 1988; PEIFER and BENDER 1988; SPANA, HARRISON and CORCES 1988), enhancer-promoter interactions (JACK *et al.* 1991), transcriptional termination (DORSETT 1990; DORSETT *et al.* 1989), and RNA processing and turnover (CHOU,

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ZACHAR and BINGHAM 1987; FRIDELL, PRET and SEARLES 1990; GEYER *et al.* 1991; MITCHELSON *et al.* 1993; ZACHAR, CHOU and BINGHAM 1987). In general, the products of second-site modifier loci play a role in the expression of the mutation-causing transposable element, and in modifying its activity, result in an alteration of the mutant phenotype.

We are seeking to understand the functions these modifiers serve in retrotransposon and gene expression. Modifiers of w^a comprise the most extensive set affecting a single transposon-induced allele known in a metazoan. Several of them have overlapping effects on transposon-induced mutations at other loci, and some affect additional transposon-induced alleles of *white* (BIRCHLER and HIEBERT 1989; BIRCHLER, HIEBERT and RABINOW 1989; GREEN 1959; RABINOW and BIRCHLER 1989). Each modifier affects a different process, since they are genetically additive and each has a different quantitative and qualitative effect on w^a RNA (BIRCHLER and HIEBERT 1989; BIRCHLER, HIEBERT and RABINOW 1989; LEVIS, O'HARE and RUBIN 1984; MOUNT, GREEN and RUBIN 1988; PIRROTTA and BROCKL 1984; RABINOW and BIRCHLER 1989, 1990; ZACHAR *et al.* 1985). Mutations in one of these, the *Darkener of apricot* (*Doa*), dominantly suppress (darken) w^a , and enhance (lighten) w^{sp55} (RABINOW and BIRCHLER 1989), an allele induced by a transposon distinct from *copia* (ZACHAR and BINGHAM 1982). The *Doa* product presumably interacts with sequences contained within the transposons inserted at w^a and w^{sp55} , since *white* alleles with point mutations do not respond (RABINOW and BIRCHLER 1989). *Doa* acts upon w^a as an inverse function of its own dosage, *i.e.*, is lightened by additional copies of wild-type *Doa*. Conversely, w^{sp55} is directly affected by *Doa*, addition of wild-type copies producing progressively darker eyes. These unusual dosage interactions suggest that the *Doa* product is involved in a stoichiometrically sensitive process of gene expression. *Doa* also plays a vital role in host gene expression, since mutant alleles are almost invariably recessive lethal. Rare surviving individuals which are *trans*-heterozygotes for a few specific *Doa* alleles possess wild-type pigmented eyes in a w^a background, and therefore the *Doa* product defines a process essential for *copia*'s mutagenic effect. A role for *Doa* in normal eye development is indicated by the roughened eye phenotype of *Doa* heteroallelic flies. These *trans*-heterozygotes show a reduction of stable transcripts initiated at the *white* promoter and terminating in the *copia* LTRs, while the amount of wild-type-sized transcript is increased. The *Doa* product is thus implicated in transcription termination in the *copia* LTR, but this explanation does not account for the entirety of phenotypic effects, since among other observations, the structure of *copia* mRNA is unaltered.

We wished to further our understanding of the role *Doa* plays in expression of the *copia* retrotransposon. Using "natural" fusion transcripts initiating in the LTRs of the *copia* element at w^a and terminating in *white*, as well as those from the population of elements, we show here that *Doa* mutants increase the accumulation of *copia* transcripts, probably through altering rates of transcription. We also show that the transcript initiating in the 5' LTR of the element at w^a is structurally altered. Although *Doa* mutations increase the amount of functional *white* mRNA, they decrease transcript accumulation of an anti-parallel *copia*-induced allele, Hw^{Ua} , consistent with observed phenotypic responses, although contrasting with previous observations at w^a .

