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Functional analysis of the transcriptional control regions of the *copia* transposable element

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The introduction of *copia*-based vectors in *Drosophila hydei* cells results in their high-level transient expression and the subsequent establishment of stably transformed cell lines containing multiple copies of vector integrated into host genomic DNA. Using transformation frequency and transient expression analysis as assays of promoter strength, we have defined the regions of *copia* essential for expression. We find that the essential sequences reside within the long terminal repeat, but 3' to the site of initiation of *copia* RNA. Deletion of the consensus enhancer-like sequences from *copia* appears to have no effect on vector expression.

Key words: transposable element/*copia*/transformation/deletion analysis

Introduction

The transposable element *copia* is present at about 100 copies per cell in cultured *Drosophila melanogaster* cell lines (Potter *et al.*, 1979). It exists, as DNA, in two forms: as linear molecules integrated into the genome flanked by the two long terminal repeats (LTRs) and as extrachromosomal molecules, similar in structure to the DNA integration intermediate of vertebrate retroviruses (Flavell and Ish-Horowicz, 1981). Indeed, the structural features of *copia* and its insertion sites are closely paralleled by the integrated proviruses of retroviruses (Varmus, 1983), and cultured *Drosophila* cells contain abundant virus-like particles containing *copia* RNA and reverse transcriptase (Shiba and Saigo, 1983).

Copia is transcribed at high levels in cultured *D. melanogaster* cells resulting in two major RNA species which are initiated within the 5' LTR of the element (Flavell *et al.*, 1981) and analysis of the coding sequences of these RNAs has revealed weak homologies to a number of retroviral proteins (Emori *et al.*, 1985; Mount and Rubin, 1985). Recently, assays of avian retroviral (Luciw *et al.*, 1983; Laimins *et al.*, 1984; Cullen *et al.*, 1984) and yeast *Ty* element vectors (Roeder *et al.*, 1985) by *in vitro* mutation and *in vivo* analysis have defined DNA sequences involved in enhancement of gene expression. We have previously shown that cloned *copia* circles containing the dominant selectable marker xanthine guanine phosphoribosyl transferase (*gpt*) are expressed to high levels in *D. hydei* cells (a *Drosophila* species that contains no endogenous *copia* elements) (Burke *et al.*, 1984a). By assaying the level of *gpt* expressed transiently in *D. hydei* cells after introduction of *copia:gpt* deletion variants, we have asked which sequences of the *copia* transposable element are required for its high-level expression. As *copia:gpt* is a dominant selectable marker and a direct correlation between stable transformation frequency and promoter strength has been shown for selectable expression vectors in vertebrate cells (Berg and French-Anderson, 1984), we have also used a similar approach to assay for promoter strength of *copia* vectors by measuring the stable transformation frequency of *D. hydei* cells.

We show that the regions required for *copia*-vector expression and high-frequency transformation reside entirely within the LTRs of *copia* but 3' to the start of RNA transcription. Further, deletion analysis of unique *copia* transcripts around the LTRs suggest the absence of enhancer-like elements which have been suggested on the basis of homology to SV40 and the yeast transposon *Ty*1 (Mount and Rubin, 1985). Finally, comparison of deletion derivatives of the *copia*-vectors suggest that there is good correlation between high levels of transient expression and high stable transformation frequency.

Results

Transient expression of deletion vectors

We have described a protocol for the assay of *gpt* activity using [^{3}H]guanine incorporation (Burke *et al.*, 1984a). pCV2*gpt* (Figure 1), which contains the bacterial *gpt* gene inserted 820 bp downstream of the LTRs of the cloned *copia* circle pBB5 (Flavell and Ish-Horowicz, 1981), is expressed at high levels in *D. hydei* DH33 cells (Burke *et al.*, 1984a). Consequently, we carried out assays for transient *gpt* expression in DH33 cells after transfection with various deletion derivatives of pCV2*gpt*.

Deletion derivatives of pCV2*gpt* made by *Bal*3I recession have been described previously (Sinclair *et al.*, 1983; Burke *et al.*,

