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Regulated Expression of a *Drosophila melanogaster* Heat Shock Locus after Stable Integration in a *Drosophila hydei* Cell Line

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DNA-mediated cotransformation has been used to transfer a *Drosophila melanogaster* heat shock locus into cultured *Drosophila hydei* cells by use of the copia-based selectable vector pCV2gpt and of pMH10A, a cloned 87A7 heat shock locus encoding a mutant heat shock protein (hsp). Transformed lines contain between 50 and 200 copies of both plasmids, each separately organized as a head-to-tail concatemer which is stably maintained in the transformed lines. Exposure of the cotransformants to heat shock temperatures induces the regulated expression of the hsp RNA and the mutant hsp in all the lines analyzed.

DNA-mediated cotransformation has been used successfully to stably introduce nonselectable DNA sequences into mammalian cells, permitting an analysis of their structure and function (23). A similar capability in cultured *Drosophila* cells would also be extremely useful for the analysis of gene expression in *Drosophila*. While we (6) and others (9, 14) have introduced recombinant vectors into *Drosophila* cells and analyzed their transient expression, there have been no reports of the expression of nonselectable genes which have been stably cointroduced with a dominant selectable marker into *Drosophila* cells. Recently we described how the dominant selectable marker pCVgpt can be used to stably cointroduce a *Drosophila melanogaster* heat shock locus into cultured *D. melanogaster* cells (5), but we were unable to detect any expression of the cotransfected DNA, even though we have obtained efficient transient expression of recombinant heat shock genes in this cell line (6). The reason for the lack of expression of integrated heat shock genes in these *D. melanogaster* cells is unknown, but similar differences in the ability of different mammalian cell lines to express various transfected genes have been observed (15). Consequently, we carried out similar experiments with a *Drosophila hydei* cell line which also transiently expresses recombinant heat shock genes efficiently but takes up more DNA and transforms to higher frequencies (6). By cotransfection with pCV2gpt (27), we have recovered cell lines containing between 50 and 200 copies of a mutant heat shock locus and equivalent numbers of pCV2gpt. Both plasmids are present as head-to-tail concatemers which were stably inherited when selection was relaxed, a fact that argues for their integration into host genomic DNA. Exposure of the selected cell lines to heat shock temperatures resulted in the regulated expression of the cointroduced heat shock loci at the level of both RNA and protein.

These experiments, the first example of the regulated expression of a nonselectable cotransfected gene stably introduced into *Drosophila* cultured cells, demonstrate the feasibility of using *Drosophila* cells as a system for the analysis of the regulated expression of genes of interest. Moreover, because of the lack of sequence homology between *D. hydei* and *D. melanogaster*, the use of *D. hydei* as recipient cells allows assays for transfected *D. melanogaster* DNA and its transcribed RNA without the problem of cross-hybridization to endogenous sequences.

MATERIALS AND METHODS

Plasmid constructions. The detailed construction of pCV2gpt has been described elsewhere (27). pMH10A has been described previously (4) and is a pAT153 clone of the 87A7 locus of the Df(3R)Kar^{D1} strain of *D. melanogaster*.

Cell culture and transformation. The *D. hydei* cell line, DH33, has been described previously (28). Cells were routinely maintained in M3 medium (26). For transfection, approximately 1×10^6 to 2×10^6 cells were cotransfected with 5 μg of pCV2gpt and 15 μg of pMH10A by calcium phosphate precipitation, as previously described (11). Originally, selective medium, M3X (27), contained mycophenolic acid and adenine. More recently, however, we have found that there is no need to include mycophenolic acid in the selective medium, as *Drosophila* cells cannot salvage hypoxanthine (2, 35; Sinclair, unpublished observations). Selective medium was replaced weekly, and after 4 to 5 weeks, colonies were picked and expanded.

DNA and RNA analyses. DNA and RNA were extracted from transformed cell lines as described previously (4). Formaldehyde-denatured total cell RNA was analyzed by dot blots (18) on Gene Screen Plus (New England Nuclear Corp.). Total cell DNA was digested with restriction endonucleases (Amersham Corp.) under conditions specified by the suppliers. Restricted or unrestricted DNA was analyzed on agarose gels by Southern transfer (30) or dot blots (18) on nitrocellulose.