MATERIALS AND METHODS

Drosophila culture, stocks and new *Doa* alleles: Crosses were maintained at 25° on Carolina Biological Instant Drosophila Medium. γ -Ray mutagenesis was performed by irradiating w^a males with 4000 rad from a ^{137}Cs source. Males were crossed to w^a females, and removed after 3 days. F₁ individuals with altered eye color were selected for further characterization. In the hybrid dysgenic screen, a P element vector carrying G418 resistance and a pUC plasmid (COOLEY, KELLEY and SPRADLING 1988) was mobilized by the $\Delta 2-3$ P element at 99B (ROBERTSON *et al.* 1988). F₁ dysgenic males were crossed to w^a females, and individuals with altered eye color were again selected. In both mutageneses, isolates failing to complement the recessive lethality of other *Doa* alleles and segregating with the third chromosome were identified as new *Doa* alleles. Five new γ -ray-induced alleles were recovered, and one new one resulted from the dysgenic screen. One of them, *Doa* ^{$\gamma 3B$} , shows a small cytological aberration of undetermined nature at the previously determined location of the gene, 98F1-2. As for previously described *Doa* alleles, all darken w^a and are recessive lethal, both as stocks and fail to complement the recessive lethality of other *Doa* alleles. The dysgenic allele, *Deus ex machina* (*Dem*), darkens w^a , and is recessive lethal as a stock and with all *Doa* alleles previously shown not to produce *trans*-heterozygotes. However, *Doa*^{*Dem*} survives at a frequency approaching 50% in combination with other *Doa* alleles known to produce individuals escaping recessive lethality (*HD1*, *HD2*, *105* and *CC*). Interestingly, surviving *trans*-heterozygotes of *Doa*^{*Dem*} and other *Doa* alleles do not demonstrate the roughened eye phenotype otherwise invariably found. We interpret the high frequency of flies escaping recessive lethality and lack of the roughened eye phenotype as indicative of a less severe lesion at *Doa*. We exploited this high frequency of flies escaping recessive lethality to produce progeny in crosses described below. Deficiencies of the region have not yet been identified to allow characterization of these alleles as hypomorphs or nulls.

To determine if *Doa* mutations altered the quantity or structure of transcripts initiating in the LTRs of the specific *copia* element inserted at w^a , RNA was prepared from adults, pupae and larvae segregating from a cross between male w^a ; *Doa*^{*HD1*}/*TM6b*, *Tb* and female w^a ; *Doa*^{*105*}/*TM6b*, *Tb*. The *Tubby* (*Tb*) marker carried on the *TM6b* balancer chromosome distinguished *Doa/Doa* pupae and larvae from heterozygous siblings. Adults were separated by eye color, *Doa/Doa* adults possessing wild-type, brick-red eyes.

Maternal contribution to survival of *Doa* heteroallelic flies: A gain in the survival rate of *Doa* trans-heterozygotes escaping lethality by a factor of approximately 5-fold is obtained when either of two *Doa* alleles used in producing trans-heterozygotes, *Doa*¹⁰⁵ and *Doa*^{CC}, are used as females when crossed with *Doa*^{HD1} or *Doa*^{HD2}, as opposed to the reciprocal cross. This finding was surprising, because *Doa*¹⁰⁵ and *Doa*^{CC} are both due to translocations, and are substantially less fecund than *Doa*^{HD1} and *Doa*^{HD2}. Given the characterized cytological breakpoints in the 105 and CC alleles, it is likely that both are due to position effect. Both in fact show slight reproducible variegation, with the posterior quarter of the eye darker than the anterior three fourths at various ages. The variation in the percentage of individuals escaping lethality further suggests the existence of a maternal effect of *Doa* mutations. Using this crossing scheme, generation of *Doa* trans-heterozygotes at approximately 10% of the expected Mendelian frequency is attained.

Additional effects of the genetic background influencing the rate of survival of *Doa* heteroallelic individuals were also noted. Chief among these is that when the balancer chromosome *TM6b*, *Tb* is used in crosses for production of *Doa* trans-heterozygotes, the rate of survival is increased 2–5-fold. In crosses of this sort (e.g., *Doa*^{HD1}/*TM6b* × *Doa*¹⁰⁵/*TM6b*), lethality of most *Doa* heteroallelic individuals occurs during pupation, as evidenced by many dead *Tb*⁺ pupae. Use of the *TM6b* balancer has no effect on the lethality within any *Doa* stock or in crosses between alleles not previously observed to yield trans-heterozygotes, however.

Other *copia*-induced alleles: *Hairy-wing*^{Ua} (*Hw*^{Ua}) (CAMPUZANO *et al.* 1986), is a hypermorph, caused by an anti-parallel *copia* insertion in an exon, generating a truncated RNA. The protein produced is functional, and is present at elevated levels relative to wild type. To examine *Doa* effects on *Hw*^{Ua} RNA, male *y*² *sc* *Hw*^{Ua} *w*^a; *Doa*^{HD1}/*TM6b*, *Tb* were crossed to *y*² *sc*¹ *Hw*^{Ua} *w*^a; *Doa*^{DM}/*TM6b*, *Tb* females. RNA was prepared from *Doa* trans-heterozygotes, heterozygous siblings, and a stock carrying the appropriate X chromosome (*Hw*^{Ua} or +).