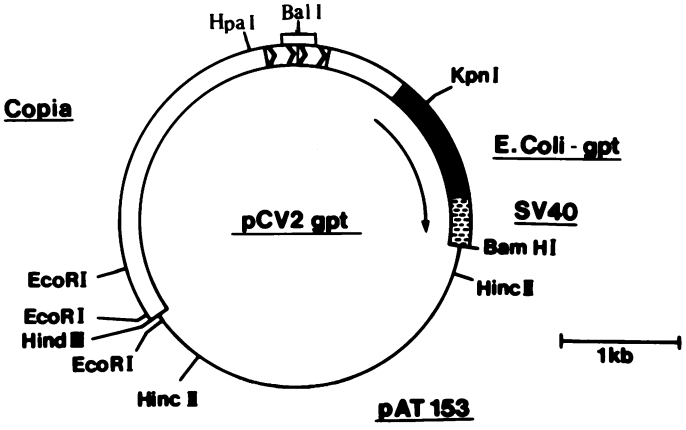

Fig. 1. Restriction map of pCV2*gpt*. Construction of the pCV2*gpt* vector has already been described in detail (Sinclair *et al.*, 1983). □ Represents *copia* DNA sequences in BB5 (Flavell and Ish-Horowicz, 1981); ▨ represents a long terminal repeat (LTR) of *copia* and indicates the direction of transcription from the *copia* promoters; ■ is the *Escherichia coli gpt* gene; ▤ are SV40 sequences containing the small antigen splice sequences and the poly(A) addition site. The thin line represents pAT153. Throughout the text we refer to the right-hand LTR of the *copia* circle as the 5' LTR and the left-hand LTR as the 3'.

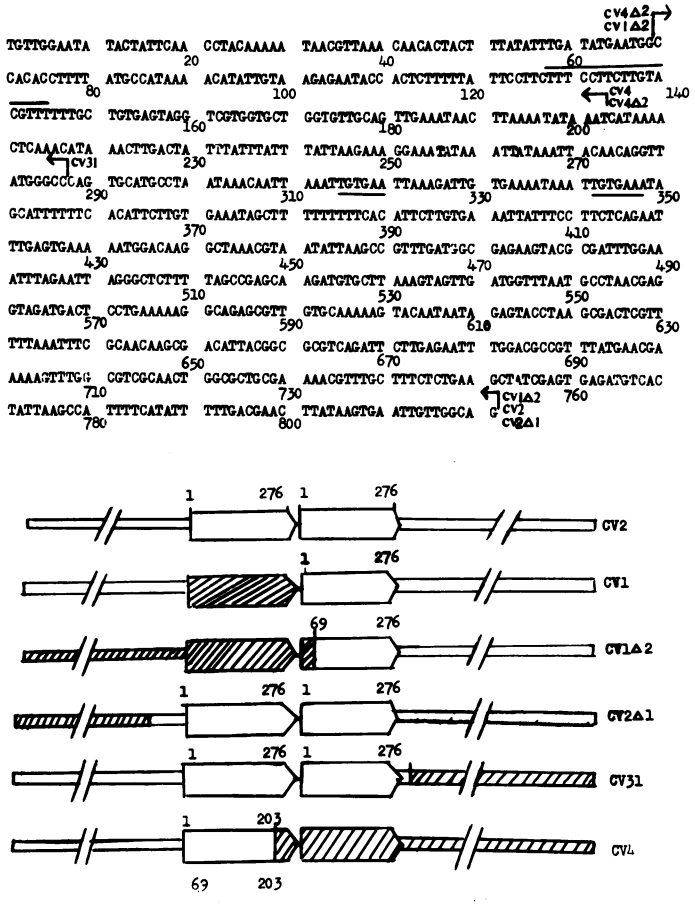

Fig. 2. Copia-vector deletions. **(A)** The sequence of the *copia* LTR and 3′ flanking sequences contained in *copia*-vectors are shown (from Flavell *et al.*, 1981). The putative start site of *copia* transcription is overlined. The SV40 enhancer-like sequences are underlined. *Copia* deletions are displayed as L-shaped arrows which define LTR and 3′ flanking sequences remaining after the deletion. pCV2Δ1*gpt* contains an extra 100 bp 5′ to the start of the LTR up to the *Hpa*I site of pBB5 (see Flavell *et al.*, 1981; and Figure 1). pCV4*gpt* and pCV31*gpt* also contain ~2.4 kb of unique *copia* sequence 5′ to the start of the LTR (see Flavell *et al.*, 1981; and Figure 1). pCV2*gpt*, pCV2Δ1*gpt* and pCV31*gpt* contain two complete LTRs each. pCV1*gpt* is similar to pCV2*gpt* (see Figure 1) except that it contains only one LTR. This was constructed by removing the 276 bp *Ball* fragments from the 3′ end of the left-hand LTR and the 5′ end of the right-hand LTR (see Figure 1) followed by religation to generate a 1-LTR *copia* vector. pCV1Δ2*gpt* and pCV4Δ2*gpt* are based on the vectors of pCV1*gpt* and pCV4*gpt*, respectively, except they are deleted for unique *copia* sequences upstream of their LTRs by a *Hin*dIII/*Bal*I deletion followed by blunt-end religation. **(B)** The deletion vectors are shown diagrammatically. Pointed boxes are the LTRs showing the direction of the transcription. Shaded areas denote the deleted sequences and numbers above the LTRs represent the number of nucleotides remaining in the LTRs after the deletion. For clarity pCV1*gpt* and pCV1Δ2*gpt* are shown as containing the complete right-hand LTR but it should be noted that a complete single LTR was generated by religation of the 5′ end of the left-hand LTR and the 3′ end of the right-hand LTR (see above).

