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Suppression in *Drosophila*: *su(Hw)* and *su(f)* gene products interact with a region of *gypsy (mdg4)* regulating its transcriptional activity

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The *gypsy (mdg4)* mobile element of *Drosophila* contains two closely spaced regions which bind proteins from nuclear extracts. One of these is an imperfect palindrome having homology with the *lac*-operator of *Escherichia coli*; the other contains a reiterated sequence (5'PyPu$^{T}/_{C}$ TGCATA$^{C}/_{T}$PyPy) homologous to the octamer that is the core of many enhancers and upstream promoter elements. Transient expression of deletion mutants has shown that these DNA regions are negative and positive regulators of transcription. As was demonstrated earlier by other authors, mutations induced by the presence of *gypsy* in different loci are suppressed owing to either repression or activation of *gypsy* transcription in *Drosophila* strains carrying unlinked mutations in *su(Hw)* or *su(f)* genes. We have shown that binding to a negative regulator (silencer) is weakened in nuclear extracts isolated from fly stocks carrying *su(f)* mutations which activate *gypsy* transcription; therefore the *su(f)* gene seems to code for a protein capable of *gypsy* repression. Furthermore, binding to a positive regulator is weakened in nuclear extracts isolated from fly stocks carrying *su(Hw)* gene mutations which decrease the level of *gypsy* transcription; therefore, the *su(Hw)* gene most likely encodes a protein which activates *gypsy* transcription.
Key words: Drosophila/regulation of *gypsy (mdg4)* transcription/suppression/transposable elements

Introduction

The phenomenon of suppression implies that a mutation at the suppressor locus reverses the effect of other mutations at unlinked loci and restores the wild-type phenotype. In *Drosophila melanogaster*, suppressible alleles are usually caused by the insertion of a retrotransposon (*copia*-like element) into or near a mutant gene (for references, see reviews by Kubli, 1986; Parkhurst and Corces, 1986a). *Drosophila* is known to have five genes, *su(f)*, *su(Hw)*, *su(pr)*, *su(s)* and *su(w^a)*, whose mutations suppress the effects caused by the insertion of mobile elements into other loci in the genome. Two of these suppressor genes reverse the effect of mutations induced by *gypsy*. The first is *su(Hw)* (3-54.8) which suppresses the mutations caused by a *gypsy* insertion into *Hw*, lz^1, f^1, f^5, Bx^2, *bxd* and other genes (for references, see Lindsley and Grell, 1968, and Modolell *et al.*, 1983). The *su(Hw)* gene mutation decreases the level of *gypsy* transcription (Parkhurst and Corces, 1986b). The mutation of the second gene, *su(f)* (1-65.9), restores the phenotype of lz^1 f^1 and f^5 mutations (Lindsley and Grell, 1968). Mutation of the *su(f)* gene results in an increase in the amount of *gypsy* RNA (Parkhurst and Corces, 1986c). Therefore, suppressor gene mutations change the level of *gypsy* transcription, which is always maximal at the pupal stage (Parkhurst and Corces, 1985, 1986a,b). The above data suggest that the products of the cellular genes *su(Hw)* and *su(f)* regulate *gypsy* transcription. To prove this contention, it is necessary to demonstrate that the products of these genes interact with *gypsy* regulatory sequences.

However, the evidence on the regulation of retrotransposon transcription and the localization of sequences responsible for these functions is rather scarce and contradictory. The sequence essential for transcription of the *Drosophila* retrotransposon *copia* is located in the LTR (Burke *et al.*, 1984; Sinclair *et al.*, 1986), but it has not been shown to interact with a *trans*-acting factor. On the other hand, it has been reported that the regulatory sequences of the yeast retrotransposon *Ty* are located between the upstream LTR and the beginning of the reading frame (Roeder *et al.*, 1985). Several retroviral proviruses which are very similar in structure to retrotransposons in general and to *gypsy* in particular, have been found to contain enhancer sequences in the upstream LTR (Khoury and Gruss, 1983).

Therefore, we first localized the *gypsy* regulatory sequences which interact with nuclear factors, and then determined whether these factors are indeed associated with the suppressor genes. Our study has allowed us to identify *gypsy* sequences located adjacent to one another in the non-translated region downstream of the 5'-LTR that are responsible for positive and negative control of *gypsy* transcription. The binding of these sequences to nuclear extracts isolated from wild-type strains and from strains mutant at the *su(Hw)* or *su(f)* genes was compared. The results indicate that these genes code for positive and negative regulators of *gypsy* transcription.

Results

Localization of the gypsy region which binds to nuclear proteins

In the present study we used the plasmid Dm 111 (Bayev *et al.*, 1984) which contains a *gypsy* element inserted into unique DNA sequences of *D.melanogaster*. This sequence was cloned from the cell line 67D27G into pBr322. This