*Beadex*⁴⁶ (*Bx*⁴⁶) was previously described as a *copia* insertion (MATTOX and DAVIDSON 1984). The orientation of the *copia* element relative to the host gene was deduced from the restriction map of this allele. The *copia* insertion lies near the 3' end of a transcript thought to be *Bx*, and yields a truncated RNA (W. MATTOX, personal communication). *Beadex* alleles are hypermorphs, since duplications of the wild-type locus yield a recessive *Beadex* phenotype (LIFSCHYTZ and GREEN 1979). *w*^a *Bx*⁴⁶; *Doa*^{CC}/*CyO* females were crossed with *y*^a; *Doa*^{HD1}/*TM6*, *Tb* male flies, and the amount of wing-structure was compared among the four classes of segregants in the F₁ generation. Since the *Bx* locus is X-linked, F₁ males were hemizygous for *Bx*⁴⁶, and females were heterozygous for wild-type and *Bx*⁴⁶ alleles. Similar tests were performed with *Bx* alleles generated by the insertion of B104 elements (*Bx*¹⁻³), and insertion of dual gypsy elements (*Bx*²), with no effects of altered *Doa* dosage observed.

RNA isolation, Northern blots and RNA probes: Total RNA was extracted from frozen organisms by guanidine-HCl extraction (COX 1968). Collections were made from stocks and crosses from the developmental stages of wandering third instar larvae, 0–24-hr-old pupae, and 0–24-hr-old adults. Ten micrograms per lane of total RNA were separated on 1.5% formaldehyde-agarose gels (LEHRACH *et al.* 1977), after which the RNA was transferred to Biotrans nylon filters. The RNA was fixed to the filter by both UV crosslinking and baking under vacuum at 80° for 2 hr.

Anti-sense RNA probes were synthesized with T3 or T7 RNA polymerase. T4 and T5 probes of the AS-C were

cDNA clones kindly provided by JUAN MODOLLEL. Probes for *white* and *copia* were previously described (RABINOW and BIRCHLER 1989). Loading controls were performed by re-hybridizing the blots with a probe for *rp49* (O'CONNELL and ROSBASH 1984). Blots were hybridized at 60°, under described conditions (DORSETT *et al.* 1989), at a probe concentration of >2 × 10⁶ cpm/ml. Filters were washed with 0.1 × SSC, 0.4% SDS at 75° after overnight hybridization. Estimation of RNA levels on the autoradiograms was performed with an LKB 2202 Ultra-Scan laser densitometer, and values were normalized to levels of *rp49*.

RESULTS

Interaction of *Doa* with other *copia*-induced mutations: To study the interactions of *Doa* alleles with *copia*-induced alleles with varied insertion sites and orientations relative to the host gene, we examined the response of the *copia*-induced alleles *Hairy-wing*^{Ua} (*Hw*^{Ua}) and *Beadex*⁴⁶ (*Bx*⁴⁶). The structure of *w*^a and *Hw*^{Ua} and their major transcripts are shown in Figure 1.

*Beadex*⁴⁶: The *copia* insertion in *Bx*⁴⁶ results in loss of posterior wing-margins, typical of the *Bx* phenotype. The *copia* insertion is transcriptionally anti-parallel to the host gene, as deduced from restriction maps. Like all *Beadex* alleles, *Bx*⁴⁶ is a hypermorph (LIFSCHYTZ and GREEN 1979). In crosses to generate *Doa* heteroallelic flies, the F₁ generation was scored. F₁ males were hemizygous for the X-linked *Bx*⁴⁶ allele, (genotype *y* *w*^a *Bx*⁴⁶/*Y*), while females were heterozygous for the wild-type and *Bx*⁴⁶ alleles (genotype *y* *w*^a *Bx*⁴⁶/*w*^a), and segregating for the two *Doa* alleles and balancer chromosomes. Sexes were scored separately, and the phenotypes of the four segregant classes of siblings were compared. In *Bx*⁴⁶/+ females from this cross, a slight suppression of the *Beadex* phenotype is observed in *Doa* mutant heterozygotes relative to wild-type siblings, while in heteroallelic *Doa* mutant females, it is completely suppressed (Figure 2). In males from this cross, the dose of functional *Doa* product also varies the amounts of wing tissue, although *Bx*⁴⁶ is never completely suppressed: wild type has the least wing tissue, *Doa* mutant heterozygotes intermediate amounts, and *Doa* heteroallelic flies the most. Other *Bx* alleles similarly tested (*Bx*-a B104 element, *Bx*²-an insertion of dual gypsy elements, *Bx*³-also a B104 element) do not respond to *Doa* mutations, confirming that the interaction is based upon the *copia* element inserted in *Bx*⁴⁶.