1984a). Vector pCV1*gpt*, which contains only one LTR, was constructed by partial *Bal*I digestion and blunt end re-ligation (see Materials and methods). The vectors fall into three classes: those that delete unique *copia* sequences upstream of the LTRs (e.g. pCV2Δ1*gpt*), those that delete unique *copia* sequences downstream of the LTRs (e.g. pCV31*gpt*), and those which delete sequences within the LTRs (e.g. pCV4*gpt*) (see Figure 2). We have already shown (Burke *et al.*, 1984a) that deletions on either side of the *copia* LTRs (e.g. pCV2Δ1*gpt*, pCV31*gpt*) have little

Table I. Transient expression of *copia*-deletion vectors

Deletion vector	[^{3}H]Guanine incorporation (% c.p.m. of pCV2*gpt*)
pCV2*gpt*	100
pCV1*gpt*	98.5
pCV1Δ2*gpt*	95.2
pCV4*gpt*	9.4
pCV4Δ2*gpt*	4.9

Approximately 90 h after transfection, cells were assayed for [^{3}H]guanine incorporation. The highest incorporation observed with pCV2*gpt* was 29 175 c.p.m. which is equivalent to 23 pmol of guanine incorporated over the 20-h labelling period. These are average results of at least three experiments.

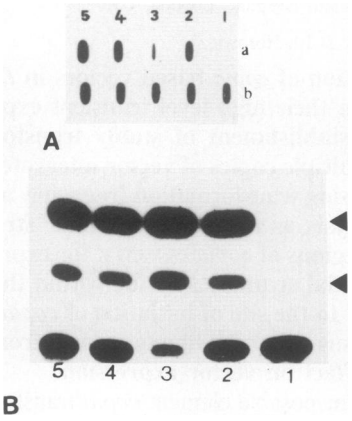

Fig. 3. Analysis of RNA in transfected cells. **(A)** 96-h post-transfection control cells (1) and cells co-transfected with pCV2*Cat* together with pCV2*gpt* (2), pCV4*gpt* (3), pCV31*gpt* (4) or pCV1*gpt* (5) were split into two. RNA was isolated from half the cells and transferred to nitrocellulose by slot-blotting. Filters were probed with a ^{32}P-labelled *gpt* specific insert (a) or a rDNA probe (b). **(B)** The other half of the cultures were assayed for CAT activity. (1) control; (2) pCV2*gpt*; (3) pCV4*gpt*; (4) pCV31*gpt*; (5) pCV1*gpt*; all together with pCV2*Cat*, transfected cells. ◀, acetylated chloramphenicol reaction products.

effect on the transient expression of *gpt* in *D. hydei* cells. Table I shows that vectors containing one LTR (pCV1*gpt*) result in similar levels of *gpt* expression as pCV2*gpt*, as does the vector pCV1Δ2*gpt* which removes the first 69 bp of an LTR. However, deletions which remove the 3′ end of the LTR, leaving only nucleotides 1–203 of a single LTR (pCV4*gpt*), reduce the transient expression of *gpt* by 10-fold. Similarly pCV4Δ2*gpt* which removes the 5′ end of pCV4*gpt*, leaving only nucleotides 70–203 of an LTR, reduces the amount of *gpt* expression to a very low level.

To show that these differences in GPT activity could be related directly to transcription of the *copia*:*gpt* constructs, we also analysed *gpt* expression at the level of RNA. Figure 3 shows that the level of *gpt* transcripts in transfected cells correlates well with GPT activity assayed by [^{3}H]guanine incorporation. DH33 cells were co-transfected with deletion variants together with pCV2*Cat* (J.H.Sinclair, unpublished) as an internal control for equal transfection efficiencies. pCV4*gpt* transfected cells (Figure 3a, track 3) contain 10- to 20-fold less *gpt* RNA than cells transfected with pCV2*gpt*, pCV31*gpt* or pCV1*gpt* (tracks 2, 4 and 5, respectively), whereas no variation in CAT activity was observed (Figure 3b). However, it is possible that the levels of RNA could be due to differential stability of pCV4*gpt* RNA. Consequently, we also analysed the relative rates of pCV4*gpt* and