Filters were hybridized in 50% formamide–9% dextran sulfate for 24 to 36 h at 42°C (32).

Analysis of heat shock proteins. Cells were labeled with [^{35}S]methionine as described previously (1), except that the labeling medium was M3 with 10% heat-inactivated serum but lacking yeastolate, lactalbumin hydrolysate, and methionine. Total cell proteins were extracted and analyzed by sodium dodecyl sulfate-gel electrophoresis on 10% polyacrylamide gels (13) and detected by fluorography with Amplify (Amersham).

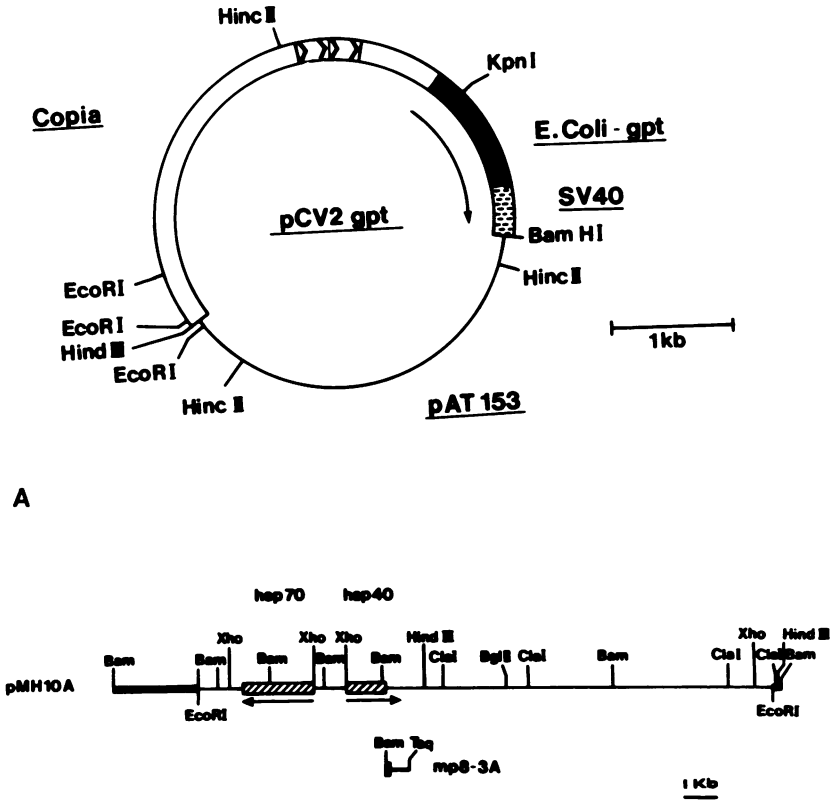

FIG. 1. Plasmid restriction maps. (A) Restriction map of pCV2gpt. Construction of the pCV2gpt vector has already been described in detail elsewhere (27). ▭, copia DNA sequences in BB5(10); ▭, long terminal repeat copia, indicating the direction of transcription from the copia promoters; ▬, *E. coli gpt* gene; ▭, simian virus 40 (SV40) sequences containing the small antigen splice sequences and the poly(A) addition site. The thin line represents pAT153. The copia sequences contain an origin of replication, and the long terminal repeats contain the copia promoters. (B) Restriction map of heat shock plasmid pMH10A and subclones. Plasmid pMH10A contains the mutant heat shock locus from Df(3R)kar^{D1}. ▬, vector sequences; ⊢⊣, *Drosophila* genomic sequences; ▨, hsp-70 coding sequences; →, direction of transcription. Subclone mp83A includes 60 nucleotides of hsp-70 coding region proximal to the deletion breakpoint. This subclone is cloned into M13mp8.