Hairy-wing^{Ua}: The *Hw*^{Ua} phenotype is caused by a *copia* element inserted transcriptionally anti-parallel to the T4 gene of the AS-C (CAMPUZANO *et al.* 1986). The *copia* element is inserted one-third of the way upstream of the 3' end of the mRNA, and causes overaccumulation of a 1.3-kb transcript truncated in the 3' LTR of the inserted element, which yields a functional protein. *Hairy-wing* mutations are also classified as hypermorphs (CAMPUZANO *et al.* 1986). In

A. white (apricot) **B. Hairy-wing (*Ua*)**

FIGURE 1.—Structure of two *copia*-induced mutations and their transcripts. (A) The summarized structure of *w^a* is based upon reports cited in the text and on additional data presented in this report. The gene is oriented to agree with genetic precedent, telomere to the left, entomere to the right, and is aligned at the exons of *white*. The *copia* element is inserted in a transcriptionally parallel orientation to the *white* gene. (B) The structure of the *Hw^{Ua}* allele of T4 of the AS-C, as excerpted from CAMPUZANO *et al.* (1986). The *copia* element is inserted transcriptionally anti-parallel to T4 and produces a truncated mRNA. The resultant protein retains activity, and this allele is classified as a hypermorph, which is due to an overaccumulation of T4 mRNA (CAMPUZANO *et al.* 1986).

Doa mutant heteroallelic flies, suppression of *Hw^{Ua}* results in loss of all scutellar bristles (RABINOW and BIRCHLER 1990). However, since 50% of *Hw⁺*; *Doa* trans-heterozygotes lack one or more scutellar bristles (RABINOW and BIRCHLER 1989), this interaction was possibly due to effects of *Doa* mutations on expression of the wild-type AS-C.

To determine whether the phenotypic suppression of *Hw^{Ua}* by *Doa* was specific to this *copia*-induced allele or an interaction with the host gene, RNA was prepared from adults, pupae, and larvae segregating from a cross between male *y² sc Hw^{Ua} w^a; Doa^{HD1}/TM6b Tb* and female *y² sc Hw^{Ua} w^a; Doa^{Dem}/TM6b, Tb*. *Hw⁺* samples were generated in a cross of male *w^a; Doa^{HD1}/*

A. **B.** **C.**

FIGURE 2.—*Doa* mutations suppress the phenotype of a *copia*-induced *Beadex* allele. A *copia* element inserted in *Bx⁴⁶* causes a typical *Beadex* phenotype of scalloped wings. This element is inserted in a transcriptionally anti-parallel orientation relative to the host gene, based on its restriction map (MATTOX and DAVIDSON 1984). *Doa* mutations suppress the dominant *Bx* phenotype, restoring wild-type wings in the most extreme cases. Shown are wings of female progeny segregating from a cross between male *y w^a Bx⁴⁶; Doa^{HD1}* and female *w^a; Doa^{CC}/CyO*. These females are heterozygous for *Bx⁴⁶* and a wild-type *Bx* allele, and segregate as indicated for *Doa* genotype. (A) *y w^a Bx⁴⁶/+; +/+*; (B) *y w^a Bx⁴⁶/+; Doa^{CC}/+*; (C) *y w^a Bx⁴⁶/+; Doa^{CC}/Doa^{HD1}*. The phenotype of the *Doa* heteroallelic escapers is essentially wild-type.

TM6b, Tb with female *w^a; Doa¹⁰⁵/TM6b, Tb*. The 105 allele used to generate control samples is more hypomorphic (less functional) than the *Dem* allele, based on the rate at which trans-heterozygotes are generated, stronger bristle effects and eye-roughening in 105/*HD1* than in *Dem*/105 trans-heterozygotes. Thus,

FIGURE 3.—Effect of heteroallelism for *Doa* mutations on the accumulation of RNA from the T4 gene of the AS-C in *Hw^{Ua}* and wild-type backgrounds. Pupal RNA is shown in both panels. (A) The *Hw^{Ua}* allele is due to the overaccumulation of T4 RNA, truncated in the transcriptionally anti-parallel *cop*ia, inserted in the single exon of the gene near the its 3' end. Heteroallelism for *Doa* mutations causes a reduction of approximately twofold in accumulation of this transcript. Antisense RNA for ribosomal protein 49, *rp49* (O'CONNELL and ROSBASH 1984), was used to probe the same blot as a loading control. The unmarked band between the T4 transcript and the *rp49* loading control is an uncharacterized transcript detected in our experiments specifically with the T4 cDNA probe. It is expressed throughout development, and is unaffected in either expression or size by the *cop*ia insertion in *Hw^{Ua}* compared to wild type. This transfer shows a relative difference in accumulation of T4 transcript of *Hw^{Ua}*; +/+ compared to *Hw^{Ua}*; *Doa/Doa* of 2.3-fold. (B) RNA derived from *Hw⁺* flies generated in crosses to produce the blots in Figure 4A was size-fractionated and probed with the T4 cDNA used in (A). The *rp49* probe was used as a loading control on the same transfer. No differences in accumulation of the T4 transcript were found to correlate with the genotype at the *Doa* locus.

