Alteration of *hsp82* gene expression by the gypsy transposon and suppressor genes in *Drosophila melanogaster*

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Several mutations in *Drosophila* result from insertion of the gypsy retrotransposon. Gypsy insertion mutagenesis and its modulation by allele-specific modifier genes were investigated by inserting gypsy or fragments of it into the intron of the *Drosophila hsp82* heat shock gene. With gypsy in the parallel orientation, nearly all transcripts in transfected cells and transformed pupae were truncated in the 5' long terminal repeat (LTR). Truncation also occurred in or near the 3' LTR. The 5' LTR polyadenylation signal was strongly potentiated by a downstream 326-bp internal gypsy segment in either orientation. Anti-parallel gypsy reduced the amount of normal transcript to a much smaller extent, and a low level of truncation occurred within gypsy. No evidence was found for effects of the gypsy insertions on the *hsp82* promoter. Mutations in the allele-specific modifier genes *su(f)* and *su(w^a)* had effects on the amounts of readthrough transcripts consistent with their genetic behavior, whereas the effects of mutations in *su(Hw)* were only partly in accord with genetic expectations.

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Numerous spontaneous mutations at various loci in *Drosophila melanogaster* result from the insertion of retrotransposons. The mutant phenotypes of many of these insertion alleles are made less severe (suppressed) or more severe (enhanced) by mutations in certain allele-specific modifier genes. An example of these phenomena is provided by the 7.5-kb gypsy element, insertions of which are associated with nearly all of the alleles that are suppressed by mutations of *suppressor of Hairy wing* [*su(Hw)*] (Modolell et al. 1983). Some of the gypsy-insertion alleles suppressed by *su(Hw)* mutations are also suppressed by other modifier mutations, such as *suppressor of forked* [*su(f)*], and are enhanced by still others, including *suppressor of white-apricot* [*su(w^a)*] (Rutledge et al. 1988).

It may be asked what mechanisms are responsible for the disruption of gene expression by modifiable insertion mutations and for the modulation of such effects by modifier genes. Studies of the copia-insertion allele *w^a* (Levis et al. 1984; Zachar et al. 1985) the 412-insertion allele *v¹* (Searles and Voelker 1986), and the gypsy-insertion alleles at the *achaete-scute* (Campuzano et al. 1986), *forked* (Parkhurst and Corces 1985; McLachlan 1986), and *yellow* (Parkhurst and Corces 1986) loci have indicated that the mutant phenotypes of these alleles and their modification result from effects on the formation of transcripts, including transcript truncation and

promoter effects. The identification and investigation of specific mechanisms, however, have been limited by uncertainties regarding the complex patterns of transcripts of the loci that have been studied.

To overcome these difficulties, we have made use of a simple inducible gene with a single intron, the *hsp82* heat shock gene. Without gypsy, the *hsp82* gene is transcribed in response to heat shock to give two prominent RNAs, the full-length transcript and its spliced product. We investigated the effects on transcript formation, in transfected cells and in transformed *Drosophila* pupae, of inserting the gypsy element or specific fragments of it into the *hsp82* intron. We also examined the effects of mutations in the modifier genes *su(Hw)*, *su(f)*, and *su(w^a)*. Here we present these observations and discuss their implications for the mechanisms of mutation by retrotransposon insertions and for the effects of modifier genes.

Results

The plasmid pCAT82SVSX constructed for use in these experiments contained the *D. melanogaster hsp82* gene with two marker DNA segments, SV inserted at bp 428 of the *hsp82* intron, and SX placed 156 bp farther downstream. SV and SX are simian virus 40 late gene coding sequences (see Methods) and do not contain known transcription or processing signals. Another marker segment, CAT, was substituted in the second exon. Derivatives of pCAT82SVSX were constructed in which the *bx^{34e}* gypsy element or fragments of the element were

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inserted at the *SalI* site at the 3' junction of the SV marker. The control plasmid pLAC82SU contained the *hsp82* gene with the marker segment SU in the intron and the marker segment LAC substituted in exon 2. The structures of the heat shock genes in pCAT82SVSX and pLAC82SU and of the gypsy element are depicted in Figure 1.

Gypsy insertions reduce the amount of normal transcript

In the first series of experiments, cultured *Drosophila* cells were transfected with plasmid pCAT82SVSX or with pCAT82SVSX containing the *bx^{3de}* gypsy element inserted in either direction. The cultures were simultaneously cotransfected with the control plasmid pLAC82SU. After 48 hr, each transfected culture was divided into two aliquots, one of which was heat shocked for 20 min at 34.5°C. Total cell RNA from each aliquot was extracted and analyzed on a Northern blot hybrid-

ized sequentially with CAT, SV, and LAC anti-sense RNA probes. All RNAs detected with these probes were increased by heat shock. The mild heat shock also increased the ratio of unspliced to spliced transcript, although it was considerably less severe than the heat shock required to inhibit splicing (Yost and Lindquist 1986).

A Northern blot of RNA from each of the three heat-shocked cultures in a typical experiment is shown in Figure 2. The plasmid pCAT82SVSX (lane 1) was transcribed to give a 3.4-kb RNA that hybridized to the SV and CAT probes and a 1.8-kb RNA that hybridized only to the latter, as expected for the unspliced and spliced transcripts from the *hsp82* promoter, respectively. The LAC probe hybridized to a 5.1-kb RNA and a 3.9-kb RNA, corresponding to the unspliced and spliced transcripts of the pLAC82SU control plasmid.

In repeated experiments, the plasmid with an anti-parallel gypsy insertion (the gypsy antisense strand inserted into the *hsp82* sense strand), pCAT82SVSX-Ga (Fig. 2, lane 3), consistently gave 2- to 3-fold less of the 1.8-kb spliced transcript than did the plasmid without gypsy, as estimated by normalization to the spliced transcript of the pLAC82SU control. The expected 10.8-kb unspliced transcript was detected with CAT and SV probes. Its amount relative to that of the unspliced transcript of pCAT82SVSX could not be estimated, however, due to the large but unknown difference in blotting efficiencies expected for RNA molecules differing so greatly in size.

Insertion of gypsy in the parallel orientation (Fig. 2, lane 2) reduced the amount of the 1.8-kb spliced transcript by a factor of approximately 20, a much greater reduction than found for anti-parallel gypsy. The amounts of the 1.8-kb and 10.8-kb transcripts given by the plasmid containing parallel gypsy, pCAT82SVSX-Gp, were both approximately 10-fold less than the amounts given by pCAT82SVSX-Ga.

Northern blot analysis of RNA from cells not subjected to heat shock showed reductions in the amount of spliced transcript from plasmids with anti-parallel and parallel gypsy insertions similar to those found for the corresponding heat-shocked cultures (not shown). Thus, with and without heat shock, gypsy insertions in the *hsp82* gene reduced the amount of normal mature transcript and the reduction was much greater when gypsy was in the parallel orientation.

Parallel insertion of a gypsy element causes accumulation of transcripts truncated in the 5' LTR

In addition to the spliced and full-length unspliced transcripts from the *hsp82* promoter, pCAT82SVSX-Gp gave an abundant 1.4-kb RNA to which the SV probe hybridized but the CAT probe did not (Fig. 2). In contrast, the only discrete species observed after transfection with pCAT82SVSX-Ga were the spliced and unspliced transcripts already noted. The amount of the 1.4-kb transcript of pCAT82SVSX-Gp was compared with the amount of the 1.8-kb spliced transcript from the same plasmid by normalizing the SV and CAT probe effi-

Figure 1. Maps of marked *hsp82* genes and the reconstructed *bx^{3de}* gypsy element. The CAT82SVSX gene is marked in the intron with sequences from Simian virus 40 (SV and SX) and in the second exon with a bacterial sequence coding for chloramphenicol acetyl transferase (CAT). The *SalI* site in the intron used to insert gypsy or fragments of gypsy is indicated. Dashes indicate flanking pUC13 sequences; continuous lines indicate endogenous *Drosophila* sequences flanking the *hsp82* gene on the 5' and 3' ends and the natural *hsp82* intron sequences; open rectangles indicate natural *hsp82* exon sequences; cross-hatched rectangles represent SV and SX markers; CAT sequences are represented by a stippled rectangle. The LAC82SU gene is marked with a simian virus 40 sequence in the intron (SU) and the *lacZ* (LAC) sequence in the second exon. The sequences are represented as for the CAT82SVSX gene with the exceptions that the stippled rectangle represents LAC sequences and the filled rectangle represents a *Drosophila* *sgs-8* gene sequence that contains a polyadenylation site. Gypsy is the reconstructed *bx^{3de}* gypsy element. The positions of some of the restriction sites referred to in the text are shown. Dashed lines indicate vector (pGEM-1) sequences; continuous lines represent internal non-long-terminal-repeat gypsy sequences; open rectangles represent LTR sequences. The LTR sequences present at both ends of gypsy genomic transcript (R regions) are indicated by filled rectangles. The polyadenylation sites are the 3' ends of the R region.