RESULTS

Cotransfection of the Df(3R)Kar^{D1} hsp-70 locus into *D. hydei* cells. The pCV2gpt plasmid (Fig. 1A) carries the *Escherichia coli gpt* gene under the control of copia long terminal repeats. The pMH10A plasmid (Fig. 1B) carries a variant of the *D. melanogaster* 87A7 locus for the 70,000-molecular-weight heat shock protein (hsp-70) differing from the wild-type (which has two back-to-back hsp-70 genes, each encoding a 2.4-kilobase [kb] transcript) due to a chromosomal deletion, Df(3R)Kar^{D1}. This deletion truncates one of the hsp-70 genes to give a 1.8-kb transcript, encoding an hsp-40 initiated at the original hsp-70 promoter (4).

D. hydei DH33 cells transfected with equimolar amounts of the two plasmids were selected for *gpt* expression in HAT (hypoxanthine-aminopterin-thymidine) medium. Clones were established at a frequency of 5×10^{-4} to 10×10^{-4}, and the cells grew well, with a doubling time of 24 h.

pCV2gpt and pMH10A sequences in transfected lines. Total cell DNAs from several cotransfected lines were analyzed for plasmid sequences by Southern hybridization. Transfected lines contain multiple copies of pCV2gpt (Fig. 2A), and none are found in control, untransfected cells (Fig. 2A, track C). From the intensity of hybridization to the tracks and to the DNA dot blots (Fig. 2B), we estimate that the different cell lines contain from 50 to 200 copies of pCV2gpt. These sequences are associated only with high-molecular-weight, genomic DNA (Fig. 2A, tracks 1 through 6), suggesting that either the transfected DNA has formed large multimers, indistinguishable from genomic DNA, or that these multimers have integrated into the host cell chromosomes.

The selected cell lines maintain both plasmids in the absence of selection (data not shown), which argues strongly for plasmid integration in the genome, as is already found when the same plasmids are transfected into *D. melanogaster* cells (5).

In most of the transfected cell lines (Fig. 2A, tracks 2 through 6), the predominant *Eco*RI and *Bam*HI fragments (8.0 and 8.4 kb, respectively) are indistinguishable from the pCV2gpt vector (Fig. 1A). The fact that *Bam*HI cuts pCV2gpt only once suggests tandemly arranged pCV2gpt molecules in these lines. Track 1 (which shows cell line 1) differs from the other tracks, since the 8.0- and 8.4-kb fragments are not the major bands and must therefore be arranged differently. Likewise, the many other minor bands may represent either rearranged pCV2gpt molecules, terminal fragments of tandem arrays, or multiple, individual inserts of pCV2gpt.

Rehybridization of these filters with mp83A, an M13 subclone of the 87D3,4 region of the Df(3R)Kar^{D1} breakpoint