any effects on the wild-type T4 transcript should have been magnified in the controls relative to the experimental samples. RNA levels were determined from Northern transfers, probed with a cDNA probe of the T4 gene of AS-C, and quantitated on a scanning densitometer.

A reduction of approximately twofold in the amount of *cop*ia-truncated T4 RNA in *Hw^{Ua}* was found in both larval (Figure 3A), and pupal stages from *Doa trans*-heterozygotes relative to heterozygous siblings, and also a non-sibling *y² sc Hw^{Ua} w^a; Doa⁺* stock. Since *Hw^{Ua}* is due to overaccumulation of the T4 transcript, a reduced level in *Hw^{Ua}*; *Doa* mutants is consistent with the observed phenotypic suppression. Unlike *w^a*, no wild-type transcript was restored. T4 transcript levels in a wild-type AS-C background do not vary in response to mutations at *Doa* (Figure 3B). No effects were found on expression of the AS-C T5 transcript, which was tested by reprobing the same blot with a cDNA probe for T5. Thus, interaction of *Hw^{Ua}* with *Doa* mutants is due to interaction with the inserted *cop*ia element, and results in a reduction of host gene transcript accumulation, in contrast to our findings at *w^a*, although in both cases the interaction suppresses the effects of the insertion.

Effects of *Doa* mutations on transcripts of the

***cop*ia element at *w^a*:** Using hybridization probes derived from *white* exons downstream of the *w^a-cop*ia insertion, we detected transcripts of about 7 kb and 2 kb initiating in the LTRs of this specific element and reading-through into *white* (Figure 1A). The structure and expression of transcripts initiating in this element therefore serve as reporters for transcription of the single *w^a-cop*ia element, which necessarily interacts with *Doa*. The 7-kb transcript initiates in the 5' LTR of *cop*ia and terminates at or near the normal site in the *white* gene, based on its patterns of hybridization to *white* probes, its length, and modulations in size by insertions into the 5' and 3' *cop*ia LTRs in *w^a* revertants (BIRCHLER and HIEBERT 1989; BIRCHLER, HIEBERT and RABINOW 1989; our unpublished data). The 2-kb transcript initiated in the *w^a-cop*ia was hypothesized to originate in the 3' LTR and terminate in *white*, based on its size and hybridization pattern (Figure 1A) (ZACHAR *et al.* 1985). It is primarily expressed during larval and pupal stages, periods of high *cop*ia expression (PARKHURST and CORCES 1987; SCHWARTZ, LOCKETT and YOUNG 1982).

In heteroallelic *Doa* mutant backgrounds, a new transcript approximately 500 bases smaller than the 5' LTR-initiated product is found, at a roughly equal intensity to it (Figure 4A). This transcript has a pat-

FIGURE 4.—*Doa* mutations alter the structure of the copia 5' LTR-initiated read-through transcript and the quantity of a transcript initiating in the 3' LTR. (A) Heteroallelism for *Doa* mutations results in the formation of a doublet band in the copia 5' LTR-initiated read-through transcript, and also increases transcript accumulation of the copia 3' LTR-initiated transcript, which is found primarily in larvae and pupae. Pupal RNA is shown. Note that the *Doa* heteroallelic lane is slightly underloaded relative to *w*^a; +/+, and thus the increase in LTR-initiated transcripts is underestimated. The *rp49* probe was used as a loading control on the same transfer. On this autoradiogram, the 7-kb copia 5' LTR-initiated doublets in *w*^a; *Doa/Doa* compared to the single band in *w*^a; +/+ are increased 1.7-fold, and the 2-kb copia 3' LTR-initiated transcript is increased 1.3-fold. Other transfers show that *Doa* mutations increase levels of the 3' LTR-initiated transcript up to 2.4-fold. The 1.8-kb transcript is observed with *white* probes, is expressed throughout development, and is enriched in males. Its origin and structure have not been determined. (B) The larval and pupal 2-kb transcript driven from the 3' copia LTR in *w*^a is absent in *w*^{aR84h}. This *w*^a revertant is due to an 83-bp I element insertion into the 3' LTR (MOUNT, GREEN and RUBIN 1988), apparently inactivating transcription from it. The 2-kb transcript is also found in a solo-LTR revertant of *w*^a, *w*^{aR59k1}. Thus, the 2-kb transcript must initiate in the 3' copia LTR, which apparently encodes all information necessary for temporal specificity of its expression, since it is found in *w*^{aR59k1}. Pupal RNA is shown. The *rp49* probe was used to reprobe the same blot, as a loading control.