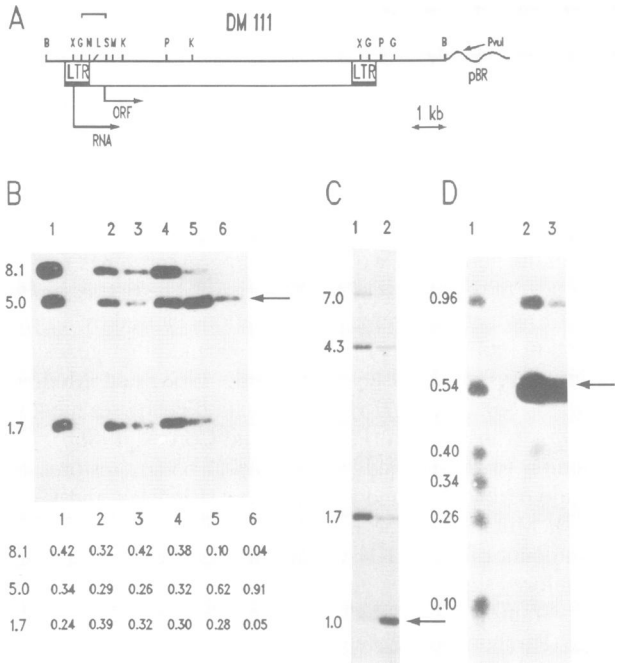


Fig. 1. The detection of *gypsy* sequences which specifically bind nuclear proteins. (**A**) On the restriction map of clone Dm 111 (Bayev *et al.*, 1984), brackets indicate the *Sau*3A fragment which specifically binds nuclear proteins. The symbols for the restriction enzymes are: B, *Bam*HI; G, *Bgl*II; X, *Xho*I; K, *Kpn*I; S, *Sau*3A; L, *Bal*I; N, *Nar*I; M, *Xma*III; P, *Pst*I. *Bal*I, *Sau*3A and *Nar*I sites are indicated only on the left, non-translated part of *gypsy*. (**B,C,D**) Binding of *gypsy* fragments to nuclear extracts from cell culture. (**B**) The plasmid DM 111 was digested wtih *Pvu*I and *Kpn*I, ^{32}P-labeled (**lane 1**) and used for filter binding assay. **Lane 1**, mixture of fragments; **lanes 2–6**, incubation with different quantities of 0.4 M NaCl protein extract prepared from Schneider cells: **2**, 80 μg of protein; **3**, 10 μg; **4**, 6 μg; **5**, 4 μg; **6**, 3 μg. Competitor DNA was present in 10-fold excess (180 ng). The arrow indicates the binding fragment. The autoradiograph of the gel was scanned. Numerical values of band intensities are presented in the table. (**C**) The plasmid Dm 111 was digested with *Bgl*II and *Kpn*I (**lane 1**). **Lane 2**, 16 ng DNA, 4 μg of protein, 10-fold excess of non-specific competitor DNA. The shortest *Bgl*II–*Bgl*II fragment has run off the gel. (**D**) *Bgl*II–*Kpn*I fragment of Dm 111 was recloned into pUC19 plasmid, digested with *Eco*RI, *Sau*3A and *Nar*I and end-labeled (**lane 1**). **Lane 2**, 5 μg of protein; **lane 3**, 2.5 μg. The short plasmid fragments have run off the gel.

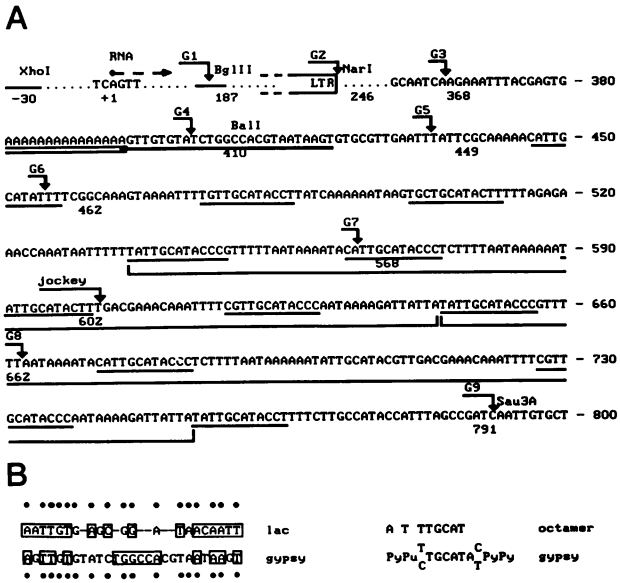


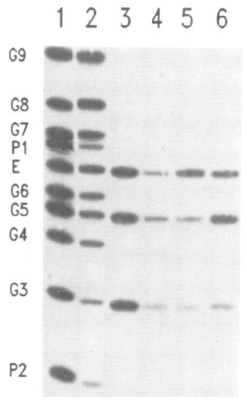
Fig. 2. The nucleotide sequence of the region of *gypsy* that binds nuclear proteins. (**A**) The imperfect palindrome is underscored by a solid bar; 12mer repeats are underlined; 109 bp repeats are in brackets; A_{15} block is double underlined. The end points of constructs G1–G9 are indicated by arrows. The nucleotides are numbered from the transcription initiation point (Arkhipova *et al.*, 1986). In the position of the A_{15} block in this sequence (Ilyin *et al.*, 1986), Marlow *et al.* (1986) found A_{19} block in their copy of *gypsy*. (**B**) Homologies between the 12 bp repeat from *gypsy* and the enhancer octamer (Falkner *et al.*, 1986) (on the right) and between the imperfect palindrome of *gypsy* and the *lac*-operon of *E.coli*. Common nucleotides are marked with dots; complementary nucleotides in the palindrome are boxed.

copy of *gypsy* is transcriptionally active (Ilyin *et al.*, 1984; Arkhipova *et al.*, 1986). The restriction map of the cloned sequence is shown in Figure 1A.

Two strategies can be used to search for regulatory sequences: (i) deletion of different regions to analyze whether the resultant mutants are capable of either *in vivo* or *in vitro* transcription; and (ii) localization of regions which can bind to nuclear proteins, and then demonstration of their regulatory nature. We decided upon the second approach because of two considerations. First, the strategy of deletion was complicated due to the large size of *gypsy* and a possible compensating effect of two LTRs (Laimins *et al.*, 1984). Second, in our opinion, it is necessary to detect the binding of regulatory sequences to nuclear proteins in order to study the molecular mechanisms of suppression.

We used the method of retention of DNA–protein complexes on nitrocellulose filters (Jack *et al.*, 1981), modified to include non-specific competitor DNA in the incubation mix. The basic procedure is as follows: restriction fragments of DNA are end-labeled with ^{32}P and incubated with serial dilutions of crude nuclear extracts in the presence of non-specific competitor DNA. Optimal concentrations of crude nuclear extract and competitor DNA minimize non-specific protein–DNA binding. A specific DNA fragment retained on nitrocellulose filter as a DNA–protein complex is eluted and subjected to gel electrophoresis. As can be seen in Figure 1B, by serial dilution of crude nuclear extract, i.e. by decreasing protein concentration, the optimal conditions for revealing specific binding of DNA fragment(s) to protein(s) can be determined (lanes 5 and 6). It is evident that the limiting parameter of specific binding under optimal conditions is the quantity of a specific protein in the incubation mix. Thus this assay provides an estimate of the quantity (or quality) of a specific binding protein in nuclear extracts. However, when analyzing the results obained by this method it is necessary to compare the *ratio* of bands in one lane with those of another lane, but not the absolute intensities of bands in different lanes, because DNA–protein complexes are retained on nitrocellulose filters to different extents (see, for instance, Figure 1B, lanes 3 and 4). Nevertheless, the ratio of band intensities within one lane is highly reproducible under the given conditions.