Figure 2. Gypsy transposon insertions in the *hsp82* intron alter transcript production. S2 cells were cotransfected with the control pLAC82SU (Fig. 1) (1 μ g/ml) and one of the CAT82SVSX (Fig. 1) construct plasmids: (lane 1) pCAT82SVSX (1 μ g/ml); (lane 2) pCAT82SVSX-Gp (CAT82SVSX with a parallel-oriented gypsy insertion, 2 μ g/ml); (lane 3) pCAT82SVSX-Ga (CAT82SVSX with an antiparallel gypsy insertion, 2 μ g/ml). The amounts of CAT82SVSX constructs used were equimolar and the total DNA concentration in each transfection was below saturation. Each lane contained 10 μ g of total cellular RNA from transfected cells heat shocked (20 min) 48 hr after transfection. Panels are autoradiograms of the same Northern blot hybridized to (A) CAT, (B) SV, and (C) LAC 32 P-labeled anti-sense RNA probes. Transcript sizes are indicated in kilobases. The autoradiographic exposures presented were insufficient to detect the 10.8-kb transcript of CAT82SVSX-Gp and only the spliced (3.9-kb) transcript of LAC82SU is shown. Heat shock increased the level of unspliced precursor RNA (3.4-kb) 5- to 10-fold and the level of truncated (1.4-kb) and spliced (1.8-kb) transcripts 2- to 3-fold above the levels observed in the absence of heat shock (not shown).

ciencies according to their relative intensities of hybridization to the unspliced 3.4-kb precursor transcribed from pCAT82SVSX. This comparison revealed that the amount of the 1.4-kb transcript was approximately 40 times greater than that of the 1.8-kb spliced transcript from the same plasmid and was approximately equal to the total amount of spliced and unspliced transcript from the plasmid without gypsy. Thus, there was no indication of any strong effect of the gypsy insertion on the activity of the *hsp82* promoter.

The 1.4-kb transcript of pCAT82SVSX-Gp was induced by heat shock and was retained by an oligo(dT) column, indicating that it initiated at the *hsp82* promoter and was polyadenylated. Its length suggested that polyadenylation was directed by the polyadenylation signal sequence AATAAA located at nucleotide 257 in the gypsy 5' long terminal repeat (LTR) (Freund and Meselson 1984; Arkhipova et al. 1986). To map the 3' end of

the 1.4-kb transcript more exactly, S1 nuclease protection experiments were performed, using as probe the 525 nucleotide *SalI*–*Sau96I* DNA fragment 3'-end-labeled at the *SalI* site 62 bp upstream of the gypsy 5' LTR. As shown in Figure 3, transcripts of pCAT82SVSX-Gp protected a single fragment of the probe approximately 350 nucleotides in length, as expected for transcripts polyadenylated 20–30 nucleotides downstream of the signal sequence in the 5' LTR. A similar S1 protection analysis was done with RNA from cells transfected with pCAT82SVSX-Ga, using as probe the 487 nucleotide *SalI*–*HpaI* DNA fragment 3' end-labeled at the same *SalI* site upstream of the gypsy insertion. In agreement with the results of the Northern analysis, S1 protection revealed no transcripts ending in the proximal LTR of anti-parallel gypsy (data not shown).

Truncation of transcripts at other sites in gypsy

Although the 1.4-kb RNA transcribed from pCAT82SVSX-Gp was the only discrete truncated transcript seen on Northern blots, we used RNA probe protection analysis to look for truncation that might not have been detected, possibly due to a low blotting efficiency of large RNA molecules or a broad distribution of RNA sizes. For this purpose we constructed plasmids with the SV marker at its usual position just upstream of the gypsy insertion site but with the SX marker at various posi-

Figure 3. The 3' ends of transcripts truncated in the gypsy 5' LTR map just downstream of the polyadenylation signal. Total cellular RNA from S2 cells transfected with either 1 μ g/ml pCAT82SVSX (lane 1: 20-min heat shock) or 2 μ g/ml pCAT82SVSX-Gp (lanes 2–5: 0-, 5-, 10-, 20-min heat shock, respectively) was analyzed by S1 nuclease protection. Each hybridization contained 20 μ g of RNA and an excess of a 525-bp *SalI*–*Sau96I* fragment of pCAT82SVSX-Gp, 3'-end-labeled at the *SalI* site. Renaturation of the probe is responsible for most of the 525-nucleotide protected probe fragment observed in the autoradiogram. The expected protected probe fragment (350 $\pm$ 5 bases) due to RNA with 3' ends at the gypsy LTR polyadenylation site is indicated. The transcript responsible for the protected probe fragment is indicated by a wavy line; CAT82SVSX and gypsy sequences are depicted as in Fig. 1.

tions within gypsy or downstream of the site where gypsy was inserted. The last 205 nucleotides of SV are identical to the first 205 nucleotides of SX, so that RNA transcribed through the SV marker protects a 205-nucleotide fragment of the SX probe. RNA transcribed through the SX marker protects the entire 290-nucleotide SX sequence of the probe. The molar ratio of the 290-nucleotide to the 205-nucleotide protected fragments therefore represents the proportion of transcripts extending through SV that do not have 3' termini between SV and SX. The results of such protection-scanning analyses of RNA extracted from heat-shocked transfected cells are shown in Figure 4. As may be seen in lanes 1 and 2, the amount of SV protected by RNA transcribed from constructs with anti-parallel gypsy was essentially indistinguishable from the amount protected by RNA transcribed from pCAT82SVSX, indicating that the gypsy insertion did not affect the strength of the *hsp82* promoter.

As expected, RNA from cells transfected with pCAT82SVSX, the plasmid lacking gypsy, protected equimolar amounts of SV and SX. Equimolar amounts of SV and SX were also protected when SX was placed in anti-parallel gypsy, at the *NdeI* site 1.7 kb downstream of the proximal end of the element. As SX was moved farther downstream in gypsy, however, the ratio of SX to SV protected decreased, reaching a value of approximately one-third with SX at the *NcoI* site 6.3 kb into the gypsy element. There was no further change in the ratio with SX at the *XhoI* site of the *hsp82* intron, 156 bp beyond the distal gypsy LTR. It therefore appears that approximately two-thirds of the observed transcripts are truncated between the two LTRs of the anti-parallel gypsy and that few, if any, are truncated within the LTRs themselves.

RNA from constructs containing parallel gypsy protected 3–4 times more SV than did RNA from pCAT82SVSX. Such a difference was expected because SV is included in the abundant 1.4-kb polyadenylated truncated transcript of pCAT82SVSX-Gp but is spliced out and degraded with the CAT82SVSX intron. Given that *hsp82* promoter activity is essentially unaffected by the gypsy insertion, this SV ratio is consistent with the observation that after a 20-min heat shock, unspliced precursor accounts for one-third to one-fourth of the transcripts from pCAT82SVSX (Fig. 2, lane 1A).

When SX was placed at the *NcoI* site of parallel gypsy, 1.1 kb downstream of the proximal end, the ratio of SX to SV protected was approximately 1 : 20. It remained at this value for SX insertions at 2.6, 4.2, and 5.6 kb (corresponding to *PvuII*, *AsuII*, and *NdeI* sites, respectively) into the gypsy element. The SX/SV ratio then dropped an additional factor of 3–4 when SX was placed at the *XhoI* site 156 bp beyond the 3' LTR. This *XhoI* site is the position of SX in pCAT82SVSX, the plasmid without a gypsy insertion. If splicing did not occur, the ratio of SX/SV protection would correspond to the proportion of transcripts that escape truncation in the SV–SX interval. Transcripts extending through the entire intron, however, are subject to splicing while the 1.4-kb truncated transcript is relatively stable. Therefore, the SX/SV

Figure 4. Transcripts of *hsp82* genes with parallel and antiparallel gypsy elements in the intron have a variety of 3' termini. S2 cells were cotransfected with pLAC82SU control DNA (1 μ g/ml) and one of several CAT82SVSX constructs: (lanes 1 and 7), pCAT82SVSX (no gypsy, 1 μ g/ml); (lanes 2–6), a series of CAT82SVSX-Ga constructs with an antiparallel gypsy (2 μ g/ml each) differing in the position of the SX marker; (lanes 8–12), a series of CAT82SVSX-Gp constructs with a parallel gypsy (2 μ g/ml each) differing in the location of SX marker. The positions of the SX marker are: (Lane 2), 1.7 kb (*NdeI*) into anti-parallel gypsy; (lane 3), 3.1 kb (*AsuII*); (lane 4), 4.7 kb (*PvuII*); (lane 5), 6.3 kb (*NcoI*); (lane 6), 156 bp downstream of the gypsy element; (lane 8), 1.1 kb (*NcoI*) into parallel gypsy; (lane 9), 2.6 kb (*PvuII*); (lane 10), 4.2 kb (*AsuII*); (lane 11), 5.6 kb (*NdeI*); and (lane 12), 156 bp downstream of gypsy. Total cellular RNA was isolated from cells heat shocked for 20 min 48 hrs after transfection and analyzed by RNA probe protection. Each hybridization contained 20 μ g of RNA and excess 32 P-labeled antisense SX RNA probe. RNA containing SV protects 205 nucleotides of this probe, SX protects 290 nucleotides, and SU protects 95 nucleotides. The amount of probe protected by SU was equal in all lanes (not shown). Positions of the SV and SX marker sequences in the constructs are shown schematically below the autoradiogram with the lane numbers for the anti-parallel and parallel gypsy constructs they represent. CAT82SVSX and gypsy sequences are depicted as in Fig. 1.

ratio exaggerates the truncation frequency. The protection ratios nevertheless show that transcripts of pCAT82SVSX-Gp are truncated both in the 5' LTR and in or near the 3' LTR.