tern of hybridization with *white* probes identical to the 7-kb 5' copia LTR-initiated read-through transcript, and must be structurally closely related to it. Transcriptional initiation or splicing of the the 7-kb copia-initiated *white*-terminated transcript is thus partially altered by *Doa* mutations. Transcriptional termination and polyadenylation are presumably unchanged, since these processes occur in *white* sequences, and *Doa* has specificity for *copia* and not *white*. The new transcript varies in quantity relative to the the normal read-through product as a function of developmental stage. It is most prominent in larvae and pupae, and only faintly detectable in *w*^a; *Doa* mutant adults. When the quantities of the two read-through transcripts are summed, their amounts are increased twofold by homozygosity for *Doa* mutations, relative to heterozy-

gous *Doa* or wild-type backgrounds (Figure 4A).

Levels of the 2-kb copia-*white* transcript are also increased twofold in heteroallelic *Doa* individuals (Figure 4A), as confirmed in four repetitions of the experiment. We reasoned that if the structure of this transcript was as proposed, then the *Doa* target is likely located within the 276-bp copia LTR. We therefore tested its point of transcription initiation. Since insertions into LTRs of the *w*^a-copia might inactivate transcription from them, we examined levels of the putative 3' LTR initiated transcript in several *w*^a revertants (MOUNT, GREEN and RUBIN 1988). These included an insertion of 2.3 kb into or near the 5' LTR (*w*^{aRM}), an insertion of 83 bp into the 3' LTR (*w*^{aR84h}), and a recombination event leaving a solo LTR

in the second *white* intron (CARBONARE and GEHRING 1985).

The results confirm that the 2-kb read-through transcript initiates in the 3' LTR of the *w^a-copia*. This transcript is present in *w^a*, *w^{aRM}*, and the solo LTR revertant *w^{aR59kl}*, but lacking in *w^{aR84h}* (Figure 4B). The insertion in *w^{aR84h}* occurred in a region necessary for high levels of expression from the *copia* LTR in transfected tissue culture cells (SNEDDON and FLAVELL 1989). Thus, the *w^{aR84h}* insertion disrupts the LTR's ability to direct transcription, and our results confirm *in vivo* the *in vitro* results cited above. Levels of the 2-kb transcript are comparable between *w^a* and the solo LTR revertant, *w^{aR59kl}* (Figure 4B), and vary coordinately during third-instar larval, pupal and adult stages. This further suggests that all sequences necessary for developmentally regulated *copia* transcription are contained within the LTR. Since *Doa* mutations increase accumulation of this transcript in *w^a*, it is likely that the site responsible for interaction with *Doa* also lies within the LTR.

Effects of *Doa* mutations on *copia* RNA levels: Increased accumulation of transcripts initiating in the LTRs of the *w^a-copia* in *Doa* mutants led us to determine whether levels or structure of the transcripts from the population of elements in the genome were similarly affected. Although no dramatic changes in adult *copia* transcript levels due to *Doa* mutations were described in our initial report, a slight increase was observed in *Doa* mutant *trans*-heterozygotes (RABINOW and BIRCHLER 1989). By reprobing transfers used in characterizing *w^a* and *Hw^{Ua}* transcripts with probes for *copia* RNA, we examined effects of *Doa* mutations on *copia* transcript accumulation in third instar larvae, pupae, and adults. Comparisons reproducibly show elevations in *copia* transcript accumulation of twofold throughout these developmental stages, relative to the loading control *rp49* (Figure 5). These experiments were repeated five times for larval and pupal samples, and eight times for adult samples. Qualitatively identical results were obtained for each repetition, independent of the genetic background (three different backgrounds tested). No doublet bands as described for *w^a-copia* 5' LTR initiated transcripts, were observed in RNA from the population of *copia* elements (Figure 5). These would have been observed if they existed at levels corresponding to those found at *w^a*, given the demonstrated resolution of our gels of even higher molecular weight RNA species.

