Transient expression of genes introduced into cultured cells of *Drosophila*

(calcium phosphate transfection/Schneider cells/chloramphenicol acetyltransferase assay/heat shock)

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ABSTRACT   Recombinant plasmids in which the sequence encoding the bacterial chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) has been placed under the control of *Drosophila* heat shock protein 70 (hsp 70) or *copia* promoters have been introduced into cultured cells of two *Drosophila* species (Schneider II line of *Drosophila melanogaster* and *D. immigrans*) as calcium–phosphate complexes. Within 1–2 days after transfection functional CAT enzyme was detected in cells exposed to either CAT recombinant. The expression of the bacterial information depends on the activity of the *Drosophila* promoters because plasmids in which the *Drosophila* DNA fragments were fused to the CAT coding sequence in inverted orientation did not support the synthesis of CAT enzyme activity. Low levels of CAT activity and of hybrid mRNA were detected in cells transformed with hsp-cat recombinants when the cells were maintained at room temperature, and both mRNA levels and CAT activity increased substantially after a brief exposure to 37°C. hsp-cat mRNA has the same 5′ terminus as authentic *Drosophila* hsp 70 messenger. These experiments document a practical system for the introduction and expression of isolated genes in cultured cells of *Drosophila*.

Isolated genes, often subjected to modification *in vitro*, have been introduced into homologous or heterologous cells in a variety of ways to define the requirements for their expression *in vivo*. DNA sequences can be introduced into *Escherichia coli*, yeast, and various cultured cells by transformation (1–4) and have been delivered into the nuclei of *Xenopus* oocytes or mammalian cells by microinjection (5, 6). An elegant protocol has been devised to integrate any DNA segment into the germ line of *Drosophila* embryos by exploiting the mobile properties of P elements (7). To date, there has been no report of uptake and expression of exogenous DNA sequences in cultured cells of *Drosophila*, although infection of *Drosophila* cells by RNA from black beetle virus mediated by DEAE-dextran has been achieved (8).*

The chloramphenicol acetyltransferase (CAT; acetyl-CoA: chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) assay developed by Gorman *et al.* (9) offers a rapid and sensitive method to detect the functional activity of different promoter sequences. The system allows both the analysis of RNA transcripts and measurement of the activity of the protein product. Because CAT is a bacterial enzyme there is no corresponding background activity in animal cells (9). To evaluate the applicability of the CAT expression system to *Drosophila* cells we have fused two homologous promoters, the heat shock protein 70 (hsp 70) and *copia* long terminal repeat (LTR) promoters, to the CAT coding region. This paper reports experiments on the introduction of these recombinant DNA molecules into *Dro-*

sophila melanogaster and *D. immigrans* cells by the calcium phosphate coprecipitation method. Expression of the recombinant genes was observed, showing that DNA-mediated gene transfer can be used effectively to introduce and express DNA segments of interest in cultured *Drosophila* cells.

MATERIALS AND METHODS

Recombinant DNA Constructions. Segments of *Drosophila* DNA were introduced into the Sma I site of pSVO-cat. This vector is a promoterless derivative of pSV2-cat^s (9) that lacks any simian virus 40 (SV40) sequences upstream from the CAT coding region and contains a unique Sma I site 39 base pairs (bp) upstream from the AUG of the CAT gene (C. M. Gorman and B. H. Howard, personal communication; see also Fig. 4). A 1.2-kilobase (kb) *Bgl* II–*Pvu* II fragment from pPW 229, a plasmid carrying a hsp 70 gene (10), and a 1.6-kb *Eco*RI–*Apa* I fragment from cDM 5002, a plasmid carrying a copy of the *copia* element (11), were purified by gel electrophoresis. Flush ends were generated by treatment with T4 DNA polymerase (12), and each fragment was ligated to Sma I-digested pSVO-cat. Ligation products were digested with Sma I to linearize vector molecules having no inserts and were used to transform *E. coli* HB101. Ampicillin-resistant colonies containing *Drosophila* inserts were detected by colony hybridization (13). The orientations of *Drosophila* segments were distinguished by restriction analysis (see *Results and Discussion*).

Plasmid Preparation. Recombinant DNA to be introduced into *Drosophila* cells was isolated by the alkali procedure (14) or by the cleared lysis method (15) and was purified in two cycles of CsCl/ethidium bromide gradients, followed by Sepharose 6B gel filtration. Impurities affecting cell transfection present in some DNA preparations were removed by spermine precipitation (16).