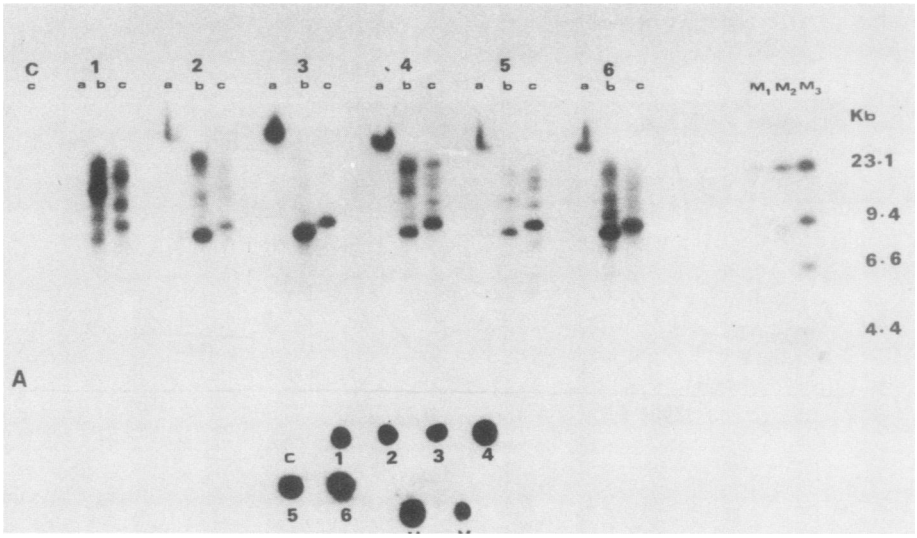

FIG. 2. pCV2gpt in transformed cells. (A) Approximately 2 to 5 μg of DNA from control untransformed cells (track C) and six transformed cell lines (tracks 1 through 6) were either left undigested (lanes a) or digested with *Eco*RI (lanes b) or *Bam*HI (lanes c) and separated on an 0.6% agarose gel. After transfer to nitrocellulose, the filter was probed with the *Hin*dIII-*Bam*HI fragment of pCV2gpt (21) under stringent conditions. This fragment is specific for the *E. coli gpt* gene. Markers are 0.1 (M_1) and 0.5 (M_2) ng of pCV2gpt and *Hin*dIII-digested λ DNA (M_3). (B) DNAs from transformed cells (dots 1 through 6) and control untransformed cells (c) were also analyzed by dot blots. Approximately 5 μg of DNA in 5 μl of 0.4 M NaOH–1 M NaCl–1 mM EDTA was heated at 100°C for 3 min and spotted onto nitrocellulose which had been presoaked in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and dried. After being baked, the filter was probed with the same *E. coli gpt* insert as in panel A under stringent conditions. Markers are 100 (M_1) and 1.0 (M_2) ng of pCV2gpt.

(Fig. 1B), shows that the cell lines have also taken up the pMH10A sequences (Fig. 3A). As with pCV2gpt, the plasmid is associated with high-molecular-weight DNA (Fig. 3A, tracks 1 through 6), and the intensity of hybridization and the DNA dot blots (Fig. 3B) show that the cell lines carry between 50 and 200 copies of pMH10A. Despite the differences in copy number between cell lines, it seems that individual lines contain nearly equal numbers of the two plasmids. No hybridization was observed in the DH33 control track, indicating the lack of homology between the probe and *D. hydei* DNA under stringent conditions.

The major pMH10A-containing genomic DNA fragments in all the cell lines are similar to the input DNA (Fig. 3). *Eco*RI and *Bam*HI digestion gives 20.0- and 8.4-kb fragments, respectively, corresponding exactly to the fragments expected from the plasmid (Fig. 1B). Thus, all the trans-