If truncation in or near the 3' LTR of parallel gypsy requires the polyadenylation signal, then removal of the region containing the signal should allow greater accumulation of the full-length unspliced precursor RNA and of the mature spliced transcript. To test this hypothesis, the construct pCAT82SVSX-XGp was made, containing the 7-kbp internal *XhoI*–*XhoI* fragment of gypsy rather than the full-length element. Because the *XhoI* sites lie 59 bp upstream of the polyadenylation

signal sequences in each LTR, the remaining 283 bp of the 5' LTR in pCAT82SVSX-XGp include the polyadenylation signal, but the remaining 199 bp of the 3' LTR lack it. As shown in Figure 5, this construct gave a 5'

Figure 5. Insertion of a parallel gypsy element lacking the 3' LTR polyadenylation site allows greater accumulation of precursor and spliced RNA than does insertion of a full-length gypsy. S2 cells were cotransfected with the pLAC82SU control (1 μ g/ml) and a CAT82SVSX construct: (lanes 1 and 4), pCAT82SVSX (no gypsy, 1 μ g/ml); (lanes 2 and 5), pCAT82SVSX-Gp (parallel full-length gypsy, 2 μ g/ml); (lanes 3 and 6), pCAT82SVSX-XGp (parallel insertion of the 7-kbp *XhoI*–*XhoI* fragment of gypsy, 2 μ g/ml). Each lane contained 10 μ g of total cellular RNA. RNA in lanes 1–3 was from nonshocked cells and RNA in lanes 4–6 was from cells heat shocked for 20 min 48 hr after transfection. The panels are autoradiograms of the same Northern blot hybridized to (A) CAT, (B) SV, and (C) LAC 32 P-labeled antisense RNA probes. Lengths of the major transcripts are indicated in kilobases. The faint band just above the 1.8-kb spliced CAT82SVSX RNA in all lanes in panel A is an artifact due to ribosomal RNA. The faint band migrating at 8.5 kb in the CAT82SVSX-Gp lanes of panel B is not induced by heat shock and its origin is unknown. The minor band migrating at 5 kb in the CAT82SVSX-XGp lanes of panels A and B is induced upon heat shock and may be an alternatively spliced transcript. CAT82SVSX-Gp and CAT82SVSX-XGp are compared schematically and the positions of the LTR polyadenylation sites [poly(A)] are indicated. X indicates the lack of the 3' LTR polyadenylation site in CAT82SVSX-XGp. CAT82SVSX and gypsy sequences are depicted as in Fig. 1.

LTR-truncated transcript that was, as expected, approximately 200 nucleotides shorter than the 1.4-kb truncated transcript of pCAT82SVSX-Gp. While these two truncated transcripts accumulated to the same level, pCAT82SVSX-XGp gave more of the unspliced precursor and 3–4 times more of the spliced transcript than did pCAT82SVSX-Gp. These results are consistent with a role of the 3' LTR polyadenylation signal in the truncation of transcripts in or near the 3' LTR, as suggested by the SV–SX RNA probe protection experiments.

Parallel insertion of a segment of the LTR followed by insertion of a nearby gypsy region in either orientation gives a high level of truncated transcript

To locate sequences required for truncation in the 5' LTR of parallel gypsy, transfections were done with plasmids containing fragments of gypsy extending from nucleotide 199 to various positions downstream. Each fragment was inserted in the parallel orientation at the *SalI* site of the SV marker in pCAT82SVSX. All fragments contained the 5' LTR polyadenylation signal at gypsy nucleotide 257, now located 58 bp downstream of the *SalI* insertion site. Cultured cells were transfected with the resulting plasmids and with the pLAC82SU control. After 48 hr, whole-cell RNA from nonshocked cells was analyzed on a Northern blot sequentially hybridized to SV and LAC probes. Figure 6 shows the results for the fragments *XhoI*–*BstXI* (199–967), *XhoI*–*HincII* (199–835), *XhoI*–*BalI* (199–641), *XhoI*–*NarI* (199–484), and *XhoI*–*BglII* (199–425). Each fragment gave rise to a 1.2-kb transcript, the size expected for truncation directed by the polyadenylation signal. The two longer fragments gave 5–10 times as much of the truncated transcript as did the three shorter ones. Indeed, insertion of either of the longer fragments gave approximately as much truncated transcript as did insertion of the entire gypsy element, as found by normalization to transcripts of pLAC82SU (not shown).

We conclude that the amount of truncated transcript is increased by sequences between 641 and 835. This is supported by the observation that, as shown in Figure 7, insertion of the *BalI*–*BstXI* internal fragment (641–967, abbreviated BaBx) immediately downstream of the parallel *XhoI*–*BglII* fragment (199–425, abbreviated XB) gave 5–10 times more truncated transcript than did parallel insertion of XB alone. It is noteworthy that BaBx was effective in increasing the amount of the 1.2-kb transcript when inserted in either orientation following XB.

The parallel insertion of BaBx by itself had no evident effect on transcripts of the heat shock gene. In contrast, anti-parallel insertion of BaBx gave rise to a prominent 1.3-kb transcript that hybridized to SV, as seen in Figure 7. The fact that this truncated transcript is approximately 0.1 kb larger than that produced by the insertion of XB suggests that its 3' terminus is within the BaBx fragment itself. It is noteworthy that anti-parallel BaBx has a polyadenylation signal AATAAA 187 nucleotides from the *SalI* insertion site, 129 nucleotides farther downstream than the polyadenylation signal of XB.

Figure 6. Internal gypsy sequences near the 5' LTR are necessary for high levels of truncated transcript. S2 cells were co-transfected with pLAC82SU (1 μ g/ml) and one of a series of plasmid DNAs (1 μ g/ml each) that contain parallel-oriented fragments of gypsy in the intron of CAT82SVSX: (lane 1), gypsy nucleotides 199–967; (lane 2), 199–835; (lane 3), 199–641; (lane 4), 199–484; (lane 5), 199–425. Each lane contained 10 μ g of RNA isolated from non-shocked cells 48 hr after transfection. Panels are autoradiograms of the same Northern blot hybridized to (A) SV and (B) LAC 32 P-labeled antisense RNA probes. Sizes of some of the transcripts are indicated in kilobases. The unspliced precursors vary in size with the lengths of the gypsy DNA insertions as expected. The gypsy sequences in the different insertions are indicated by lines under a diagram of the gypsy element, and the site of insertion in CAT82SVSX is indicated with a vertical line. The appropriate lane numbers for the different insertions are indicated. CAT82SVSX and gypsy sequences are depicted as in Fig. 1.

Transcripts in transformed *Drosophila*

To examine the effects of modifier gene mutations on transcripts of the *hsp82* gene containing gypsy insertions, constructs similar to those used in transfection experiments were introduced into the *Drosophila* germ line by P-element-mediated transformation. The marked *hsp82* gene from the plasmid pCAT82SVSX was inserted into the Carnegie-20 P-element transformation vector (Rubin and Spradling 1983), giving the plasmid pC20CAT82SVSX. The plasmids pC20CAT82SVSX-Ga and pC20CAT82SVSX-Gp were obtained by inserting gypsy in the anti-parallel and parallel orientations, respectively, into the *Sa*I site at the 3' end of the SV marker segment of pC20CAT82SVSX. The gypsy element used for transformation differed slightly from the element used for transfection in that it lacked the first 4 bp of the 5' LTR and the short duplication of internal gypsy DNA just upstream of the LTR.