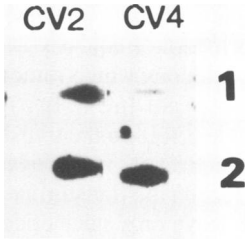

Fig. 4. Analysis of rates of transcription of *gpt* in transfected cells. DH33 cells were transfected with pCV2*gpt* or pCV4*gpt*. 96-h post-transfection nuclei were isolated and labelled with [α-^{32}P]UTP. RNA was isolated and hybridized to nitrocellulose filters which had been slot-blotted with 5 μg of pRSV*gpt* (1) plasmid DNA or 5 μg of DH33 DNA (2). Filters were hybridized to ~50 000 c.p.m. of [^{32}P]UPT-labelled RNA.

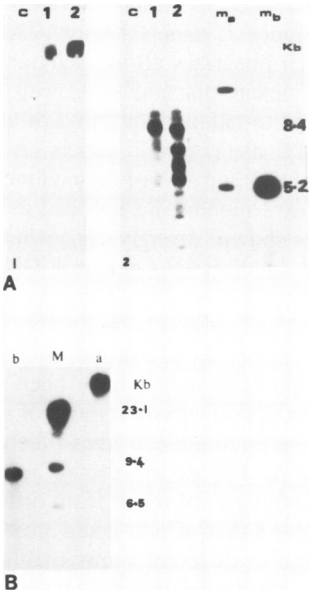

Fig. 5. (A) Southern analysis of pCV2*gpt* transfected *D. hydei* cells. DNA from two cloned lines (**tracks 1** and **2**) of pCV2*gpt* transfected cells either undigested (**panel 1**) or digested with *Bam*HI (**panel 2**) were separated on 0.6% agarose gels and blotted onto nitrocellulose. The filters were probed with a nick-translated *Hin*dIII/*Bam*HI *gpt* insert of pSV2*gpt* (Mulligan and Berg, 1981). **Tracks C** are control un-transfected cells and Ma and Mb, marker tracks containing 100 pg of undigested pCV2*gpt* and 1 ng of *Hin*dIII/*Bam*HI digest of pCV2*gpt* respectively. **(B)** Southern analysis of uncloned pCV2*gpt* transfected cells. DNA from transformed uncloned cells transfected with pCV2*gpt* but selected in HAT for >8 weeks was separated on 0.6% agarose gels either undigested (**track a**) or digested with *Bam*HI (**track b**). After transfer to nitrocellulose the filters were probed with nick-translated *gpt* insert of pSV2*gpt*. Marker (M) is a *Hin*dIII digest of λ DNA.

pCV2*gpt* transcription in nuclei from transfected cells, by measuring the amounts of *gpt* transcripts by DNA excess hybridization (Marzluff and Huang, 1984). Figure 4 shows that *gpt* transcription is approximately 10- to 20-fold lower in cells transfected with pCV4*gpt* compared with pCV2*gpt* transfected cells.

Stable transformation of DH33 cells with pCV2gpt

In contrast to the introduction of pCV2*gpt* into *D. melanogaster* D1 cells which results in low frequencies of unstable transformants (Sinclair *et al.*, 1983), introduction of this vector into DH33 cells resulted in a high frequency of stable transformants. DH33 cells transfected with pCV2*gpt* by calcium phosphate precipitation (Sinclair *et al.*, 1983) were selected in HAT medium (Sinclair *et al.*, 1985) and transformed clones were visible after 3–4 weeks. The transformation frequency of DH33 cells with

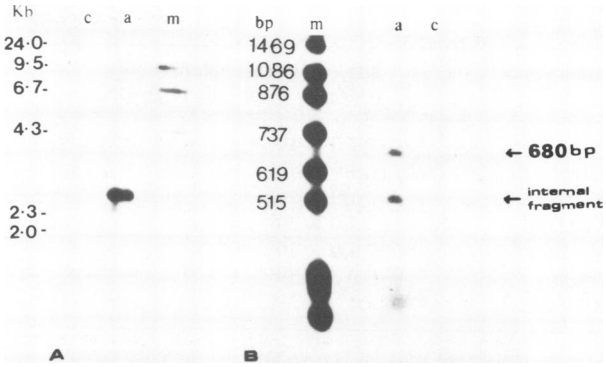

Fig. 6. Analysis of CV2*gpt* RNA transcripts in cells. **Panel A:** total RNA from pCV2*gpt* transfected cells (**track a**) or control un-transfected cells (C) was separated on 1.2% agarose, formaldehyde gels. After transfer to Gene Screen Plus (New England Nuclear), the filter was probed with nick-translated pCV2*gpt*. **Track M** is *Hin*dIII digested λ DNA. **Panel B:** total RNA from pCV2*gpt* cells (**track a**) was analysed by S1 analysis (see Materials and methods). **Track M** is a ^{32}P-end-labelled *Sau*3AI digest of pCV2*gpt* and **track C** is control un-transfected cells.