DISCUSSION

The diverse mechanisms of phenotypic modification of insertion-induced alleles by second-site modifier loci encompass many steps of gene expression, including transcription, splicing and transcription ter-

mination. Further definition of second-site modifier loci can be expected to characterize both known and unknown processes. We are investigating the *Doa* locus, which was previously shown to have unusual dosage-sensitive properties. Previous results showed that *Doa* mutations reduce the accumulation of transcripts initiating at the *white* promoter and terminating in both *copia* LTRs, while the quantity of wild-type *white* mRNA is increased (RABINOW and BIRCHLER 1989). This potentially implicated *Doa* in transcription termination within the *copia* element, but did not account for the failure to observe structurally altered *copia* transcripts from the population of elements. The large and variable number of *copia* elements in the genome introduces complications in any analysis of their expression, because individual elements may vary in response to a given modifier. We avoided these complications by using sequences downstream of the *copia* insertion in *w^a* as probes for transcription of the individual element. These probes reveal that accumulation of transcripts initiating in both LTRs of the *w^a-copia* element are increased roughly twofold in *Doa* mutant backgrounds compared to wild type. About 50% of the transcripts initiating in the 5' LTR of this specific *copia* are also slightly smaller, although this is not generalizable to transcripts from the population of elements. Quantitation of total *copia* transcript accumulation shows approximately a twofold increase in *Doa* mutant backgrounds, consistent with the effects seen at the specific *w^a*-element.

The "doublet" band formed by the *w^a-copia* 5' LTR-initiated transcript in *Doa* mutants is not due to altered transcription termination in *white* sequences, since *Doa* interacts specifically with the *copia* element, and not with *white* itself. Alteration in the splicing or site of transcription initiation of the *w^a-copia* element must therefore be responsible for formation of the "doublet" band. Since this transcript is extremely rare, and oligonucleotide primers to study its structure via amplification with a thermostable polymerase would necessarily be at least 5.5 kb apart, we have not performed further structural analysis of this transcript.

However, *Doa* mutations suppress the effects of both the transcriptionally anti-parallel *copia* insertions in *Bx⁴⁶* and *Hw^{Ua}* and the parallel insertion in *w^a*. They also have no effect on the relative proportions of spliced *versus* unspliced *copia* RNA. Since components of splicing and polyadenylation processes are presumably strand-specific, the probability that *Doa* is involved in splicing or polyadenylation of *copia* RNA seems remote. Based on these facts, it is thus most likely that *Doa* functions to modify the initiation of *copia* transcription. The significance of an altered site of transcription initiation in the *w^a-copia* by *Doa* mutations is obscured by the lack of a similar finding for

FIGURE 5.—Accumulation of the two major *copia* transcripts is increased in *Doa trans*-heterozygotes. RNA obtained from a *w^a* stock for the *w^a*; $+/+$ samples, and from a population segregating for *Doa¹⁰³*, *Doa^{HD1}* and *TM6b Tb*, for the *Doa trans*-heterozygotes and heterozygotes, was compared on Northern transfers. Quantities of both the full-length 5.2-kb and the 2.1-kb spliced *copia* transcripts are affected, approximately equally. The same transfers were reprobed with antisense *rp 49* RNA as a loading control. No doublets, as found originating from the *copia* element at *w^a* are observed in transcripts from the population of *copia* elements. The same transfer used in demonstrating the doublet induced by *Doa* mutants in the *copia* 5' LTR-initiated read-through transcript terminating in *white* (Figure 4A) served for the adult and pupal samples. We presume that *w^a-copia* read-thru transcripts were not visible in these reprobings because of differences in exposure time (hours instead of days). This is attributable to increased signal strengths of transcripts from the population of *copia* elements, compared to that of the specific single element at *w^a*. A different transfer was used to present the larval RNA data. Note that the wild-type pupal sample is slightly overloaded relative to the *Doa* homozygotes, and thus this sample represents an underestimate of *Doa* effects on *copia* transcription at this developmental stage. Based on densitometric scans, in which sample loadings were normalized using the *rp49* signal, these autoradiograms show the following quantitative increase in *copia* full-length transcript accumulation, comparing *w^a*; *Doa* homozygous samples to those of the *w^a*; $+/+$ stock: 4 $\times$ (adults); 1.4 $\times$ (pupae); 2.8 $\times$ (larvae), for a mean value of 2.6 $\times$.

the transcripts from the population of elements. Similar findings concerning some of the suppressors of *Ty* element insertions (*SPT*) in yeast have been reported (WINSTON *et al.* 1984, 1987). This subset of the *SPT* mutations cause *Ty* transcription to initiate 3' to its normal site in the LTR of the element, similar to what may be occurring to transcription of the *copia* element at *w^a*, but not to the population of elements. Increased transcription from the *copia* LTR in *Doa* mutants may correlate with altered initiation of transcription in the *w^a* element, perhaps by activating an otherwise cryptic promoter.