Embryos of the *rosy* stock *ry*⁵⁰⁶ were injected with plasmids containing parallel or anti-parallel gypsy or lacking gypsy. Seven transformed lines made homo-

zygous for insertions of the wild-type *rosy* allele were chosen for RNA analysis. Lines C9 and C11 had insertions of pC20CAT82SVSX, lines A19 and A20 had insertions of pC20CAT82SVSX-Ga, and lines P19, P36, and P57 had insertions of pC20CAT82SVSX-Gp. All of the insertions were in chromosome 2, except for those in

Figure 7. The internal gypsy sequences required for high levels of 5' LTR truncated transcript are contained within a 326-bp fragment and function in either orientation. S2 cells were cotransfected with pLAC82SU (1 μ g/ml) and a series of CAT82SVSX constructs (1 μ g/ml each): (lanes 1 and 7), pCAT82SVSX; (lanes 2 and 8), pCAT82SVSX-XBp (XB is the 226-bp *Xho*I–*Bgl*II fragment of the LTR and contains the polyadenylation signal); (lanes 3 and 9), pCAT82SVSX-BaBxp (parallel insertion of the 326-bp BaBx internal fragment of gypsy); (lanes 4 and 10), pCAT82SVSX-BaBxa (anti-parallel BaBx); (lanes 5 and 11), pCAT82SVSX-XBp-BaBxp (BaBx inserted immediately 3' to XB); (lanes 6 and 12) pCAT82SVSX-XBp-BaBxa. Each lane contained 10 μ g of total cellular RNA isolated 48 hr post-transfection: (lanes 1–6), RNA from non-shocked cells; (lanes 7–12), RNA from cells heat shocked for 20 min. The panels are autoradiograms of the same Northern blot hybridized to (A) CAT (B), SV and (C) LAC 32 P-labeled antisense RNA probes. The sizes of the major transcripts are indicated in kilobases. The lengths of the unspliced transcripts correlate as expected with the sizes of the insertions in the intron. The gypsy sequences present in each construct are indicated by lines under a map of the gypsy element, and the site of their insertion in CAT82SVSX is indicated with a vertical line. The BaBx fragment is represented as an arrow to show its orientation. Lane numbers corresponding to the constructs are also shown. CAT82SVSX and gypsy sequences are depicted as in Fig. 1.

P36 and P57, which were in chromosome 3, as determined by segregation analysis. The uniqueness and integrity of the insertion in each line was verified by Southern blot analysis of genomic DNA (not shown).

RNA from heat shocked and nonshocked mid to late pupae of each of the seven transformed lines was analyzed by Northern blotting. Pupal RNA was selected for analysis because several modifiable gypsy-insertion alleles are expressed at this stage. Each blot was probed with SV and CAT antisense RNA and also with antisense RNA from a region of the endogenous *hsp82* exon 2, providing a control for normalization of the amounts of transcripts of the marked *hsp82* gene in different transformed lines. All lines transformed by a given plasmid gave transcripts of the marked *hsp82* gene that were qualitatively and, except for P57, quantitatively indistinguishable. Line P57 gave about twice as much of each transcript, relative to the endogenous control, as did lines P19 and P36. Representative Northern blot analyses of transcripts from transformants with parallel, anti-parallel, and no gypsy insertions are shown in Figures 8 and 9.

The principal transcripts of the marked *hsp82* gene in the various transformed lines were the same as the transcripts produced by the corresponding plasmids in transfected cells. In particular, both lines transformed with the plasmid lacking gypsy had the 3.4-kb unspliced transcript and the 1.8-kb spliced product. All five transformants with gypsy insertions had the 1.8-kb spliced product and the 10.8-kb precursor. All three transformants with gypsy in the parallel orientation had large amounts of the 1.4-kb truncated transcript.

In addition, the transformed lines with gypsy insertions gave minor transcripts, possibly due to alternative splicing patterns. Transformants with the parallel gypsy insertion gave a minor 3.8-kb species that hybridized to the CAT probe but not to SV. Transformants with the anti-parallel gypsy insertion gave two minor transcripts, one of 3.8 kb that hybridized to CAT but not to SV, and another of 3.0 kb that hybridized to both probes. All transcripts of the transformed *hsp82* gene, both major and minor, were induced by heat shock.

Parallel insertion of gypsy was associated with a clear reduction in the amount of spliced transcript. Two of the three transformants with parallel insertions had only about one-fifth as much of the 1.8-kb spliced transcript as did the two transformants without gypsy and the third transformant, P57, had about one-third as much. In all three lines with parallel gypsy insertions, the ratio of 1.4-kb truncated transcript to 1.8-kb spliced transcript was approximately 10. The transformants with insertions of anti-parallel gypsy elements appeared to have somewhat less spliced transcript than transformants without gypsy on some blots but not on others. This effect was not sufficiently consistent to be judged significant.

Effects of modifier gene mutations

Lines P19, A19, and C11, containing marked *hsp82* genes with parallel, anti-parallel, and no gypsy inser-

Figure 8. Mutations in the *su(w^a)* and *su(f)* genes modify transcript production from an *hsp82* gene with a parallel-oriented gypsy element in the intron. Total cellular RNA was isolated from heat shocked mid to late pupae of transformed fly lines with different suppressor genotypes: (lane 1) *y² w^a ct⁶ f¹*; C11; (lane 2) *y² su(w^a) w^a ct⁶ f¹*; C11; (lane 3) *y² w^a ct⁶ f¹ su(f)¹*; C11; (lane 4) *y² w^a ct⁶ f¹*; P19; TM2/MKRS; (lane 5) *y² su(w^a) w^a ct⁶ f¹*; P19; (lane 6) *y² w^a ct⁶ f¹ su(f)¹*; P19; (lane 7) *y² w^a ct⁶ f¹ su(f)^{br}*; P19; (lane 8) *y² w^a ct⁶ f¹*; A19; (lane 9) *y² su(w^a) w^a ct⁶ f¹*; A19; (lane 10) *y² w^a ct⁶ f¹ su(f)¹*; A19. The panels are autoradiograms of the same Northern blot (2.5 μ g RNA/lane) hybridized to (A) SV (5-day exposure), (B) CAT, (C) SV (6-hr exposure, the density of the 3.4-kb transcript is apparently in the nonlinear range of the film's response curve), and (D) endogenous *hsp82* ³²P-labeled antisense RNA probes. Lengths of the major transcripts are indicated in kilobases. The differences in levels of the 10.8-kb transcript between lanes 8, 9, and 10 observed in this Northern blot are not reproducible. Additional analysis of these lines, an A19 line with *su(wa)²⁰*, and A20 lines with *su(f)¹* and both *su(w^a)* alleles indicate that these modifier mutations do not alter the levels of the observed A19 and A20 transcripts. The major transcripts observed in the different types of transformant lines are shown diagrammatically as wavy lines; dashed lines indicate excised intron sequences. CAT82SVSX and gypsy sequences are depicted as in Fig. 1.

tions, respectively, were tested for effects of the suppressor of forked alleles *su(f)¹* and *su(f)^{br}*. These lines and line A20 were tested for effects of the suppressor of white-apricot alleles *su(wa)¹* and *su(wa)²⁰* [Rutledge et

Figure 9. Mutations in the *su(Hw)* gene modify transcript production from *hsp82* genes with gypsy elements in the intron. Total cellular RNA was isolated from heat-shocked (lanes 1–6) and nonshocked (lanes 7–12) mid to late pupae of transformed fly lines with different *su(Hw)* genotypes: (lanes 1 and 7), $y^2 w^a ct^6 f^1$; C11; (lanes 2 and 8), $y^2 w^a ct^6 f^1$; C11; *su(Hw)²/su(Hw)^f*; (lanes 3 and 9), $y^2 w^a ct^6 f^1$; P19; TM2/MKRS; (lanes 4 and 10), $y^2 w^a ct^6 f^1$; P19; *su(Hw)²/su(Hw)^f*; (lanes 5 and 11), $y^2 w^a ct^6 f^1$; A20; (lanes 6 and 12), $y^2 w^a ct^6 f^1$; A20; *su(Hw)²/su(Hw)^f*. Each lane contained 2.5 μ g of total cellular RNA. The panels are autoradiograms of the same Northern blot hybridized to (A) SV (6-day exposure), (B) CAT, (C) SV (12-hr exposure), and (D) endogenous *hsp82* ³²P-labeled antisense RNA probes. Lengths of the major transcripts are indicated in kilobases. Maps of the major transcripts are presented in Fig. 8.

al. 1988). Lines P19 and C11 and the anti-parallel insertion lines A19 and A20 were tested for effects of the modifier genotype *su(Hw)²/su(Hw)^f*. All of the modifier stocks containing the marked *hsp82* gene carried the modifiable alleles y^2 , w^a , ct^6 , and f^1 , which provided confirmation of the modifier genotype in each case. Northern blots of RNA from heat-shocked and nonshocked pupae of a representative set of these various stocks are shown in Figures 8 and 9.