pCV2*gpt* was ~1 × 10^{-3}/10 μg of plasmid, and the cells grew well with a doubling time of 18–24 h. No clones were obtained with cells transfected with pRSV*gpt* or pSV2*gpt* where the *gpt* is under the control of the Rous Sarcoma virus LTR or the SV40 early promoter, respectively.

Southern hybridization analysis of DNA isolated from two clones of pCV2*gpt* transfected cell lines is shown in Figure 5. In contrast to *D. melanogaster* cells (Sinclair *et al.*, 1983), DH33 cells contain multiple copies of pCV2*gpt* which migrate with high mol. wt genomic DNA (Figure 5a, panel 1, tracks 1 and 2). After digestion with *Bam*HI, an enzyme that cuts pCV2*gpt* once, a unit size fragment equivalent to linear pCV2*gpt* was observed in clone 1 (Figure 5a, panel 2, track 1) which is consistent with head-to-tail concatemerization of unrearranged plasmid DNA. Clone 2 (Figure 5a, panel 2, track 2), however, shows numerous *gpt*-containing fragments suggesting plasmid rearrangements or multiple insertion sites in the host genome. DNA from transformed uncloned cells transfected with pCV2*gpt* also shows plasmid DNA to be associated with high mol. wt genomic DNA which, on digestion with *Bam*HI to linearize pCV2*gpt*, results in *gpt*-containing fragments of 8.4 kb (Figure 5b). This suggests that the transformed cell population consists mainly of cells containing tandem arrays of unrearranged pCV2*gpt*.

Growth of cloned or uncloned cells in non-selective medium for more than 2 months led to no loss of plasmid DNA (data not shown), arguing strongly for the stable integration of plasmid DNA into host chromosomes.

Northern analysis of RNA isolated from *D. hydei* cells transfected with pCV2*gpt* shows a predominant species of RNA, 2.5 kb in size (Figure 6, panel A). This is consistent with the transcript initiating in the *copia* LTRs and terminating at the SV40 poly(A) addition site of pCV2*gpt*. To determine more accurately the site of initiation, the CV2*gpt* transcript was also analysed by S1 mapping. A *Sau*3AI digest of pCV2*gpt* generates several fragments which will hybridize to an RNA species initiating in the LTRs and terminating at the poly(A) site of pCV2*gpt*. *Sau*3AI fragments of pCV2*gpt* labelled with polynucleotide kinase were hybridized to total cell RNA from pCV2*gpt* transfected cells. This RNA protects two large DNA fragments (Figure 6, panel B). The smaller 520 bp fragment maps within the *gpt* gene (see Richardson *et al.*, 1983). The larger fragment of 680 bp extends

Table II. Transformation of DH33 cells with *copia*-deletion vectors

Deletion vector	Stable transformation frequency (clones/5 × 10^6 cells/10 μg DNA)
pCV2*gpt*	1.0×10^3
pCV1*gpt*	1.0×10^3
pCV1Δ2*gpt*	1.0×10^3
pCV2Δ1*gpt*	1.0×10^3
pCV31*gpt*	1.0×10^3
pCV4*gpt*	0.8×10^2
pCV4Δ2*gpt*	<1

Cells were transfected with deletion vectors and surviving colonies were counted after 5 weeks selection in HAT. These are the average results of three experiments.