As can be seen in Figure 1B, the *Kpn*I–*Pvu*I fragment specifically binding to proteins in the crude nuclear extract contains the left part of *gypsy* along with adjacent genomic DNA and part of the plasmid DNA (see Figure 1A). Only one of the two LTR-containing fragments was preferentially retained on the filter. This suggests that the LTRs do not contain protein binding sites. In further experiments we



	1	2	3	4	5	6
G9	0.11	0.21				
G8	0.09	0.19				
G7	0.10	0.13				
P1	0.11	0.05				
E	0.09	0.11	0.34	0.42	0.63	0.35
G6	0.10	0.09				
G5	0.11	0.09	0.32	0.43	0.28	0.52
G4	0.09	0.06				
G3	0.09	0.05	0.34	0.15	0.09	0.13
P2	0.11	0.04				

Fig. 3. Binding of constructs to crude nuclear extracts from tissue culture cells and band competition assay. (**A**) The ^{32}P-labeled fragments of the G3−G9 constructs and the E fragment were obtained after digestion of corresponding constructs with *Ava*II and *Hin*dIII; control fragments P1 and P2 were obtained after digestion of pUC19 DNA with *Msp*I. Labeled fragments (10 000 c.p.m. each) were mixed. Since these fragments have different lengths their equimolarity was achieved by addition of necessary quantities of corresponding unlabeled fragments. The mixture was subjected to filter-binding assay using nuclear extract (see Materials and methods) and analyzed on 4% polyacrylamide gel, 7 M urea. **Lane 1**, the mixture of fragments; **2**, the fragments after filter binding (60 ng of fragment DNA, 8 μg of protein and 1 μg of λ DNA); **3**, the mixture of fragments G3, G5 and E; binding of the mxiture (22 ng DNA) in lane 3 to protein extract (8 μg of protein added) in the presence of corresponding competitor: 1 μg of λ DNA (**4**), 1 μg of construct G5 DNA (**5**) and 1 μg of construct E DNA (**6**). The autoradiograph was scanned. Numerical values of band intensities are presented in the table.

found that the 1.0 kb *Bgl*II−*Kpn*I fragment of Dm 111 plasmid (Figure 1A and C) was retained after binding to a nuclear extract. This fragment was recloned into pUC19 plasmid and the binding region was localized to a 0.54 kb *Nar*I−*Sau*3A fragment (Figure 1A and D).

The binding region is composed of a palindrome and direct repeats

The sequence of the fragment that binds to nuclear proteins had been determined previously (Ilyin *et al.*, 1986) and was found to be identical to the sequence of the same region of a *gypsy* copy inducing the mutation f^1 (Marlor *et al.*, 1986). Analysis of this sequence (Figure 2A) revealed several interesting stretches: a 15 nt poly(A) block followed by a 23 nt imperfect palindrome having homology with the *Escherichia coli lac*-operon (see Figure 2B), and a 12 nt sequence repeated 12 times within 310 nt. The consensus sequence of this repeat is 5′PyPu$^{T}/_{C}$TGCATA$^{C}/_{T}$PyPy. Three of the 12-bp repeats are 5′ to a 109-bp duplicated sequence (each containing four such repeats) and the very first repeat after the palindrome differs from all the others by a $C_{10}\rightarrow T_{10}$ substitution (Figure 2A). The 12 nt sequence has a region which is homologous to the conserved octanucleotide sequence (Figure 2B) found in the enhancer and upstream promoter elements of many eukaryotic and prokaryotic genes (see Falkner *et al.*, 1986). These homologies may be considered as indirect evidence of the regulatory nature of these regions. The reiteration of a certain motif is a characteristic feature of enhancers and a prerequisite for strong enhancer effect in all of the studied cases (Falkner *et al.*, 1985; for references, see Sassone-Corsi and Borrelli, 1986). It is likely that the frequent reiteration of the 12 nt motif is also indicative of its enhancer nature. The *Drosophila* genome contains *gypsy* copies in which the 109 bp sequence is not duplicated (L.J.Mizrokhi, unpublished results; Peifer and Bender, 1986). The shortened copy of *gypsy* exerts less effect on the transcription of the adjacent gene than does a complete copy (Peifer and Bender, 1986), apparently because the short copy is weakly transcribed. Therefore, all these data suggest that the region of binding to nuclear proteins indeed contains regulatory sequences.

Binding of nuclear proteins to deletion mutants

The binding of the nuclear proteins to the region under study was analyzed in detail using a set of deletions constructed as follows. Plasmid Dm 111 was digested with nuclease *Bal*31 after cleavage at the *Xma*III site (Figure 1A). The products of hydrolysis were treated with *Bam*HI, labeled with ^{32}P and fragments having the desired lengths were selected and cloned. The resultant deletion constructs contained the 5′-LTR with the adjacent genomic DNA and different amounts of flanking 3′ region. The precise boundaries of the insertions in seven clones used in further experiments (constructs G3−G9) were determined by sequencing (Figure 2A).

As the size of the construct increases (from G3 to G9), more elements of the region under investigation are present. Therefore, these constructs should permit us to detect the ability of the palindrome to bind independently and the participation of the 12mer repeats in binding. To establish the ability of the latter to bind independently of the palindrome, we prepared construct E. It contains the right part of construct G8 beginning from the *Bal*I site, i.e. the right part of the palindrome and eight 12mer repeats. The cloned insertions and the control fragments of plasmid pUC19 (P1 and P2) were labeled with ^{32}P, isolated, mixed in equimolar quantities and bound to nuclear extracts using standard procedures. Differences in fragment lengths were taken into consideration and equimolarity was achieved by addition of the necessary quantity of the corresponding unlabeled fragment. The results are presented in Figure 3, lane 2 and the table. Fragment G3, which contains none of the repeated sequences, is bound at the background level. Although this fragment comprises the 5′-LTR and a considerable portion of the adjacent *gypsy* sequence, it does not bind to nuclear proteins. The slight but specific binding of fragment G4 may be due to the presence of a portion of the palindrome with a sequence homologous to the 12mer repeats (see below). The presence of a complete palindrome strengthens the binding (cf. the fragments G4 and G5). The addition of repeats also noticeably increases the binding (fragments G8 and G9).

The binding of fragment E, which contains only part of the palindrome and the same number of repeats as the G8 construct, suggested that 12mer repeats can bind independently of the palindrome. Moreover, the higher intensity of binding to G8 as compared to the E fragment shows the contribution of the complete palindrome to the general ability of this region to bind proteins. The results of these experiments imply that the palindrome as well as the 12mer repeats are capable of independent binding; in addition, increasing the number of repeats in the constructs correspondingly increases their binding effectiveness.