None of the mutations in *su(w^a)* or *su(f)* had any reproducible effect on transcripts of the marked *hsp82* gene without gypsy insertions. Neither was there any consistent effect of mutations in either modifier on transcripts of the gene containing anti-parallel gypsy. In contrast, the amounts of spliced and unspliced transcripts of the marked *hsp82* gene containing the parallel gypsy insertion were clearly and consistently affected by each of the mutations in *su(w^a)* and *su(f)*. Both of these transcripts were decreased by a factor of approximately three by the mutations in *su(w^a)* and were increased by approximately the same factor, giving nearly wild-type levels, by the mutations in *su(f)* (Fig. 8 and data not shown). The amount of the 1.4-kb truncated transcript was not noticeably affected by either of the modifiers. A small change in the frequency of truncation, however, could conceivably cause a large change in the amounts of the 1.8- and 10.8-kb transcripts, without noticeably altering the level of truncated transcript.

As shown in Figure 9, the amounts of spliced and unspliced transcripts of the marked *hsp82* gene containing gypsy in the anti-parallel orientation are approximately doubled by *su(Hw)²/su(Hw)^f*, an effect in the direction expected from the known suppression of gypsy-insertion alleles by *su(Hw)*. Some of the effects of *su(Hw)* on transcripts of the gene with the parallel gypsy insertion, however, were not expected. Not only was the truncated transcript reduced (3- to 10-fold) but also the spliced and unspliced readthrough transcripts were decreased (1- to 2-fold). The P19 stock with *su(Hw)²/su(Hw)^f* is poorly viable and may therefore tend to accumulate rearrangements. A number of controls were done to exclude the possibility that the observed reductions of readthrough transcripts resulted from loss or rearrangement of the transformed gene. The stock used for RNA analysis was found to be homozygous for the transformed gene, as determined by quantitative Southern analysis of its DNA. Moreover, the same decrease was found in RNA from the progeny of an independently constructed stock of the transformed second chromosome of P19 with *su(Hw)²/su(Hw)^f*.

Discussion

A principal objective of the experiments reported here was to determine the effects on transcript production of inserting the gypsy retrotransposon and specific fragments of it into the transcribed region of a gene. A further objective was to characterize the effects of certain allele-specific modifier genes. The experimental system and the methods of RNA analysis were chosen to avoid ambiguous or misleading results: gypsy was inserted into the intron of *hsp82*, a simple heat-inducible gene of known structure and well-characterized transcripts; RNA was extracted immediately after a short heat shock, to increase the likelihood of detecting unstable RNA species transcribed from the heat shock promoter; and RNA probe protection analysis was used to detect transcripts that might escape detection on Northern blots.

It was found that with the gypsy element in the parallel orientation in the *hsp82* intron, nearly all transcripts were polyadenylated 20–30 nucleotides downstream of the polyadenylation signal in the 5' LTR of gypsy. Some of the transcripts not truncated in the 5' LTR were truncated in or near the 3' LTR.

Parallel insertion of a segment of the gypsy LTR (XB, nucleotides 199–425 containing the polyadenylation signal), followed in either orientation by a specific segment of internal gypsy DNA (BaBx, nucleotides 641–967 containing eight direct repeats of the sequence PyPuPyTGCATAPyPyPy) into the *hsp82* intron was sufficient to give a high level of transcript polyadenylated in the LTR segment. Transcript polyadenylated in the XB segment was 5- to 10-fold less in the absence of a downstream BaBx segment. The effect of the BaBx segment did not appear to result from stimulation of the *hsp82* promoter, since readthrough transcripts were decreased. Also, by itself BaBx had no effect on the amount of spliced or unspliced transcript when inserted in the

parallel orientation. When inserted in the anti-parallel orientation, however, the BaBx fragment caused accumulation of a transcript truncated within the fragment itself, which in this orientation contains a polyadenylation signal (AATAAA) just upstream of three copies of the repeat. It therefore appears that a downstream BaBx segment, in either orientation, potentiates the polyadenylation signal.

Most of our observations do not distinguish whether the 1.4-kb transcript truncated in the 5' LTR of parallel gypsy arises by transcription termination or by rapid processing of a longer molecule. However, because the BaBx segment was effective in either orientation even though it does not contain any palindromes longer than 4 bp, we think it likely that it acts at the DNA level to promote transcription termination rather than at the RNA level to promote processing. If this is so, the BaBx segment may be analogous to the termination region downstream of the mouse β^{maj} -globin gene, which also works in either orientation and requires an upstream polyadenylation signal (Logan et al. 1987).

The effects of parallel retrotransposon insertion within the transcribed region of a gene on transcript production have been investigated in a number of cases involving spontaneous insertion mutations in *Drosophila*. The principal polyadenylated transcripts of the *white-apricot* gene, in which the copia element is inserted in the second intron, are truncated within copia at a site thought to be in the 3' LTR (Levis et al. 1984; Zachar et al. 1985; Mount et al. 1988). In experiments similar to those conducted with *hsp82* gypsy constructs, we examined transcripts produced in cells transfected with an *hsp82* gene containing the w^a copia element inserted in the intron in the parallel orientation. Among the principal transcripts observed were species polyadenylated in the 5' LTR and in or near the 3' LTR (D. Dorsett, D. Lin, and M. Meselson, unpubl.).

Genes with naturally occurring gypsy insertions are compared with the *hsp82* genes in Table 1. Accumulation of a truncated transcript polyadenylated within a parallel gypsy element has been noted in the gypsy-insertion mutant *Hw¹*, which results from a parallel gypsy insertion in a nonintronic transcribed portion of the *achaete-scute* complex (Campuzano et al. 1986). In this case, the amount of truncated transcript was 5–20 times greater than the amount of a transcript found in wild-type flies that it replaced, possibly due to an increase of promoter activity in the insertion mutant or to increased processing efficiency or stability of the truncated product. The relatively elevated abundance of the truncated transcript was concluded to be responsible for the dominant phenotype of this gypsy-insertion allele, which contrasts with the recessive phenotype of nearly all other such alleles (Campuzano et al. 1986).

Parkhurst and Corces (1985 and pers. comm.) examined polyadenylated transcripts from *f¹*, a parallel gypsy-insertion mutant of the *forked* locus (Table 1). Three different transcripts from the region of the insertion were reduced in amount but no novel species that might indicate truncation were observed. The effect of the

gypsy insertion was therefore attributed to promoter interference. In the gypsy-insertion mutant *f^s*, probably identical to *f¹*, McLachlan (1986) observed a reduction in the amount of a single transcript from this region, again with no evidence for a truncated species. Interpretation of these results is complicated because the pattern of transcription and processing at the *forked* locus is not well defined. It may be, for example, that the gypsy element of *f¹* gives rise to a truncated transcript that is either too small or too large to have been detected by the methods used. In any case, the available evidence from studies of *f¹* and *f^s* stands in contrast to the evidence from w^a , *Hw¹*, and our own studies of gypsy and copia insertions in the *hsp82* intron that show that parallel retrotransposon insertion within a transcribed region gives transcripts polyadenylated in the LTRs.

The strongest evidence in favor of some sort of promoter effect as a mechanism of mutation by gypsy insertion is the position of the gypsy element in the y^2 mutation (Biessmann 1985; Parkhurst and Corces 1986; Table 1). This allele is suppressible by *su(Hw)* and contains an anti-parallel gypsy element 5' to the transcription start site for the major transcript. Barring the possibility that the gypsy element is situated in an intron of another, undetected transcript in this region, this observation rules out transcript termination as the mechanism by which the gypsy element alters the expression of y^2 , unless it is assumed that termination can be mediated by interactions involving sites outside of the transcribed region.

In contrast to the large effect of parallel gypsy insertion, the insertion of gypsy in the anti-parallel orientation at the same site in the *hsp82* intron was associated with only a 2- to 3 fold reduction in the amount of spliced transcript in transfected cells and with no definite reduction of spliced transcript in transformed pupae. An effect of orientation of the gypsy insertion is consistent with the finding of Peifer and Bender (1986) that parallel insertions of gypsy at the *bithorax* locus are phenotypically more severe than anti-parallel insertions in the same region of the gene.

Even in transfected cells, where the anti-parallel gypsy insertion did cause a clear reduction in the amount of spliced transcript, discrete truncated transcripts were not observed. Nevertheless, RNA probe protection scanning revealed the presence of *hsp82* transcripts with 3' ends within the internal region of the gypsy element, sufficient to account for the limited reduction in the amount of spliced transcript that was observed. Indeed, anti-parallel gypsy should bring about some level of truncation at a number of sites, in view of the ability of the anti-parallel BaBx region to bring about truncation within itself and to potentiate an upstream polyadenylation signal. The failure to observe discrete truncated transcripts on Northern gels or to detect 3' ends in the region of the distal LTR of anti-parallel gypsy by RNA protection scanning may therefore have resulted from a number of factors, including additional processing or instability of long truncated RNAs and a relatively low amount of any single truncated molecule.