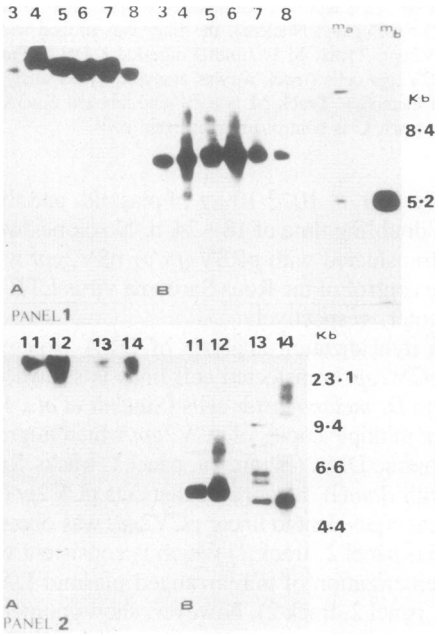

Fig. 7. Southern analysis of *copia*-deletion vectors in transformed cells. **Panel 1:** DNA isolated from two clones each of pCV4*gpt* transfected cells (**tracks 3** and **4**); pCV31*gpt* transformed cells (**tracks 5** and **6**) and pCV1*gpt* transfected cells (**tracks 7** and **8**) were separated on agarose gels either as undigested DNA (**A**) or after digestion with *Bam*HI (**B**). The gels were blotted onto nitrocellulose and probed with a nick-translated *gpt* insert from pCV2*gpt*. Markers (Ma, Mb) are 100 pg of undigested pCV2*gpt* and 1 ng of *Hin*dIII/*Bam*HI digested pSV2*gpt*, respectively. **Panel 2:** DNA isolated from two clones each of pCV2Δ1*gpt* (**tracks 11** and **12**) and pCV1Δ2*gpt* (**tracks 13** and **14**) were separated on agarose gels as undigested (**A**) or *Hin*dIII digested (**B**). Markers were *Hin*dIII digested λ DNA.

from the *Bam*HI/*Bgl*II site at the start of the *gpt* coding sequence to nucleotide 140 of the 5′ LTR (Figure 2). This site of initiation of pCV2*gpt* transcription is the same as that determined for *copia* transcription in *D. melanogaster* cells by Flavell *et al.* (1981).

Stable transformation with deletion vectors

In vertebrate cells the stable transformation frequency of a cell line by a particular expression vector is known to correlate well with the level of transcription of the vector (Berg and French-Anderson, 1984). The high frequency of stable transformation of DH33 cells with pCV2*gpt* allowed an analysis of the effect of *copia* deletion on the stable transformation frequency of DH33 cells. Transformation with each of the deletion vectors resulted in some different stable transformation frequencies (Table II). Transformation with plasmids containing only one LTR (pCV1*gpt*) had no effect on transformation frequency when compared with pCV2*gpt*. Similarly, deletions which removed unique upstream (pCV2Δ1*gpt*) or downstream (pCV31*gpt*) sequences from the LTRs had no observable effect on the transformation frequency. Vector pCV1Δ2*gpt* which leaves nucleotides 70−276 of a single LTR also had little effect on the transformation frequency. However, deletions which leave only nucleotides 1−203 of a single LTR (pCV4*gpt*) reduce the transformation frequency by 10-fold, and pCV4Δ2*gpt* which removes both 5′ and 3′ sequences of the LTR leaving only nucleotides 70−203 failed to result in any transformed colonies.

DNA isolated from cloned cells transfected with various deletion derivatives of pCV2*gpt* were analysed by Southern blot hybridization (Figure 6). In all cases cells containing multiple copies of plasmid sequences which migrate with high mol. wt genomic DNA (Figure 7, panels 1A and 2A). When digested with *Bam*HI, which linearizes all the deletion derivatives, unit size fragments of linear plasmid sizes were again observed, consistent with head-to-tail tandem arrays of unrearranged plasmids in all the cell lines (Figure 7, panels 1A and 2B). As with pCV2*gpt* transformants, growth of these clones in non-selective medium for at least 2 months did not lead to a loss of plasmid sequences (data not shown) strongly suggesting stable integration of these large concatemers into genomic DNA.

Discussion

Transient gene expression assays have been used successfully to analyse the transcription control regions of a number of genes in vertebrate and invertebrate cell lines precluding the need to assay levels of RNA. The *gpt* expression assay in DH33 cells is very sensitive and does not result from differential uptake or replication of vector DNA which could give variations in the levels of expression of different vectors (Burke *et al.*, 1984a) though, formally, we accept that this type of analysis cannot distinguish between *bona fide* transcriptional control and, perhaps, mRNA stabilization. As a good correlation exists between the stable transformation frequency of vertebrate cell lines with expression vectors and the levels of transient expression of the vector, one can also analyse the levels of expression of vectors by stable transformation frequency. The introduction of the *copia* expression vector, pCV2*gpt*, into *D. hydei* cells resulted in high frequency transformation with transformed cells containing multiple copies of unrearranged plasmid present as head-to-tail concatemers, apparently stably integrated into host genomic DNA. Similar tandem arrays of plasmid have been observed by Bourouis and Jarry (1983) after transfection of a *copia* dihydrofolate reductase gene fusion into *D. melanogaster* cells. We do not know why pCV2*gpt* is integrated at high copy numbers in DH33 cells, yet is present only episomally at low copy in *D. melanogaster* D1 cells (Sinclair *et al.*, 1983). It is possible that differences in the cell lines are the cause of this; for example, *D. hydei* cells contain no endogenous *copia* elements and take up and express pCV2*gpt* to much higher levels than D1 cells (Burke *et al.*, 1984a). We are at present investigating this phenomenon.