Drosophila Cell Lines and Media. *D. melanogaster* Schneider II (17) and *D. immigrans* (18) cells were grown at 25°C in Falcon flasks in Schneider medium supplemented with L-glutamine (GIBCO 350-1720), containing 1% penicillin/streptomycin solution (GIBCO 600-5070) and 10% fetal bovine serum (GIBCO 200-6140) heat inactivated at 50°C for 45 min. The Schneider cells used in this work adhere weakly to the culture plates, grow partly on the surface and partly in suspension, and can be harvested by pipetting. The *D. immigrans* cells grow firmly attached to the substratum; for harvesting or replating they were released with trypsin. Schneider cells were transfected at densities ranging from 0.5 to 5 × 10^6 cells per ml, and *D. immigrans* cells were used before reaching confluence.

Abbreviations: CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; hsp, heat shock protein; RSV, Rous sarcoma virus; bp, base pair(s); kb, kilobase(s); SV40, simian virus 40.
* See *Note Added in Proof*.

Drosophila **Cell Transfection.** Aliquots of 0.3–0.5 ml of 0.25 M CaCl$_2$, containing 5–30 μg of purified plasmid DNA, were added dropwise to an equal volume of 2× HeBS [2× HeBS contains (in g/liter): NaCl, 16; KCl, 0.7; Na$_2$HPO$_4$, 0.4; dextrose, 2; Hepes, 10, at pH 7.1] in a 35-mm tissue culture dish that was gently rocked by hand. After 30–60 min at room temperature the suspension of calcium–phosphate complexes was gently pipetted into the flasks, which contained 5 ml of cell culture. The medium was not changed before or after the addition of DNA, and the cultures were left undisturbed until harvest 1–5 days later.

CAT Assay. Cells were harvested, washed once in saline, and suspended in 0.25 M Tris·HCl (pH 8.0). The cells were broken by sonication, and debris was removed by centrifugation for 5 min in a Microfuge. CAT activity was assayed in aliquots of the supernatant as described (9).

RNA Preparation. Total RNA was purified from cultured cells by phenol/chloroform extraction or lysis in guanidine/CsCl (19). Samples were treated before hybridization with 10 μg of RNase-free DNase per ml at 37°C for 60 min.

S1 Mapping. Total cellular RNA (20 μg) was hybridized at 50°C for 6–18 hr to 5′-end-labeled DNA probes in 0.01 M Hepes, pH 7.2/1 mM EDTA/0.3 M NaCl (single-stranded probe) or in 0.04 M Pipes, pH 6.4/1 mM EDTA/0.4 M NaCl/80% (vol/vol) formamide (double-stranded probe). In the latter case samples were heated at 75°C for 15 min and quickly transferred to 50°C. Samples were diluted with 10 vol of S1 buffer (0.3 M NaCl/0.05 M sodium acetate, pH 4.8/1 mM ZnSO$_4$) and treated with different amounts of S1 enzyme. After ethanol precipitation samples were loaded in 80% formamide on 5% polyacrylamide/7 M urea gels (20).

RESULTS AND DISCUSSION

hsp-cat and *copia*-cat Constructions. To obtain DNA molecules suitable for introduction and expression in *Drosophila* cells, we used two strong homologous promoters linked to the CAT coding region. The hsp and *copia* DNA segments used contain RNA start sites (cap sites) and long 5′ flanking sequences. The hybrid genes are expected to initiate RNA transcription in the *Drosophila* sequences but should initiate translation at the AUG provided by the bacterial CAT gene.

The major hsp of *D. melanogaster*, hsp 70, is encoded by several genes located at chromosome positions 87A and 87C (21). The recombinant plasmid pPW 229, which contains one entire hsp 70 coding sequence from position 87C and 1.1 kb of 5′ flanking region, can direct the synthesis of hsp 70 when introduced into mouse L cells by transfection (10). A fragment of pPW 229 containing the hsp promoter and extending 65 bp beyond the cap site has been inserted into pSVO-cat in both orientations (Fig. 1; see also Fig. 4).

Copia is a well-characterized family of transposable elements in *D. melanogaster*, originally isolated as a class of middle repetitive DNA sequences coding for abundant polyadenylylated mRNA (22). Transcription of the two major *copia* RNA classes has been shown to initiate in the left LTR (11). We have derived from cDM 5002, a clone containing a complete *copia* element (11), a fragment containing 1.3 kb of upstream flanking DNA and the intact left *copia* LTR and inserted this fragment in both orientations into the *Sma* I site of pSVO-cat (Fig. 1).