Table 1. Effects of gypsy insertions and modifier mutations on molecularly characterized genes

Allele	Gypsy		Transcripts		<i>su(Hw)</i>		<i>su(f)</i>		<i>su(w^a)</i>	
	location	orientation	normal	truncated	phenotype	transcripts	phenotype	transcripts	phenotype	transcripts
<i>y²</i>	5' nontranscribed region	anti-parallel	reduced	none	suppressed	normal	no	?	no	?
<i>Hw¹</i>	nonintrinsic transcribed region	parallel	reduced	high levels	suppressed	truncated	no	?	no	?
<i>f¹</i>	transcribed region	parallel	reduced	none	suppressed	normal	suppressed	normal	enhanced	?
<i>bx^{34e}</i>	intron	anti-parallel	?	observed	suppressed	increased	suppressed	increased	enhanced	?
CAT82SVSX-Gp	intron	parallel	reduced	high levels	NA	all	NA	normal	NA	normal
CAT82SVSX-Ga	intron	anti-parallel	slightly reduced	low levels	NA	reduced	NA	increased	NA	decreased
						normal		no		no
						increased		change		change

Information is summarized from the following sources: effects of *su(Hw)*, *su(f)*, and *su(w^a)* mutations on phenotypes, Rutledge et al. (1988); gypsy insertion and transcripts of *y²*, Biesmann (1985) and Parkhurst and Corces (1986); gypsy insertion and transcripts of *Hw¹*, Campuzano et al. (1986); gypsy insertion and transcripts of *f¹*, Parkhurst and Corce (1985 and pers. comm.); gypsy insertion in *bx^{34e}*, Peifer and Bender (1986). Information on modified *hsp82* genes (CAT82SVSX) containing gypsy insertions is from this study. The first column gives the names of the genes with gypsy insertions, the second indicates where the gypsy insertion is located, the third indicates the orientation of the inserted element relative to the gene (parallel indicates that the sense strand of gypsy is in the sense strand of the gene), the fourth indicates the effect of the gypsy insertion on the levels of the normal gene's major transcripts, and the fifth indicates whether truncated transcripts are observed. The sixth column indicates the observed effects of *su(Hw)* mutations on the levels of the normal and/or truncated transcripts of the allele. Columns eight, nine, ten, and eleven provide similar information on the effects of *su(f)* and *su(w^a)* mutations. (?) Information not available; (NA) information not applicable.

We found that mutations in *su(f)* and *su(w^a)* increase and decrease, respectively, the amounts of readthrough transcripts in pupae transformed with the *hsp82* gene containing a parallel gypsy insertion. These effects are consistent with the known suppression and enhancement of certain alleles, such as *f¹*, with gypsy insertions in transcribed regions (Rutledge et al. 1988; Table 1). Because neither of these modifier mutations coordinately altered the level of the 5' LTR truncated transcript, we conclude that they do not affect initiation of transcription. A simple explanation is that *su(f)* and *su(w^a)* mutations alter the amount of readthrough transcripts by modulating the frequency of truncation. Such alteration of the truncation frequency at the 5' LTR of parallel gypsy could easily have gone undetected since a small change in this frequency can give a large change in the amount of readthrough transcripts. Although our experiments with transformed pupae, unlike those with transfected cells, did not measure 3' LTR truncation, modulation of such truncation might also account for the effects of *su(f)* and *su(w^a)* mutations.

The allele *Hw¹* is not modified by *su(f)* and *su(w^a)* (Rutledge et al. 1988; Table 1) even though its phenotype is associated with truncation within a parallel gypsy element (Campuzano et al. 1986). This may be explained, however, by the fact that the dominant phenotype of this allele results not from the underproduction of full-length transcript but from the high-level production of truncated transcript. This is consistent with our observation that these two modifiers affected the amounts of readthrough transcripts without noticeably altering the amount of the 5' LTR truncated product.

We did not detect any effects of *su(w^a)* or *su(f)* mutations on transcripts of the *hsp82* gene containing anti-parallel gypsy. If, however, these modifiers act by altering truncation frequencies we might not expect to see effects, because the anti-parallel gypsy insertion did not clearly alter the amount of spliced transcript in pupae, indicating that in this case the frequency of truncation is low. Indeed, both of these modifiers act on an allele, *b^{x34e}*, with an anti-parallel gypsy insertion (Peifer and Bender 1986; Rutledge et al. 1988; Table 1).

Mutation of *su(Hw)* clearly reduced the levels of the 5' LTR truncated and readthrough transcripts of the *hsp82* gene containing a parallel gypsy insertion and increased the levels of the spliced and unspliced transcripts when gypsy was in the anti-parallel orientation. These observations might be taken to mean that *su(Hw)* is affecting the activity of the *hsp82* promoter. If so, we would expect the changes in transcript levels to be coordinate, when, in fact, this was not observed for the gene with a parallel gypsy insertion. Instead, the reduction of 5' LTR truncated transcript was consistently 3- to 5-fold greater than the reduction of the readthrough transcripts. Moreover, Spana et al. (1988) have recently reported that *su(Hw)* protein binds a restriction fragment that overlaps the BaBx fragment. Since we find that this region potentiates the 5' LTR polyadenylation signal and that in the absence of an upstream polyadenylation signal BaBx has no effect, we think it likely that *su(Hw)* affects truncation. This would be consistent with the

observed increase in precursor and spliced transcripts from the *hsp82* gene with anti-parallel gypsy seen in *su(Hw)* pupae. If, however, the effect of *su(Hw)* on transcripts of the gene with parallel gypsy were due only to modulation of truncation in the 5' LTR, we would expect readthrough transcripts to increase. We have no explanation of why these transcripts decreased.

Retrotransposon insertion into the intron of a cellular gene may be imagined to affect a number of steps in the production of mature message, including initiation, termination, cleavage, and splicing. The only effect for which we found clear evidence, however, was truncation of transcripts. The amounts of truncated products were sufficient to account for the reductions observed in normal spliced transcript. Truncation has also been observed in *Drosophila* genes with naturally occurring transposon insertions in transcribed regions, and in alleles of the *Shrunken* gene of maize that have a *Ds* transposon insertion in an intron (Fedoroff et al. 1983). Furthermore, transcription termination appears to be the mechanism by which *Mu1* transposon insertions in the first intron of the maize *Adh1* gene interfere with normal transcript production (Vayda and Freeling 1986). The regions of the gypsy element we found to be required for high levels of truncated transcript include an LTR fragment containing the polyadenylation signal, and an internal fragment that we think is likely to potentiate the polyadenylation signal by mediating termination. Mutations in the modifier genes *su(f)*, *su(w^a)*, and *su(Hw)* all appeared to have effects on truncation of transcripts by gypsy, although in the case of *su(Hw)*, there appear to have been other effects as well.

Methods

Plasmid constructions

Plasmid DNAs were prepared for transfection and stored in TE [10 mM Tris·HCl (pH 7.4), 1 mM EDTA] as described elsewhere (Dorsett et al. 1985). Unless otherwise indicated, restriction fragments with 5'-protruding ends were blunted with Klenow polymerase.

The *hsp82* gene of *D. melanogaster* from nucleotide -876 to nucleotide 3963 [nucleotide numbers are relative to the start of transcription (Blackman and Meselson 1986)] was cloned in the *Bam*HI and *Eco*RI sites of the polylinker of pUC13 by ligating the *Nru*I site of *hsp82* (nucleotide 3963) to the blunted *Eco*RI site. The CAT82SVSX gene (Fig. 1) was constructed from this clone [91.876]. The *Bam*HI and *Sal*II sites in the polylinker were removed by blunting and the *Bcl*II-*Bcl*II internal fragment of exon 2 (nucleotides 1399-3307) was replaced with a 795-bp fragment of bacterial DNA coding for chloramphenicol acetyl transferase (CAT). The CAT fragment was a *Bgl*III-*Mbo*I fragment of pSV0CAT, a promoterless derivative of pSV2-cat (Gorman et al. 1982) in which the *Hind*III site proximal to the CAT AUG had been converted to a *Bgl*III site. The ends were blunted so that the CAT coding sequence was in frame with the *hsp82* coding sequence. This plasmid, pCAT82, contained *Clal*I sites at the ends of the CAT segment. These were used to clone the CAT fragment into the *Acc*I site of pGEM-1 (Promega Biotec) to provide a template (pGCAT) for the synthesis of antisense CAT RNA probe. A 252-bp *Sma*I-*Hinc*II fragment (SV) containing Simian virus 40 (SV40) DNA was inserted into the