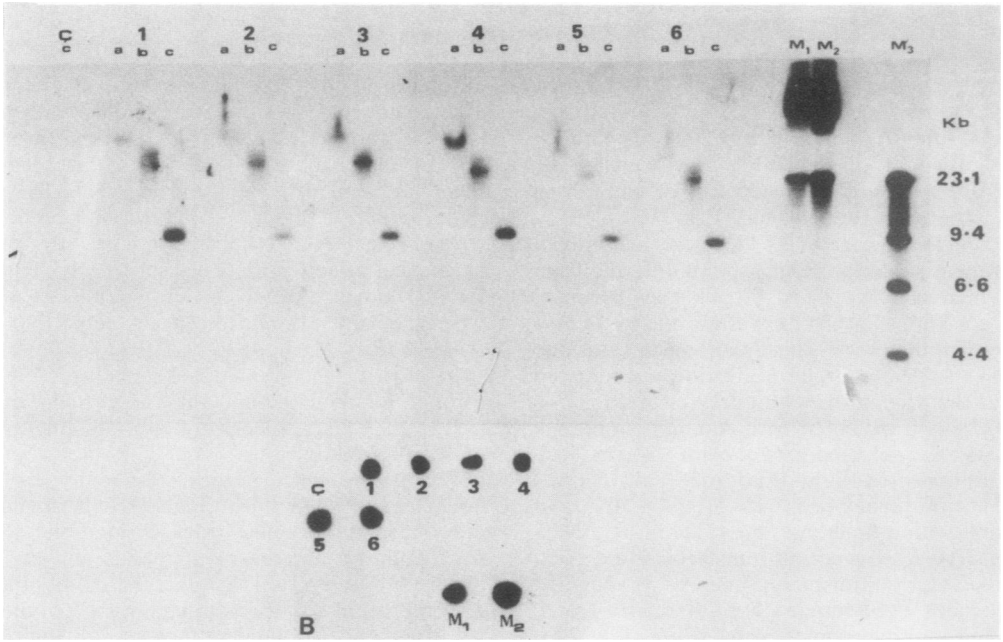

FIG. 3. pMH10A in transformed cells. The probe was removed from the nitrocellulose filters, and the filters were rehybridized with mp83A (Fig. 1) under stringent conditions. The markers in Fig. 3A are 30 (M_1) and 60 (M_2) ng of pMH10A and *Hin*dIII-digested λ DNA (M_3). For Fig. 3B, the markers are 3 (M_1) and 15 (M_2) ng of pMH10A.

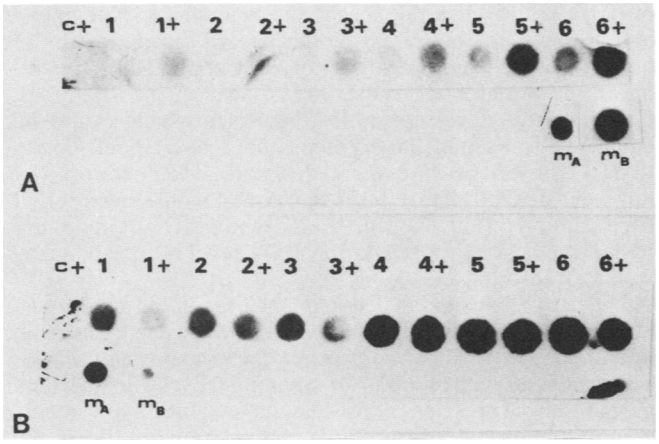

FIG. 4. RNA dot blot analysis in transformed lines. (A) Total cell RNA (5 μg) from untransformed control cells after heat shock (lane C+) at 37°C for 1.5 h or from transformed lines before (lanes 1 through 6) or after (lanes 1+ through 6+) heat shock were denatured (18) and spotted onto Gene Screen Plus (New England Nuclear) membrane. After being baked, the membrane was hybridized to nick-translated mp83A (Fig. 1) and autoradiographed. Markers are 3.0 (m_A) and 15 (m_B) ng of pMH10A. (B) After removal of probe, the same filter was rehybridized to the nick-translated *E. coli gpt* gene insert of pCV2gpt. Markers are 1.0 (m_A) and 0.1 (m_B) ng of pCV2gpt. Note that there is an underloading of RNA in dots 1+ and 3+ compared with their equivalent unshocked samples (dots 1 and 3).

formed cell lines also contain tandemly arranged pMH10A molecules. This fact suggests that the two plasmids are predominantly integrated as separate concatemers. Restriction analysis with enzymes that have single cut sites in one plasmid but not the other confirm this arrangement (data not shown).

Regulated expression of pMH10A RNA in transformed cells. To determine whether the transformed cell lines were expressing the transfected heat shock genes, total cell RNAs from the transformed *D. hydei* lines were analyzed for pMH10A RNAs by dot blots. Heat shock induces increased pMH10A expression in all the transformed cell lines, with as much as a 50-fold increase in some (Fig. 4A, lines 5 and 6). It is difficult to know, however, if there is constitutive expression of pMH10A in some lines (lines 5 and 6), or if this low-level production results from the stress of the experimental manipulation. In contrast, hybridizing the same filter with a *gpt*-specific probe (Fig. 4B) shows that the levels of *gpt* RNA are the same before and after heat shock. The response to heat shock is a property only of the genes encoded by the pMH10A plasmid.

Although roughly equal amounts of RNA were loaded in each sample, the levels of hybridization with the *gpt* probe varied among the cell lines, though this variation could be correlated with their pCV2gpt copy number (Fig. 2B, lanes 1, 2, and 3; compare lanes 4, 5, and 6). This correlation, however, was not observed for levels of pMH10A RNA and pMH10A copy number (Fig. 4A); for example, lines 4 and 6, though containing roughly equal copies of pMH10A, contained about a 20-fold difference in levels of pMH10A RNA. Northern and S1 analyses confirmed the regulated expression of the pMH10A RNA and showed the RNA to be of the correct size (data not shown).