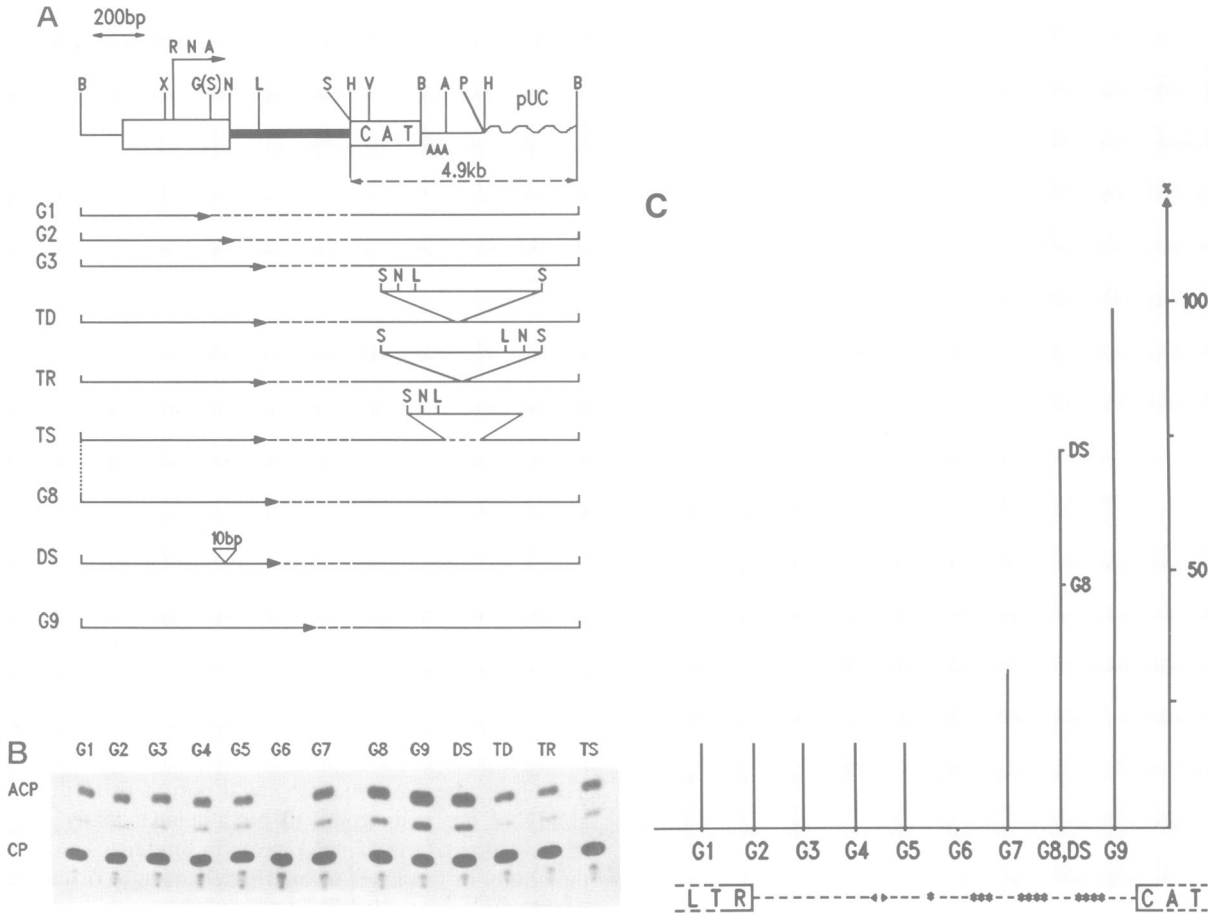


Fig. 4. Schematic representation of *gypsy*−CAT constructs and their CAT activity in transfected cells. (**A**) The exact boundaries of the deletions in constructs G1−G9 are shown in Figure 2B. Constructs DS, TD, TR and TS are constructed as described in Materials and methods. A, *Apa*I; V, *Pvu*II; other restriction sites are designated as in Figure 1A. (**B,C**) A representative CAT assay demonstrating the effect of deletion of 3′ sequences of *gypsy* on its expression in *Drosophila* cultured cells. Cells were transfected (30 mm Petri dish) with 15 μg of construct DNA and 3 μg of D88 DNA (β-galactosidase gene of *E.coli* under hsp-promoter) using the $CaPO_4$ protocol and cultured for 48 h. The relative CAT activity was normalized as described in Materials and methods. (**B**) **Lanes G1−G9, DS, TD, TR, TS**, cells transfected with corresponding construct; ACP, spots corresponding to acetylated chloramphenicol; CP, spots corresponding to chloramphenicol. (**C**) Histogram showing the mean relative CAT activity of *gypsy* deletion plasmids in *Drosophila* cells (at least five experiments). Dashed lines at the bottom of the drawing indicate sequences appearing in the plasmid 5′ to the CAT gene as the construct length increases (G1−G9). The position of the palindrome (< >) and the 12mer repeats (*) are shown. Relative CAT activity is expressed as the percentage of c.p.m. of [^{14}C]chloramphenicol acetylated in extracts from cells transfected with the test plasmid compared to that from transfectants containing the reference plasmid (G9). The SEM of each test condition was never > 12% of the test value.

The palindrome and 12mer repeats are bound to different nuclear proteins

The fact that fragments G5 and E, containing different sequences, are capable of binding raises the question of whether these sequences bind to different proteins. This problem was addressed in competition experiments in which the palindrome (fragment G5) and 12mer repeats (fragment E) compete for protein binding. The labeled inserts of constructs E and G5, as well as a fragment of construct G3 used as a control, were mixed; DNA from phage λ, construct E or construct G5 was then added in increasing quantities and binding to nuclear extracts was performed.

When analyzing the ratio of band intensities in one lane (see above) it is evident that the addition of construct E DNA (or construct G5 DNA) decreases the binding of the corresponding fragment in a specific manner (see Figure 3, lanes 5, 6 and the table) as compared to their binding in the presence of non-specific competitor DNA (lane 4). We could not completely inhibit the specific binding of the corresponding fragment because the binding disappeared entirely when the amount of added DNA was further increased. One of the possible explanations of the incomplete competition might be the existence of some homology between the left part of the palindrome and 12mer repeat (8 nt from 12) which leads to the partial competition between these fragments. When the excess of fragment E, which contains eight repeats, is added, this side-effect is stronger than in the case of fragment G5, which contains only the palindrome itself. It means that there is the decrease in the binding of both fragments (more in lane 6) but the decrease in binding of the fragment which was added is, of course, much stronger. The other explanation for the incomplete competition might be the presence of a large excess of proteins in the crude nuclear extracts which decrease the effect of the competition in cases when competitor is the same fragment which is tested in binding. Nevertheless, the results of this experiment support the suggestion that the palindrome and 12mer repeats are bound to different nuclear proteins.