By deletion analysis we have defined those sequences present in *copia* essential for transcription by assaying transient expression levels or stable transformation frequency of DH33 cells after introduction of pCV2*gpt* and we find that the level of transient expression correlates well to the stable transformation frequency of transfected cells. Our analysis shows that deletion of one of the two LTRs (Figure 2), from the *copia* vector pCV2*gpt*,

had little effect on the expression of *gpt*. This is not surprising as there is a potential polyadenylation signal at nucleotide 194 of the second LTR (Emori *et al.*, 1985) suggesting that any transcript originating from the first LTR of pCV2*gpt* would terminate at the beginning of the second LTR. This is consistent with the Northern data which showed only one species of CV2*gpt* RNA of 2.5 kb in size and not 2.8 kb, which would be the size of the RNA if it initiated in the first LTR, and consistent with the knowledge that the 5-kb RNA of *copia* initiates at the 5′ LTR and terminates in the 3′ LTR (Schwartz *et al.*, 1982).

We have previously shown that deletion of unique *copia* sequences 5′ to the LTRs has little effect on the expression of *gpt* (Burke *et al.*, 1984a). Extension of this deletion to 69 nucleotides of the 5′ end of the LTR (pCV1Δ2*gpt*) also has no effect on the expression of *gpt*. This shows that these sequences upstream of the start site of the RNA are not essential for high-level expression. Deletions into the 3′ end of the LTR, however, do effect *gpt* expression. For instance, pCV4*gpt* removes all nucleotides of unique *copia* sequence 3′ to the LTR and 73 nucleotides from the 3′ end of the LTR itself, leaving only nucleotides 1−203, and drastically reduces *gpt* expression. This deletion includes two TATA motifs present at nucleotides 256 and 263. However, as the *copia:gpt* transcript initiates at a nucleotide 140 ± 10, it is clear that these TATA sequences are not acting in their usual role. Nevertheless, it is clear that sequences present between nucleotides 203−276 of the LTR and 10 nucleotides of unique *copia* sequence 3′ to the LTR are essential for expression of *copia* vectors. We do not know why a further deletion of the first 69 nucleotides of the vector pCV4*gpt* (i.e. pCV4Δ2*gpt*) reduces the level of expression of *gpt* even further, especially when the deletion of the first 69 nucleotides of complete LTR (e.g. pCV1Δ2*gpt*) has little effect on *gpt* expression. It is possible that a small decrease in very high levels of expression is not as easy to recognize in this system as an equivalent decrease in a level of expression that is already low.

As the measurement of *gpt* expression by [^{3}H]guanine incorporation only measures GPT activity, we have also confirmed these observations by analysing RNA levels and measuring levels of transcription. Both confirm the transient *gpt* expression assays.

Recently, transcripts of complete *copia* elements (Mount and Rubin, 1985; Emori *et al.*, 1985) have shown that DNA transcripts between the end of the *copia* LTR and the beginning of the large open reading frame (ORF) (i.e. 3′ to the LTR) share homology with transcripts in the SV40 enhancer and the yeast transposon *Ty*1 (see Mount and Rubin, 1985). Mutations in these transcripts of SV40 (Weiher *et al.*, 1983) and *Ty*1 (Roeder *et al.*, 1985) are known to reduce their expression drastically. However, deletion of these transcripts (e.g. pCV31*gpt*), which removed both of the putative SV40 enhancer-like transcripts present in *copia* (see Figure 2), had no effect on the transient expression of *gpt* (Burke *et al.*, 1984a) or on the transformation frequency, arguing strongly against these sequences acting as transcriptional enhancers of *copia* expression. As the vector pCV31*gpt* contains two LTRs it is possible that the presence of two LTRs, in some way, compensates for the absence of the enhancer-like sequences. However, we feel this is unlikely due to the lack of effect of the deletion of one LTR, from pCV2*gpt*, on the expression of *gpt*. Similarly, we cannot rule out that these enhancer-like sequences play some other role in *copia* expression or transposition.