Transformed cells synthesize an hsp-40. The heat shock proteins of *D. hydei* have been well characterized elsewhere (29) and include a major 70,000-molecular-weight species indistinguishable at this level from the *D. melanogaster* hsp-70. Consequently, we can distinguish only the hsp-40 of the Df(3R)Kar^{D1} plasmid when transformed *D. hydei* cells are conventionally heat shocked. As expected, a typical heat shock response is obtained in control DH33 cells, showing an increase in the expression of heat shock proteins and a concomitant decrease of all other proteins synthesized (Fig. 5A, lanes C and C+). The hsp-40 is easily identifiable in the transfected cell lines tested (lanes 3+ and 5+) but is absent in the unshocked cells (lanes 3 and 5), as well as in the controls. A qualitative inspection of the autoradiograph suggests that cell lines 3 and 5, which contain different levels of pMH10A RNA, make similar amounts of hsp-40. Similarly, cell line 6 (Fig. 5B), which expresses high levels of pMH10A, does not make correspondingly higher levels of hsp-40 than do cell lines 3 and 4, which express much lower amounts of pMH10A RNA. These facts suggest that there is

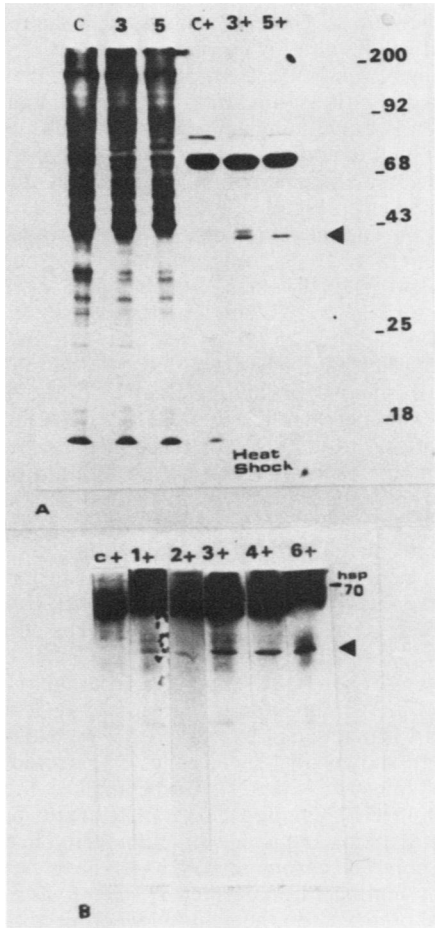

FIG. 5. hsp-40 in transformed cells. (A) Total cell protein of untransformed control DH33 cells (lane C) and transformed lines 3 and 5 before (lanes C, 3, and 5) and after (lanes C+, 3+, and 5+) heat shock at 37°C for 1.5 h were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, fluorographed, and exposed to X-ray film at −70°C. Molecular mass markers are in kilodaltons, and ◀ marks the position of hsp-40. The minor band above hsp-40 in lane 3+ is residual actin and can be seen in all heat-shocked samples (lanes C+, 3+, and 5+) under increased exposure (not shown). (B) Total cell proteins from other transformed lines after heat shock at 37°C for 1.5 h were separated on a 17.5% sodium dodecyl sulfate-polyacrylamide gel, fluorographed, and exposed to X-ray film. ◀ marks the position of hsp-40.

some repression of translation or differences in RNA stability consistent with the reported autoregulation of hsp-70 in *D. melanogaster* (8, 20). Furthermore, and also in agreement with this possibility, the transformed lines make little more hsp-70 than do the untransformed controls, although they may contain up to 200 extra copies of the hsp-70 gene.