The palindrome and 12mer repeats are negative and positive regulators of gypsy transcription

To prove that the two identified binding regions are functionally active, we have placed the bacterial CAT gene under the control of the LTR promoter and assayed its transcriptional activity by measuring the levels of CAT enzyme (Gorman *et al.*, 1982). Fragments G1−G9 were cloned into a plasmid containing the CAT gene-coding region following the SV40 DNA containing an intron and polyadenylation signal (see Figure 4A). In addition, we constructed CAT-containing clones TS, TD and TR to analyze the regulatory effect of the tested fragments when the distance from the initiation site and orientation were changed (see Figure 4A and Materials and methods). The calcium phosphate precipitation technique was used to co-transfect a *D.melanogaster* cell line (Schneider cells) with the test plasmids and D88, a plasmid containing the β-galactosidase gene under the control of the heat shock promoter. To control for differences in transfection efficiency, the level of β-galactosidase activity was used to normalize the CAT assay (see Materials and methods). Three main conclusions may be drawn from the results shown in Figure 4B and C. (i) The sequences to the left of palindrome (constructs G1−G4) provide for a certain level of transcription and correct initiation takes place (see below). (ii) The expression is almost completely blocked if a palindrome and the first 12mer repeat are present. Both of these structures are needed for negative regulation. This follows from the comparison of G5, G6 and DS expression levels. The change in the palindrome in the DS construct increases the level of transcription (as compared to G7) and the addition of the first 12mer repeat to the G5 construct (the G6 construct) results in almost complete disappearance of expression. (iii) As the number of 12mer repeats increases, the expression increases (G8 and G9) and exceeds the original level (G1−G5). The results unambiguously confirm the regulatory nature of the *gypsy* region binding to nuclear proteins; the palindrome-containing region is a negative regulator of transcription and the DNA region containing the 109-nt block with 12mer repeats is a positive regulator of transcription. Moving both positive (compare G9 with TD and TR) and negative (compare G3 with TS) regulatory elements 1.5 kb downstream from their original position results in the strong decrease of their activity under the conditions of transient expression (Figure 4B). The accuracy of transcription initiation was checked by primer extension (see Materials and methods, Figure 5A). The fact that negative regulation is observed at the level of transcription and not just translation was shown by Northern hybridization of RNA isolated from cells transfected with constructs G3 and G6 (Figure 5B).

Figure 5B demonstrates that the negative regulation is acting at the level of transcription although the correlation between the quantities of RNA and enzyme activity was not exact. We also analyzed in detail the CAT expression of the DS construct which, when compared to the G8, was lower than expected for a construct containing a damaged negative regulator. The DS construct also showed an inexact correlation between CAT expression and transcription, as compared to the G8 construct (Figure 5C). This phenomenon may be due to inhibition of translation by a hairpin loop in RNA (Kozak, 1980). Perhaps the palindrome in the G6 construct enhances repression leading to the most pronounced

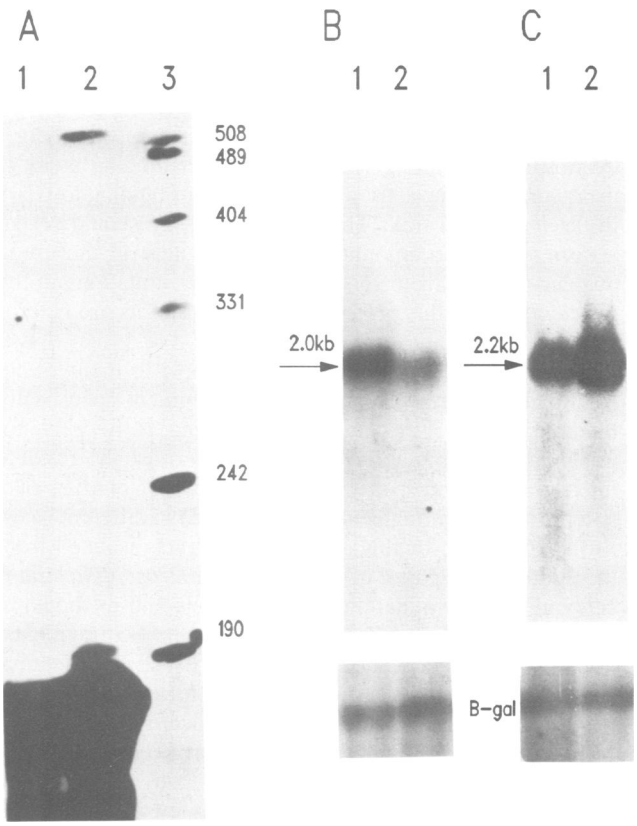


Fig. 5. Primer extension analysis and Northern blot hybridization of RNA from the cell culture transfected with DNA constructs. (**A**) ^{32}P-labeled 150 bp fragment of CAT coding sequence (see Materials and methods) was hybridized to poly(A)$^+$ RNA isolated from cells transfected with G3 construct (100 μg per 90 mm Petri dish). The primer (**lane 1**) was then extended with unlabeled dNPTs and reverse transcriptase and the product was fractionated on 5% polyacrylamide sequencing gel (**lane 2**). ^{32}P-Labeled *Msp*I fragments of pUC19 were used as nucleotide size markers (**lane 3**). The 535 nt band corresponds to the correct initiation of transcription of *gypsy* (Arkhipova *et al.*, 1986). (**B,C**) Poly(A)$^+$ RNAs were isolated from the cells transfected with constructs G3 and G6 (**B**) or G8 and DS (**C**). The quantity of RNA was normalized by β-galactosidase assay (see Materials and methods), and in each case it was estimated by Northern hybridization of the same filter to the β-galactosidase gene (see bottom panels). Northern blot hybridization was performed as described by Maniatis *et al.* (1982). (**B**) **Lane 1**, G3 RNA; **lane 2**, G6 RNA. (**C**) Lane 1, G8 RNA; **lane 2**, DS RNA.

drop of CAT activity. In the case of the DS construct, which contains a palindrome lengthened with *Sal*I linker, there is an inhibitory effect at the translational level partly masking the increase of transcription, the latter being due to the disruption of the negative regulatory region. However, this model does not explain the absence of inhibition of translation in the case of the G5 construct which also contains the palindrome.

Another, simpler, explanation of these effects may lie in the non-linear dependence of CAT product on RNA quantity (Gorman *et al.*, 1982) under our experimental conditions. However, regardless of the real cause of the inexact correlation between the results of the CAT assay and Northern experiments, the differences are not so large as to influence the main conclusion which may be drawn from both of them: they permit us to ascribe a negative regulatory function to the elements of construct G6 and a positive one to the 12mer repeats.

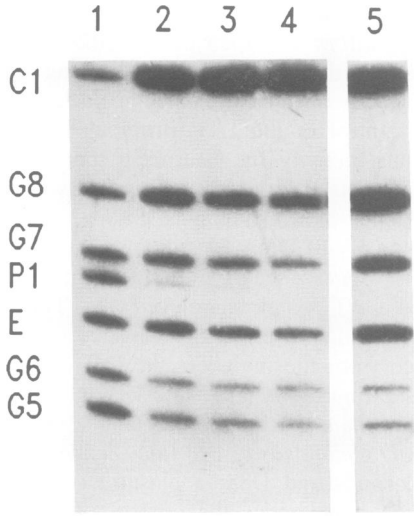
A su(Hw) mutation interferes with binding to the region of 12mer repeats

The final goal of our work was to confirm the hypothetical relationship between the products of suppressor genes and sequences of *gypsy* regulating its transcription (see Introduction). The limiting factor in the filter-binding assay is the quantity of a specific protein capable of binding to DNA in the incubation mix. Therefore, the assay should reveal a decrease in the amount of such a protein in the crude nuclear extracts from fly strains carrying mutations in the gene coding for this protein, even if the strains are heterozygous.