StuI site in the intron of pCAT82 (*hsp82* nucleotide 428); the plasmid in which the direction of transcription of the SV40 sequence in the construct was the same as in SV40 was named pCAT82SV. The SV fragment was from the pGSV plasmid which was constructed by cloning the *HindIII* F fragment (nucleotides 1493–1708) of SV40 in the *XbaI* site of pGEM-1 after partial fill in of the *XbaI* and *HindIII* ends. The pGSV plasmid was also used as a template to make antisense SV RNA. *SalI* linker (dGGTCGACC) was inserted in the blunted *BamHI* site in the SV segment of pCAT82SV to generate pCAT82SVS. The pCAT82SVSX plasmid was created from pCAT82SVS by insertion of a 300 bp *SmaI*–*HincII* fragment of DNA (SX) containing an SV40 sequence [nucleotides 1507–1782] into the blunted *XhoI* site (nucleotide 585) in the intron of the *hsp82* gene. A clone in which the SV40 sequence in SX is in the same orientation as the sequence in SV was chosen as pCAT82SVSX. The SX fragment was from the pGSX plasmid that had been created by cloning a *HaeIII* fragment of SV40 (nucleotides 1507–2259) in the blunted *XbaI* site of a pGEM-1 vector with a deleted *EcoRI* site, followed by deletion of the *EcoRI*–*BamHI* segment. The pGSX plasmid was also used as a template for synthesis of anti-sense SX probe.

The pLAC82SU plasmid was constructed from 91.876 by substitution of the sequence from the *BclI* site in the second exon (nucleotide 1399) to the *EcoRI* site (nucleotide 3963) downstream of the polyadenylation sites with a 3.75-kbp *BamHI*–*SnaBI* fragment from the pLOXba plasmid (obtained from V. Corbin, Dept. of Biochemistry and Molecular Biology, Harvard University; pLOXba is a derivative of pOX3 constructed by E. Meyerowitz, California Institute of Technology). This fragment contains the *lacZ* coding sequence (LAC) followed by a polyadenylation site from the *sgs-8* gene of *D. melanogaster*. In the construction, the *BamHI* and *BclI* sites were blunted and ligated, the *SnaBI* site was converted to a *BamHI* site with a linker (dCGGATCCG) and the complete *hsp82* gene from the *BamHI* site at –876 to the converted *SnaBI* site was recombined in the *BamHI* site of pUC13. The *HindIII*–*XhoI* fragment of this intermediate (which contains the *hsp82* sequence from –876 to 585) was replaced with the equivalent fragment from pCAT82SV, and then the SV sequence between the *AccI* and *BamHI* sites was deleted by restricting, blunting, and religating. Antisense LAC RNA probe was prepared using a template plasmid kindly provided by R.S. Cohen (Dept. of Biochemistry and Molecular Biophysics, Columbia Presbyterian Medical Center).

Plasmids used for transformation of the *Drosophila* germ line were constructed using a Carnegie-20 vector (Rubin and Spradling 1983) in which the *SalI* site had been deleted with mung bean nuclease. The *PstI*–*NdeI* fragment of pCAT82SVSX containing the *hsp82* gene was isolated, blunted with T4 DNA polymerase, and cloned in the *HpaI* site of the Carnegie-20 vector. This plasmid was named pC20CAT82SVSX.

The gypsy element cloned from the *bx^{34e}* locus (Modolell et al. 1983) was reconstructed to remove the flanking *bithorax* sequences. A 1-kb *SalI*–*NarI* fragment containing the 5' LTR of gypsy was blunted and cloned in the blunted *XbaI* site of pGEM-1. A 1.25-kb *ClaI*–*SalI* fragment containing the 3' LTR of gypsy was also blunted and cloned in the blunted *XbaI* site of pGEM-1. Both plasmids were chosen such that the LTR was in the same transcriptional direction as the T7 promoter and the small *SalI*–*XhoI* segment of the former was replaced by the small *SalI*–*XhoI* fragment of the latter. The resulting clone, pGLTR, contained a reconstructed LTR [the LTRs of *bx^{34e}* gypsy are identical in sequence (Freund and Meselson 1984)] that lacks flanking *bithorax* DNA. The internal 7-kb *XhoI* fragment of gypsy was then cloned in the *XhoI* site of pGLTR. The

BamHI site of this clone was blunted and converted to *SalI* with a linker (dGGTCGACC) to generate pGGSS. The large *SalI* fragment of pGGSS was cloned in the *SalI* site of pCAT82SVSX to construct gypsy-containing *hsp82* genes used in transfection experiments: pCAT82SVSX-Gp (in which the direction of gypsy transcription is the same as *hsp82*) and pCAT82SVSX-Ga (the opposite orientation). This fragment contained a complete gypsy in which the 2 nucleotides just downstream of the 5' LTR are duplicated downstream of the 3' LTR and the 48 nucleotides just upstream of 3' LTR are duplicated upstream of the 5' LTR.

For the RNA probe protection experiments, the *SmaI*–*HincII* fragment of pGSX containing the SV40 sequence was cloned into various sites in the gypsy element in pGGSS. These sites are the *NcoI* site [gypsy nucleotide 1085, blunted] *PvuII* site (nucleotide 2616) *AsuII* (nucleotide 4246, blunted) and *NdeI* (nucleotide 5637, blunted). Clones with both orientations of the SX sequences at each of these sites were obtained and the large *SalI* fragments of these constructs were cloned in the *SalI* site of pCAT82SVS to construct the series of plasmids containing parallel and anti-parallel gypsy elements.

The gypsy element used in the constructs for the germ line transformation experiments came from pGGHS. To construct pGGHS the *HpaI* site in pGLTR was converted to a *SalI* site with a linker (dGGTCGACC) prior to insertion of the internal *XhoI* fragment of gypsy. Conversion of the *BamHI* site of this clone to a *SalI* site with a linker generated pGGHS. The large *SalI* fragment of pGGHS contained a gypsy element that lacked the first 4 nucleotides of the 5' LTR and in which the 2 nucleotides just downstream of the 5' LTR were duplicated just downstream of the 3' LTR. This fragment was cloned in both orientations in the *SalI* site of pC20CAT82SVSX to give rise to pC20CAT82SVSX-Gp (parallel gypsy) and pC20CAT82SVSX-Ga (anti-parallel gypsy).

The internal *XhoI* fragment of gypsy (nucleotides 199–7069) was cloned in the *SalI* site of pCAT82SVSX to produce pCAT82SVSX-XGp. The gypsy sequences from the *BstXI* site (nucleotide 967) to the *NsiI* site (nucleotide 7065) in pCAT82SVSX-XGp were deleted to generate pCAT82SVSX-XBxp. The ends were blunted with T4 DNA polymerase and ligated to a *SmaI* linker (dCCCCGGGG) before reclosing. Deletions between the *SmaI* site in this clone and the *HincII* (835) *Ball*, (641) and *BglII* (425) sites were made by restricting, blunting the ends with T4 DNA polymerase, and reclosing. To construct pCAT82SVSX-XBp, which also contained the *XhoI*–*BglII* fragment of the LTR, the *BglII* site of pGLTR was converted to a *SalI* site with a linker (dGGTCGACC) and then the *XhoI*–*SalI* fragment of this clone was inserted into the *SalI* site of pCAT82SVSX. To construct pCAT82SVSX-XBp-BaBxp and pCAT82SVSX-XBp-BaBxa, the *Ball* and *SmaI* (originally *BstXI*) sites of pCAT82SVSX-XBxp were converted to *SalI* sites with a linker (dGGTCGACC) and the *SalI* fragment of the resulting clone containing the gypsy sequence between nucleotides 641 and 967 was cloned in the *SalI* site of pCAT82SVSX-XBp.

The *bx^{34e}* gypsy is 117 bp shorter than the *f¹* gypsy element in the region between the *Ball* and *BstXI* sites (Marlor et al. 1986; Peifer and Bender 1988). The nucleotide numbering system used here is based on the published sequence of gypsy cloned from the *f¹* allele (Marlor et al. 1986) modified to reflect the deletions; to obtain the equivalent number for *f¹* gypsy, 8 should be added to numbers from 628 to 832, and 117 should be added to numbers greater than 832. It should be recognized that because the *bx^{34e}* element has not been entirely sequenced, numbers greater than 1030 cannot be assumed to be exact. Restriction analysis, however, has revealed few differences between *bx^{34e}* and *f¹* gypsy elements outside this region.