Our observations on effects of *Doa* mutations on

transcription from both specific and nonspecific *copia* LTRs are completely consistent. However, the non-proportional relationship between the effects of heterozygosity for *Doa* mutations on *white* pigment accumulation and *copia* and *w^a* RNA indicates a potential discrepancy. We observe a twofold effect on eye pigmentation in *Doa* mutant heterozygotes (estimated from comparison of *w^a* duplications with *Doa*-suppressed and unmodified *w^a*), and the lack of an effect of similar magnitude on both *copia* and *w^a* RNA in these flies. Twofold effects are found on *copia* RNA in *Doa trans*-heterozygotes. This difference has several potential explanations. Tissue specificity or a lack of

response of all *copia* elements to *Doa* mutations does not adequately explain it, since effects on total *copia* RNA are paralleled by effects on transcripts from the specific *copia* element at *w^a*, which must be responding to *Doa* mutations. We suggest that the magnitude of the *Doa* phenotypic effect on *w^a* in either heterozygotes or heteroallelic flies is not proportionally linked to the effects on *copia* transcription, which may in itself be a secondary consequence of *Doa* mutation.

Phenotypic suppression of *Hw^{Ua}* by *Doa* mutations is due solely to the insertion of *copia* in this allele, since transcripts of *Hw⁺* are not altered in quantity or structure. *Doa* mutations decrease *Hw^{Ua}* T4 transcript accumulation, in keeping with the observed phenotypic suppression and characterization of this allele as a hypermorph. This result is puzzling in light of our findings at *w^a*, where *Doa* mutations result in increased host gene transcript accumulation. It may be that the orientation of the inserted *copia* alters the effects of *Doa* mutations on transcript accumulation. This explanation would imply strand specificity in long range transcriptional interference and enhancement, for which no evidence exists in other systems. A more plausible interpretation is that decreased T4 mRNA levels in *Hw^{Ua}*; *Doa* double mutants is due to failure of RNA polymerase to terminate in the *copia* LTR, leading to a less stable mRNA. It may be noted that decreased termination in *copia* LTRs was previously observed at *w^a* in *Doa* mutant backgrounds. This interpretation suggests that increased stability of prematurely truncated messenger RNA, rather than increased transcription may be the cause of excess function in at least this *Hw* allele (CAMPUZANO *et al.* 1986).

The recessive lethality of *Doa* mutations, and their interactions with *w^a*, *Hw^{Ua}*, and *Bx⁴⁶* are presumably due to interference with a single process. Based on the increase in the 3' LTR-initiated transcript from *w^a* in pupae and larvae, it is likely that the target of *Doa* interaction lies within the *copia* LTR, although interaction with other elements of the transposon cannot yet be definitively excluded. Splicing, transcription termination and polyadenylation of *copia* messengers are unlikely candidates for *Doa* function, based on findings reported here. Prominent among the possible mechanisms not excluded is an alteration in the rate of *copia* transcription.

One model unifying our findings is that *Doa* functions as part of, or modifies a stoichiometric complex present on the LTRs of *copia*. Alteration of the components of this complex would result in alteration of *copia* transcription rates. This explanation accounts for *Doa*'s dosage-sensitive properties and hypothesizes that suppression of *copia*-induced phenotypes occurs when *Doa* fails to participate in or modify this complex, unbalancing the stoichiometry of the components. This would result in increased read-through by

RNA polymerase molecules initiating in the host promoters outside the element, generating more functional mRNA. This model would also account for the failure of *Doa* mutations to interact with *copia* elements inserted in upstream regulatory sequences, such as *ct^{ns}* (RABINOW and BIRCHLER 1990).

It should be noted that an inverse relationship between transcript accumulation and gene expression has been associated with the dosage of chromosomal segments unlinked to the affected structural genes in a wide variety of organisms and experimental systems, including maize (BIRCHLER 1985; BIRCHLER and NEWTON 1981), *Drosophila* (BIRCHLER, HIEBERT and KRIETZMAN 1989; BIRCHLER, OWENBY and JACOBSON 1982; DEVLIN, GRIGLIATTI and HOLM 1984; DEVLIN, HOLM and GRIGLIATTI 1988) and mouse (KLOSE and PUTZ 1983). These results indicate that the dosage of factors encoded in disparate chromosomal locations play a role in transcript accumulation of specific eukaryotic mRNA species. A single gene with such properties affecting the *white*, *brown* and *scarlet* loci of *D. melanogaster* was recently described (RABINOW, NGUYEN-HUYNH and BIRCHLER 1991). *Doa* thus represents another individual gene with such effects, albeit its first identified target is a transposable element rather than a host gene.

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