In this type of experiment it is desirable to include into the incubation mix a control DNA fragment specifically binding to some protein whose binding activity is not impaired by the mutation being tested. Because the absolute intensities can vary, this allows the ratio of binding of a specific to control fragment to be compared in different lanes. We analyzed the binding of nuclear extracts isolated from the middle stage pupae of three different *Drosophila* strains carrying *su(Hw)* mutations (see Materials and methods) to a mixture of labeled G8, G7, P1, E, G6 and G5 fragments. The mixture also contained fragment C1 which is the internal *mdg1* fragment that specifically binds to nuclear proteins (Ilyin *et al.*, 1986). The complete absence of homology or competition for the binding of specific proteins between this fragment and the *gypsy* region under study (unpublished results) allowed its use as a control to follow changes in the binding of *gypsy* regulatory sequences to proteins of nuclear extracts isolated from strains carrying *su(Hw)* mutant genes. Figure 6 presents the results of binding of a mixture of fragments to the nuclear extracts from three different strains with mutations in the *su(Hw)* gene (lanes 2–4) and from *Canton S* (wild-type, lane 5). The visual analysis and scanning of the autoradiograph (Figure 6 and the table) reveal an obvious decrease in the binding of *gypsy* fragments containing 12mer repeats in all the extracts from the strains with mutations in the *su(Hw)* gene as compared to the fragment Cl binding in the same lanes (compare also the ratio of these fragments in lane 5). At the same time, binding of fragments which do not contain 12mer repeats (fragment G5) or contain only the first of them (fragment G6) was largely unaffected. The variation of the ratio values was $< 15\%$ in different experiments. Therefore, the decreased level of *gypsy* transcription caused by a mutation in the suppressor gene *su(Hw)* (Parkhurst and Corces, 1986b) is due to decreased binding of the positive regulatory *gypsy* region to the *trans*-acting factor. This may result from either a decrease in the amount of the factor or a disruption in its structure essential for the interaction.

Su(f) mutation prevents the binding to the palindrome region

Similar experiments were done with nuclear extracts isolated from the middle stage pupae of the strains carrying a mutation in the *su(f)* gene (see Materials and methods), in which *gypsy* transcription is increased (Parkhurst and Corces, 1986c). The results are shown in Figure 7; the fragments used were the same as in Figure 6. Figure 7 shows that the ratio of bands G5 and G6 to C1 is decreased in the lanes corresponding to the mutant strains (lanes 3, 4) as compared to the wild-type strain (lane 2). Moreover, binding of fragment G6 containing the first repeat was slightly less



C1	1.00	1.00	1.00	1.00	1.00
G8	1.10	0.45	0.28	0.28	0.92
G7	1.14	0.32	0.16	0.17	0.62
P1	1.15	0.08	0.05	0.05	0.03
E	1.16	0.34	0.21	0.20	0.60
G6	1.10	0.13	0.10	0.09	0.13
G5	1.18	0.16	0.12	0.09	0.15

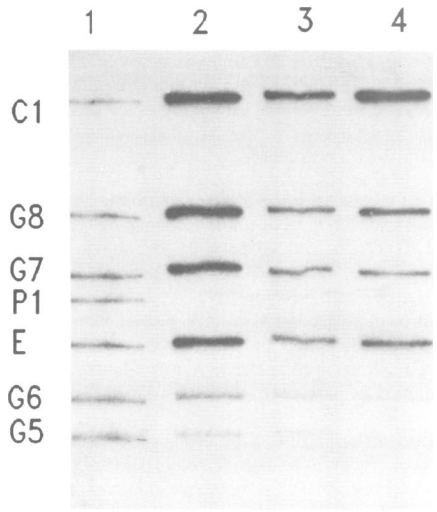
Fig. 6. Binding of constructs to crude nuclear extracts from pupae of strains carrying *su(Hw)* mutations. ^{32}P-Labeled fragments G8, G7, P1, E, G6 and G5 were obtained and analyzed as described in the legend to Figure 3A. The control fragment C1 (internal fragment of *mdg1* specifically binding to proteins of crude nuclear extract) was included into the mixture as the reference fragment which binds specifically and independently of *gypsy* specific fragments. **Lane 1**, the mixture of fragments; the fragments after filter binding with nuclear extracts from pupae of heterozygous strain *su(Hw)*^{2} (**2**); *su(Hw)*^{69K} (**3**); *su(Hw)*^{70} (**4**) and wild-strain *Canton S* (**5**) (20 ng of DNA fragments, 6 μg of protein, 1 μg λ DNA). The table presents the numerical values of relative band intensities in the corresponding lanes of Figure 6.

decreased than binding of fragment G5 containing only the palindrome (Figure 7 and the table). It is noteworthy that the effect is more pronounced in the homozygous strain (lane 4) than in the heterozygous strain (lane 3). Also fragment E shows a higher level of binding in the homozygous strain than does the palindrome-containing fragment G7 (lane 4, compare to wild-type and heterozygous strain, lanes 2 and 3). Therefore, a mutation in the *su(f)* gene weakens the binding of the *trans*-acting factor to the region of negative regulation of *gypsy* transcription and at the same time increases *gypsy* transcription (Parkhurst and Corces, 1986c). These data suggest that the *su(f)* gene is directly involved in the synthesis of this factor.

Discussion

Gypsy has a composite transcription modulator made of a positive element and a negative element (silencer)

We have found positive and negative regulators of *gypsy* transcription which comprise a block ~400 nt long starting 150 nt from the upstream LTR and terminating 50 nt before the start of the open reading frame. The approach used here



C1	1.00	1.00	1.00	1.00
G8	1.07	0.95	0.75	0.68
G7	1.12	0.81	0.71	0.47
P1	1.05	0.03	0.04	0.03
E	1.11	0.79	0.70	0.63
G6	1.12	0.29	0.19	0.13
G5	1.12	0.18	0.17	0.09

Fig. 7. Binding of constructs to crude nuclear extracts from pupae of strains carrying *su(f)* mutations. The same set of ^{32}P-labeled fragments as in Figure 6 was analyzed in identical conditions but using crude nuclear extracts of *su(f)* mutant strains: **lane 1**, the mixture of fragments; the mixture of fragments after filter-binding assay with nuclear extracts from *Canton S* pupae (**2**); heterozygous strain $su(f)^{Bx}$ (**3**); and homozygous strain $su(f)^{car}$ (**4**). The table presents the numerical values of relative band intensities in the corresponding lanes of Figure 7.

for finding regulatory sequences has not revealed any other *gypsy* sequences binding to nuclear proteins. This does not necessarily mean that *gypsy* contains no other regulatory sequences. Nevertheless, our results show that at least in the last 50 bp of 3′ part of the upstream LTR there are no other elements regulating transcription, as shown by the equal level of transcription of the constructs G1 and G2, in contrast to the finding of such elements in *copia* retrotransposon (Sinclair *et al.*, 1986).