DISCUSSION

The introduction of plasmid DNA, carrying a *D. melanogaster* truncated heat shock gene, into heterologous *D. hydei* cells by contransformation by use of another dominant selectable plasmid results in regulated expression of the cotransfected heat shock gene in all transformants analyzed. The selected lines, expressing pCV2gpt and surviving in HAT medium, are stable after many weeks out of selection (data not shown), a fact which argues for the chromosomal integration of the plasmid DNAs, as we have already shown by cloning the plasmid and genomic DNA from a *D. melanogaster* cell line similarly cotransformed (5). In the present case, up to 200 copies of both plasmids are found associated solely with high-molecular-weight DNAs. As for mammalian systems (33), both plasmids are taken up by the cells, but in various amounts. The individual cell lines contain nearly equal numbers of each plasmid, whether that number is high or low. This fact suggests that there is little difference in the efficiency of uptake and integration into cell chromosomes of plasmid sequences of up to 25 kb.

Restriction endonuclease digests of the transformed-cell DNAs generate linear, unrearranged fragments of the pCV2gpt and pMH10A sizes which would be expected if they were each separately integrated, head-to-tail concatemers. Similar tandem arrays of a dominant, methotrexate-resistant marker have been reported by Bourouis and Jarry (3) in transformed *Drosophila* Kc cells. All the transformed cell lines examined synthesize pMH10A RNA in a regulated fashion with up to a 50-fold increase in some heat-shocked lines. Although there is a general correlation between the selectable pCV2gpt copy number and RNA levels in transfected cell lines, little correlation was observed between cointroduced pMH10A and its RNA. Reconstruction experiments (not shown) suggest that transformed cell lines 5 and 6 express about 2,000 copies of pMH10A RNA per cell, or about 10 from each of the 200 copies of the plasmid they carry. *D. melanogaster* flies, on the other hand, make 10,000 copies of hsp-70 RNA per cell from their 10 copies of the gene (17). We conclude that a large part of the cotransfected DNA is inactive or expressed at a very low efficiency, as has been shown for hsp-70 genes transfected into mammalian cell lines (4, 7, 19, 22). Unfortunately, the high copy number of pMH10A in the DH33 transformants precludes an analysis of flanking sequences by cloning and in situ hybridization to polytene chromosomes, as we have carried out for a low-copy-number, transformed *D. melanogaster* cell line (5). Therefore, we do not know whether differences in chromosomal position of the integrated pMH10A result in variation in levels of expression.

A wide variability in expression levels of other cotransfected, nonselectable DNAs has also been reported routinely for cotransformed mammalian cells (12, 16, 25, 31, 34).

Nonetheless, sufficient RNA is synthesized in a regulated fashion by our transformed cell lines for us to identify the expected hsp-40 on protein gels. Expression of the truncated hsp-40 encoded by the Df(3R)Kar^{D1} insert is regulated by heat shock. We could not detect any hsp-40 in control cells or in unshocked transformed cells, even though some transformed lines (e.g., lines 5 and 6) contained detectable levels of pMH10A RNA in the absence of heat shock. One explanation for this result could be the known discrimination of heat shock ribosomes against non-heat shock RNA, resulting in the preferential translation of heat shock RNA (24). Consequently, levels of pMH10A RNA which would be efficiently translated into protein under heat shock conditions might well be outcompeted at normal temperatures by non-heat shock RNA. If this is the case, it would suggest that transcription products of the transfected pMH10A genes are under true heat shock regulation at the level of translation as well as transcription.

The use of transient and stable DNA-mediated gene transfer has been of great value in the analysis of gene expression in mammalian cells; while transient gene expression analyses have been carried out in *Drosophila* cells, systems which permit the expression of unselectable genes after stable cointroduction into *Drosophila* cells have not been described. Here we have shown how cotransfection with a dominant selectable marker can be used to introduce other DNA sequences, resulting in their regulated expression. This procedure should allow the stable introduction of virtually any DNA sequence of interest into *Drosophila* cultured cells, permitting an analysis of their structure and function.

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ADDENDUM IN PROOF

Rio and Rubin recently reported the regulated expression of a gene function in *Drosophila* cells (Mol. Cell. Biol. **5**:1833–1838, 1985).

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