The ability to introduce *copia* vectors into cells that contain no endogenous *copia* elements, where they are expressed to high levels, has permitted a functional analysis of *copia* promoter sequences. Deletion analysis has shown that the sequences essential for *copia* expression reside almost entirely within the LTR, but 3′ to the site of initiation of RNA synthesis. This observation is very similar to results obtained for yeast *Ty* transposable elements (Roeder *et al.*, 1985) but, unlike Ty, these essential sequences appear not to include the SV40 enhancer-like sequences in *copia*. Similarly Luciw *et al.* (1983) and Laimins *et al.* (1984) have suggested that some flanking viral sequences 3′ to the LTR are required for full enhancer function, though these are also required in addition to the enhancer sequences. We do not know whether the sequences we have defined here as important for the expression of *copia* are also required for *copia* transposition. However, re-introduction of deleted *copia* vectors into adult flies by germ line transformation (Spradling and Rubin, 1982) should help to answer this question.

Materials and methods

Plasmid construction

The detailed construction of pCV2*gpt*, pCV4*gpt* and pCV31*gpt* by *Bal*3I deletion has been described previously (Sinclair *et al.*, 1983). pCV1*gpt* was constructed by partial *Bal*I deletion of pCV2*gpt* and recircularization with T4 ligase. pCV2Δ1*gpt* is a *Hin*dIII/*Hpa*I deletion of pCV2*gpt* which was blunt ended with DNA polymerase I (Klenow fragment) and recircularized by T4 ligase. pCV1Δ2*gpt* and pCV4Δ2*gpt* are *Hin*dIII/*Bal*I deletions of pCV2*gpt* and pCV4*gpt* respectively, blunt-ended with DNA polymerase I (Klenow fragment) and recircularized by the T4 ligase. pCV2*Cat* (J.H.Sinclair, unpublished) was constructed by inserting the *Bgl*II/*Bam*HI *Cat* containing fragment of pSVO*Cat* (Gorman *et al.*, 1982) into pCV2 (Sinclair *et al.*, 1983).

Cell culture and transformation

The *D. hydei* cell line, DH33, has been described (Sondemeijer *et al.*, 1980). Cells were routinely maintained in M3 medium (Shields and Sang, 1977). For transfection ~4−5 × 10^6 cells were co-transfected with 10 μg of plasmid DNA using calcium phosphate precipitation, as previously described (Sinclair *et al.*, 1983). Originally, selective medium M3X (Sinclair *et al.*, 1983) contained mycophenolic acid (MPA), adenine and xanthine. More recently, however, we have found that there is no need to include any of these in the selective medium as *Drosophila* cells cannot salvage hypoxanthine (Sinclair *et al.*, 1985). Consequently selection was imposed by supplementing the medium with hypoxanthine (13.5 mg/l), aminopterin (10^{-6} M) and thymidine (25 mg/ml). Selective medium was replaced weekly and after 4−5 weeks colonies were picked and expanded.

DNA and RNA analysis

DNA and RNA were extracted from transformed cell lines as described previously (Sinclair *et al.*, 1983). Formaldehyde denatured total cell RNA was analysed on 1.2% agarose gels by Northern transfer (Thomas, 1980) on Gene Screen Plus (New England Nuclear). Analysis of CV2*gpt* RNA transcripts by S1 mapping was carried out as described previously (Flavell *et al.*, 1981) except that 40−50 μg of total cell RNA was used.

Analysis of transient levels of RNA in deletion-variant transfected cells by slot-blot analysis was carried out by the method of Meinkoth and Wahl (1984) with a slot-blot apparatus (BRL) on nitrocellulose filters.

Measurement of rates by transcription of pCV2*gpt* and pCV4*gpt* was carried out on nuclei isolated by the method of Gross and Ringler (1979) as described by Marzluff and Huang (1984) except that the incubation buffer contained 0.1 M NH_4SO_4 and 5 × 10^8 nuclei were labelled in 0.5 ml with 70 μCi of [α-^{32}O]UTP (Amersham).

Total cell DNA was digested with restriction endonucleases (Amersham) under conditions specified by the suppliers. Restricted or un-restricted DNA was analysed on agarose gels by Southern transfer (Southern, 1975). Filters were hybridized in 50% formamide, 9% dextran sulphate for 24−36 h at 42°C (Wahl *et al.*, 1979).

Gpt expression assays

The levels of *gpt* expression after transient introduction of *copia:gpt* vectors into cells were assayed as described previously (Burke *et al.*, 1984a). For stably transformed cell lines, cells were plated onto coverslips 24 h before addition of [^{3}H]guanine and assayed as described (Burke *et al.*, 1984a).

Cat expression assays

The levels of *Cat* expression were carried out as described by Gorman *et al.* (1982).

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