The positive regulator of transcription which we have found has features characteristic of enhancer elements, namely the presence of multiple copies of a 12mer sequence homologous to the octamer found in a number of enhancers and in many promoters (Falkner *et al.*, 1986) from *E.coli* to man. The enhancer nature of this element may account for the finding (Peifer and Bender, 1986) that the strength of *gypsy*-induced mutations is influenced by the distance from the *gypsy* 5′-region to the presumed target gene regulatory region rather than by the orientation of the *gypsy* region. These authors have reported also that a 109 bp deletion in the same *gypsy* region weakens the mutation it causes.

Apparently, this is the same 109mer region at issue which in some genomic copies is not duplicated (L.J.Mizrokhi, unpublished results). There are two more cases in which the level of mutagenic effect induced by *gypsy* decreases owing to changes in this region, but this is due to the insertion of *BS* (Campuzano *et al.*, 1986) and *jockey* (Mizrokhi *et al.*, 1985) mobile elements within *gypsy*. The *jockey* is inserted into *gypsy* at nt 602 (see Figure 2A, Priimagi *et al.*, 1988). The integration of *jockey* moves half of the 12mer repeat 3 kb downstream from the initiation site which leads to the strong decrease in the mutagenic effect of *gypsy*. This finding correlates with our results which show that the transcriptional activity of the *gypsy* regulatory unit is decreased when it is moved from its original position. Recently it was shown that *jockey*, which is similar to mammalian LINEs, has the internal promoter for the RNA polymerase II on its extreme 5′ end (Mizrokhi *et al.*, 1988). We suggest that such an insertion of *jockey* is not purely accidental and the new type of promoter which was found in *jockey* may use the enhancer of the target gene to increase its own transcriptional activity.

These data permit the discussion of the nature of *gypsy*-induce mutations in genes located nearby. The disappearance or weakened expression of the mutant phenotype due to suppression is accompanied by changes in the level of transcription and by a decrease in the amount of regulatory proteins. Therefore, the effect on a nearby gene may be due either to *gypsy* transcription or to interaction of regulatory sequences with nuclear proteins *per se*. The second alternative is preferable because it accounts for the following observations. The presence of a single LTR near the target gene does not cause a mutation (Mizrokhi *et al.*, 1985; Geyer *et al.*, 1986) although it does allow a certain level of RNA synthesis (our results; Burke *et al.*, 1984). Our data suggest that the insertion of *jockey* in the *gypsy* regulatory region may result in approximately the same level of transcription as does the insertion of a single LTR. However the insertion of *jockey* does induce mutation, ct^{pN} (Mizrokhi *et al.*, 1985). As these two cases show the same level of transcription but differ in the presence of DNA sequence capable of specific binding, it is evident that this interaction by itself may cause mutations. This conclusion is supported by the fact that although a mutation in *su(f)* gene increases *gypsy* transcription it nevertheless suppresses mutations caused by *gypsy* (Green, 1955; Parkhurst and Corces, 1986c).

The silencer described has a distinct homology to the *E.coli lac*-operon and is also an imperfect palindrome. This homology is not surprising since the octamer homologous to the 12mer repeats, which form the *gypsy* positive regulatory element, was also found in other regulatory sequences of *E.coli*. Nevertheless, such a high conservation of sequences has not been reported previously for negative regulatory elements.

Are the positive and negative regulatory sequences of *gypsy* independent elements whose proximity to one another is purely accidental? This question cannot be answered unambiguously, but we believe that since the first 12mer repeat is part of the negative regulator and is homologous to the left part of the palindrome, this proximity of negative and positive regulators is probably not accidental. We suggest that an interaction between negative factors, binding to the palindrome, and positive factors, binding to the first 12mer repeat, is necessary to give rise to the repressor effect. Such an interaction may result in DNA looping. The negative effect of looping on transcription may play a very important role in the mechanism of repression (for ref. see Robertson, 1987). This hypothesis is supported by the fact that mutation or reduced levels of only one of these proteins is sufficent for the suppression of the mutations induced by the insertion of *gypsy*.

Suppressor genes su(Hw) and su(f) encode regulatory proteins for gypsy

We have found a direct correlation between *su(Hw)* and *su(f)* mutations and the impaired binding of *trans*-acting factors to *gypsy* regulatory regions. Although we have not proved that the above genes code for these factors, we may postulate that they do since no genetic data are available on the existence of any other genes involved in the regulation of *gypsy* transcription, although a number of alleles are known and have been tested for *su(Hw)*- and *su(f)*-like genes in this respect (Kubli, 1986). The final solution of this problem awaits the cloning of these suppressor genes and the identification of their products. Recently, Parkhurst *et al.* (1988) have cloned the *su(Hw)* gene and analyzed its structure. They have shown that it encodes a 109 kd protein containing 12 zinc finger domains. *su(Hw)* gene product was expressed in *E.coli* and *Drosophila* cell line and was partially purified. It was shown that this protein can bind to the region of *gypsy* containing 12mer repeats (Spana *et al.*, 1988).

The fact that not all mutations suppressed by *su(Hw)* contain *gypsy* and that *su(f)* is necessary for the synthesis of glue protein (for references see Kubli, 1986) indicates that the products of these genes participate in cell metabolism. Thus *gypsy* (and probably all retrotransposons) may be considered as peculiar cell parasites whose host range is determined by the presence of proteins capable of regulating their transcription. For this reason regulation of retrotransposons should be studied only in those species which have endogenous copies and, presumably, regulatory proteins for these elements. For example, we failed to detect any CAT expression in cultured cells of *D.virilis* transfected with our constructs. From this point of view the data on *copia* transient expression in *D.hydei* (Sinclair *et al.*, 1986) which contains neither endogenous copies of this element nor, apparently, proteins regulating its expression, do not allow one to draw conclusions about the non-functional nature of the *copia* regulatory region which is highly homologous to the SV40 enhancer core and has the same relative location as the *gypsy* regulatory sequences. This point is indirectly confirmed by the finding that a homologous sequence located at the analogous site of *Ty* element functions in yeast, presumably as an enhancer (Roeder *et al.*, 1985).