Cell transfections

Schneider line 2 cells were grown in M3 medium (Shields and Sang 1977) supplemented with 10% fetal bovine serum (FBS) at 25°C in plastic dishes. Confluent cells (approximately 10^7 cells/ml) were plated at a 10-fold dilution and collected by centrifugation at half confluence (approximately 3 days after plating), washed once in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4) and once in TBS (137 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 0.5 mM MgCl_2 , 1.4 mM Na_2HPO_4 , 25 mM Tris-HCl [pH 7.5]). Transfections were carried out in TBS containing the indicated DNA concentrations and 300 $\mu\text{g}/\text{ml}$ DEAE-dextran (Pharmacia; 500,000 MW) at a cell density of 5×10^7 cells/ml for 4 hr at 25°C with occasional gentle agitation. Transfections were stopped by the addition of 5 ml of M3 (containing FBS) per milliliter of transfection. The cells were collected by centrifugation, washed once in M3, and plated at half-confluent density.

Germ line transformations

Microinjections of ry^{506} embryos were carried out as described (Cohen and Meselson 1984) using the pCAT82SVSX plasmids and p13 π wc (Cohen and Meselson 1985) as a source of P-element transposase. Determination of the linkage assignment of the transformed ry^+ genes and the selection of lines homozygous for second and third chromosomes bearing insertions were carried out as previously described (Goldberg et al. 1983; Cohen and Meselson 1984). To obtain transformant fly stocks with X-linked suppressor alleles, lines homozygous for ry^+ second chromosomes were crossed to Muller-6; In(2LR)Gla/SM5 flies and selected F_2 progeny were mated to obtain Muller-6 stocks with homozygous insertion-bearing second chromosomes. These stocks were crossed with $y^2 w^a ct^6 f^1$; In(2LR)Gla/SM5 stocks that differed in the X-linked suppressor mutation genotype (Rutledge et al. 1988). Selected F_2 progeny were mated to give rise to lines with the various X-linked suppressor alleles and homozygous for a transformant second chromosome. To obtain transformant stocks with $su(Hw)$ mutations, $y^2 w^a ct^6 f^1$; TM2/MKRS stocks with homozygous insertion-bearing second chromosomes were crossed to $y^2 w^a ct^6 f^1$; In(2LR)Gla/SM5; $su(Hw)^2/su(Hw)^f$ flies and selected F_2 progeny were mated.

RNA preparation

Transfected cells (approximately 5×10^7) were suspended by gentle pipetting 48 hr post-transfection, transferred to 15-ml polypropylene tubes (Falcon 2059), and subjected to the indicated heat shock by immersing the tube in a water bath at 34.5°C. Heat shock was terminated by immersing the tube in an ice-salt bath (-10°C) for 2 min. The cells were collected by centrifugation at 4°C, washed in 5 ml ice-cold TBS and vortexed briefly in 4 ml of Holmes-Bonner solution [7 M urea, 2% SDS, 0.35 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4); (Holmes and Bonner 1973) at room temperature. The lysate was extracted 3 times with an equal volume of PC (TE-saturated phenol : chloroform (1 : 1)) and the nucleic acids were precipitated with 2.5 volumes of cold (-20°C) ethanol. The nucleic acids were collected by centrifugation, washed with 70% ethanol, and dissolved in 200 μl TES [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, (pH 8.0), 0.2% SDS], and the RNA was precipitated by adjustment to 3 M sodium acetate (pH 5.3) and incubation on ice for 2 hr. The RNA precipitate was collected by centrifugation, washed once with 3 M sodium acetate (pH 5.3), once with

70% ethanol, dissolved in 200 μl sterile water, and stored at -20°C . Polyadenylated RNA was prepared by oligo(dT)-cellulose chromatography (Aviv and Leder 1972). Pupae were heat shocked in moistened-cotton-plugged vials as indicated, and RNA was prepared as above except that pupae were disrupted in a 1 : 1 mixture of Holmes-Bonner solution and PC with the aid of a Dounce homogenizer.

Northern blot analysis

The indicated amounts of RNA were denatured and separated by electrophoresis in containing gels formaldehyde (Maniatis et al. 1982). RNA was transferred from the gels by capillary action to a membrane (ICN Biotrans) and cross-linked to the membrane by irradiation with shortwave UV (600 $\mu\text{W}/\text{cm}^2$ for 5 min). Hybridizations with RNA probes were carried out in 50% formamide, 10% PEG-6000, 7% SDS, 0.125 M sodium phosphate (pH 6.5), 0.25 M NaCl, and 1 mM EDTA at a temperature appropriate for the specific probe. CAT probe was hybridized at 55–60°C; SV, LAC, and *hsp82* probes were hybridized at 60–65°C. Single-stranded continuously labeled RNA probes were prepared by in vitro transcription of plasmid DNAs with Sp6 or T7 polymerase (Melton et al. 1984). The blots were washed in 3–4 changes (15–20 min each) of $2 \times \text{SSC}$ (SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 mM sodium phosphate (pH 6.5), 0.02% sodium pyrophosphate, and 1% SDS at 65°C and 3–4 changes (15–20 min each) of $0.1 \times \text{SSC}$, 5 mM sodium phosphate (pH 6.5), 0.02% sodium pyrophosphate, and 1% SDS at 65°C. Autoradiograms were made by exposing X-ray film (Kodak XAR) to the blots with the aid of intensifying screens at -70°C . Exposure times ranged from a few hours to several days. Relative amounts of different transcripts were estimated by making several autoradiographic exposures of a hybridized blot and comparing the exposure times required for two radioactive bands to give the same film density. This procedure was used for signals that gave intermediate film densities with exposure times of 4–48 hr. Signals requiring significantly longer or shorter times were only roughly estimated. Probes were removed from a blot before rehybridization by boiling in distilled water.

S1 nuclease analysis

Probes for S1 analysis were prepared by digesting pCAT82SVSX-Gp and pCAT82SVSX-Ga with *SalI*, filling in the end with α - ^{32}P -labeled deoxyribonucleotides (NEN; 3000 Ci/mole) by Klenow polymerase, and then digesting with *Sau96I* for pCAT82SVSX-Gp and *HpaI* for pCAT82SVSX-Ga. The appropriate fragments (525 bp for pCAT82SVSX-Gp and 487 bp for CAT82SVSX-Ga) were separated by electrophoresis in a 4% native polyacrylamide gel and eluted from the gel (Maxam and Gilbert 1980). Probe (100 ng) was denatured at 80°C in hybridization buffer [HB is 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% formamide] for 5 min and added to 20 μg of total cellular RNA with 80 μg of yeast tRNA in HB (total volume of 50 μl) at 50°C. Hybridization was carried out overnight, and 0.3 ml of ice-cold S1 nuclease buffer [0.28 M NaCl, 0.05 M sodium acetate (pH 4.6), 4.5 mM ZnSO_4 , 20 $\mu\text{g}/\text{ml}$ sheared, single-stranded salmon sperm DNA] containing 500 units of S1 nuclease (Boehringer Mannheim) was added and digestion was carried out at 37°C for 30 min. Digestion was stopped by addition of 50 μl of 0.4 M ammonium acetate containing 0.1 M EDTA followed by extraction with an equal volume of PC. Yeast tRNA (5 μg) was added, and the nucleic

acids were precipitated with an equal volume of isopropanol. The pellet was dissolved in 3 l sequencing gel loading buffer [80% formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.15 % xylene cyanol], denatured at 90°C for 3 min, and the probe fragments were separated by electrophoresis in a 7% polyacrylamide-urea denaturing gel. Labeled *MspI* pBR322 DNA fragments were used as size markers. The probe fragments were detected by autoradiography.

RNA probe protection analysis

Total cellular RNA (20 µg) was hybridized to 500–2000 cps (measured with a mini-monitor) of single-stranded, continuously-labeled antisense SX probe (synthesized using [α -³²P] UTP at 400 Ci/mmol) in 25 µl HB (see above) overnight at 37°C. The hybridizations were rapidly cooled to room temperature and 0.35 ml of RNase buffer [10 mM Tris·HCl, (pH 7.5), 5 mM EDTA, 300 mM NaCl] containing 40 g/ml RNase A and 2 µg/ml RNase T₁ was added. Digestion was carried out at room temperature for 30 min and was stopped by addition of 10 µl of 20% SDS and 10 l of 10 mg/ml proteinase K. Proteinase K digestion was carried out at 37°C for 15 min. Yeast tRNA (5 µg) was added and the sample was extracted with an equal volume of PC. The RNA was precipitated with 2.5 volumes of ethanol, dissolved in 3 µl of sequencing dye (see above) and denatured by heating to 90°C for 3 min. Protected probe fragments were separated by electrophoresis in 7% polyacrylamide-urea denaturing gels and detected by autoradiography of the dried gel. The relative amounts of the different protected fragments were estimated by the relative exposure times required to give an equivalent autoradiographic signal. Labeled *MspI* fragments of pBR322 were used as markers.

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