Materials and methods

Drosophila strains

For reference for isolation and genetic characterization of most of the mutant strains used in this work see Lindsley and Grell (1968). We used the following strains of *D.melanogaster* (from the collection of the Division of Biology, California Institute of Technology): *sc, cv* ct^6v*:su(Hw)*^{70e}/Ubx^{130} – referred to as *su(Hw)*^{70}. sc cv ct^6v*:su(Hw)*^{69K}/Ubx^{130} – *su(Hw)*^{69K}; *su(Hw)*^{2}*sbd/T*(2:3)*Xa – su(Hw)*^{2}; c^1*Xx, yf/f mal su(f)* Bx^3 *B.G.* – *su(f)*$^{Bx:}$ 376 *car su(f) B.G.* – *su(f)*^{car}.

Preparations of extracts from the nuclei of the cells of Schneider-2 tissue culture

Drosophila melanogaster Schneider-2 tissue culture cells were grown in plastic Petri dishes (90 × 13 mm) in a CO_2-incubator at 25°C in C-46 medium (Braude-Zolotarjova *et al.*, 1986) supplemented with 10% preheated fetal calf serum (Gibco or Flow), 50 μg/ml of penicillin and 50 μg/ml of streptomycin. The cells were seeded at a density of ~1 × 10^6 cells/ml.

Cells (1–2 × 10^6) were collected by centrifugation and washed in buffer A (0.11 M NaCl, 3 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.4) and lysed by incubation at 4°C in the same buffer but containing 1 mM $MgCl_2$ and 0.2% Triton X-100 (Jack *et al.*, 1981). Nuclei were collected by centrifugation and washed twice more in the lysis buffer, followed by two washes in buffer A. The final pellet was extracted with 0.2 M NaCl, 0.01 M Tris–HCl, pH 7.4, 0.002 M DTT, 0.1 mM PMSF for 15 min at 4°C with vigorous shaking, followed by centrifugation. The supernatant was collected and the pellet was extracted in the same manner with 0.4 M NaCl. Protein concentrations of crude nuclear extracts were determined as described by Bradford (1976) and were ~1.5–2 mg/ml. Extracts were stored in aliquots at −20°C.

Preparation of extracts from 3-day old pupae

Pupae were suspended in 5 ml of solution containing 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 15 mM Tris–HCl, pH 7.4, 0.5 mM DTT, 0.35 M sucrose and homogenized in a glass homogenizer with a motor-driven Teflon pestle. The homogenate was filtered through Nytex and Miracloth. Nuclei were collected from the final filtrate by centrifugation and washed twice with buffer A. Further purification and extraction of nuclei were carried out exactly as described for the preparation of extracts from tissue culture cells.

Plasmid construction

G1–G9 were constructed as follows: the *Bam*HI–*Pst*I fragment of Dm 111 was recloned into pUC19. The resulting plasmid was cut with *Xma*III and digested with *Bal*31 exonuclease (Maniatis *et al.*, 1982). Aliquots were taken at various times during the reaction, mixed and blunted with T4 DNA polymerase. The resulting fragments were cut with *Bam*HI, ^{32}P-labeled and electrophoresed in 4% polyacrylamide gel. The fragments of appropriate lengths were cloned in pUSVO plasmid (L.J.Mizrokhi), i.e. pUC19 containing the 2.18 kb *Hin*dIII–*Pst*I fragment from the plasmid pRSV-CAT (Gorman *et al.*, 1982). The deletion end point in *gypsy* was determined by sequencing of the appropriate regions of constructs (Maxam and Gilbert, 1980). DS was constructed by insertion of the 10 bp *Sal*I linker (New England Biolabs) into the *Bal*I site of construction G8. TD and TR were obtained by insertion of the *Sau*3A fragment of Dm 111, which binds to nuclear proteins, into the *Bam*HI site of construct G3 located after the polyadenylation signal. In TD the fragment is in the same orientation as in DM 111, and in TR it is reversed. TS is a derivative of clone G3 with the *Bgl*II–*Hin*dIII fragment from construct G6 replacing the *Bam*HI–*Apa*I fragment. Plasmid D88 containing the β-galactosidase gene under heat-shock promoter was the kind gift of Dr R.Voellmy (Amin *et al.*, 1987).

Transient transfection and CAT and β-galactosidase assays

For transfection experiments, the cells were seeded at a density of 1–15. × 10^6 cells/ml in 30 mm Petri dishes. The transfection was performed 24 h later. 0.4 ml of 0.25 M $CaCl_2$ containing 15 μg of construct DNA and 3 μg of D88 DNA was added dropwise to an equal volume of 2 × Hepes solution (DiNocera and Dawid, 1983). Precipitate was allowed to form for 30–60 min at room temperature, added to the growth medium (~1/10 of its volume) and after gentle mixing the cells were grown in the conditions described above for 2 days. CAT assays were performed as described by DiNocera and Dawid (1983).

To normalize for transfection level, a portion of the cells from every dish was exposed to heat shock, followed by standard colorimetric assay to measure β-galactosidase levels (Lawson *et al.*, 1984), and the quantity of cell extracts to be added to CAT reaction mixture was determined from these data. The endogenous level of β-galactosidase activity as determined by the same method was undetectable. The effectiveness of transfection varied from 1 to 5-fold, as determined by β-galactosidase assay.

RNA analysis

Poly(A)$^+$ RNA was isolated from transfected cells and used for primer extension analysis and Northern blot hybridization according to Maniatis *et al.* (1982). The probe was ^{32}P-labeled single-stranded DNA prepared as in Davis *et al.* (1986) using the clone M13 CAT (L.J.Mizrokhi) as template. This clone has the 150 bp *Sma*I–*Pvu*II fragment of CAT gene cloned into M13.

Filter-binding assay

We used the procedure described by Jack *et al.* (1981) with the modifications briefly outlined below. Nitrocellulose filters BA 085 were from Schleicher and Shull. The ^{32}P-end-labeled DNA probe (Maniatis *et al.*, 1982) was incubated in 0.4 ml of 0.1 M NaCl, 0.05 M Tris–HCl, pH 8.0, 0.01 M $MgCl_2$, 0.001 M EDTA and 0.002 M DTT for 15–30 min at room temperature with 4–20 μg of the crude protein extract in the presence of a 10- to 50-fold excess of sonicated λ phage DNA as a non-specific competitor. The incubation mixture was filtered through a nitrocellulose filter. The filter was washed with binding buffer and the DNA retained on the filter was recovered by elution with 0.1% SDS for 3 h at 60°C, precipitated by 3 vols of ethanol, redissolved in sample buffer and subjected

to electrophoresis in agarose or polyacrylamide gels. The DNA bands were visualized by autoradiography.

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