All recombinant DNA molecules used in this work contain SV40 sequences 3′ to the CAT coding region, carrying the small t intron and the early region polyadenylylation site (9). Because all of our constructs contain this region we do not know whether they affect the expression of CAT in *Drosophila* cells. We note that the constructs do not contain SV40 sequences 5′ to the CAT
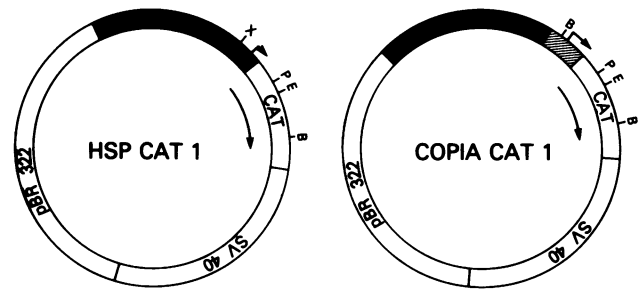

FIG. 1. *Drosophila* DNA segments cloned into pSVO-cat. Solid bars correspond to *Drosophila* DNA inserted at the unique *Sam* I site located 39 bp from the AUG of the CAT gene in the vector (see also Fig. 4D). Stippled regions in *copia*-cat refer to the *copia* LTR. Only the recombinants in which hsp and *copia* sequences are inserted in the correct orientation to direct the synthesis of hybrid *Drosophila* cat messages are shown. These recombinants are named hsp-cat 1 and *copia*-cat 1. Recombinants containing *Drosophila* segments in opposite orientations have been distinguished by *Xho* I–*Pvu* II (hsp-cat) or *Bal* I (*copia*-cat) digestion and are named hsp-cat 2 and *copia*-cat 2. Small arrows indicate the orientation of hsp and *copia* transcripts, and large arrows indicate the translational orientation of the CAT coding region. B, *Bal* I; E, *Eco*RI; P, *Pvu* II; X, *Xho* I.

region and, in particular, do not contain SV40 promoter or enhancer sequences in any position.

Transfection into Cultured *Drosophila* Cells. The hsp-cat and *copia*-cat recombinants were introduced into *D. melanogaster* Schneider II cells by calcium phosphate transfection, and CAT activity was assayed in cellular extracts after 48 hr (Fig. 2). hsp 70 and *copia* promoters are able to direct the synthesis of functional CAT enzyme only when fused to the CAT structural sequences in the proper orientation to promote synthesis of hybrid messages (lanes a and c). No CAT activity is detected in normal control cells (lane g) and in cells transfected with pSVO-cat (lane f) or with constructs carrying inverted promoter orientations (lanes b and d). CAT expression also can be directed in these cells by pRSV-cat (lane e). In pRSV-cat the CAT gene has been placed under transcriptional control of the Rous sarcoma virus (RSV) LTR promoter, and elevated levels of CAT have been measured in a variety of mammalian and avian cells
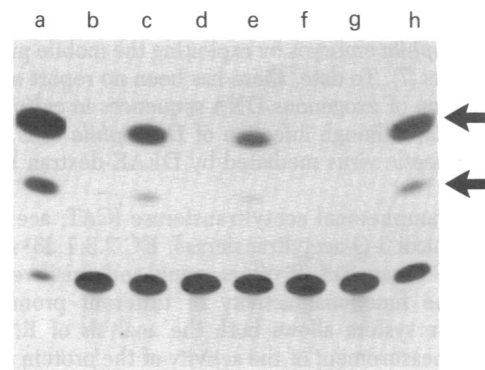

FIG. 2. Assay of CAT activity in *D. melanogaster* cells. CAT was measured in equal aliquots of Schneider II cell extracts 48 hr after transfection with hsp-cat 1 (lane a), hsp-cat 2 (lane b), *copia*-cat 1 (lane c), *copia*-cat 2 (lane d), and RSV-cat (lane e). As controls, extracts from cells transfected with SVO-cat (lane f) and from untransfected cells (lane g) were run; purified CAT enzyme (0.3 unit, P-L Biochemicals) was run as a positive control (lane h). The CAT assay was carried out at 37°C for 30 min as described (9). The reaction products were separated by ascending thin-layer chromatography separating two acetylated products (indicated by arrows), which together provide a measure of enzyme activity, from the slowly migrating unmodified chloramphenicol (9).

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transfected with this recombinant (23).

Functional CAT enzyme has been detected in hsp-cat 1 transfected cells within 12 hr after transfection. Enzymatic activities similar to those detected after 48 hr have been assayed up to 5 days after transfection.

Cultured cells of *D. immigrans* were transfected with hsp-cat 1 in the same way used for Schneider cells or after release of the cells from the substratum by trypsin treatment. In either case, CAT activity was measured in these cells 2 days after transfection at levels comparable to those in *D. melanogaster*.

CAT Synthesis Directed by the hsp 70 Promoter Is Heat Shock Regulated. The rapid increase of hsp transcripts and their preferential translation constitute the major feature of the heat shock response in *Drosophila* (21). Schneider II cells transfected with hsp-cat 1 DNA exhibit a low level of hsp-cat expression, which was increased substantially after heat shock (Fig. 3). This low basal level was obtained only when transfected cells were first cooled in ice, gently harvested, centrifuged in the cold, and assayed for CAT activity. When cells were collected by centrifugation at room temperature a much higher level of activity was found, with concomitantly lower heat shock stimulation ratios. This effect is probably due to the fact that the "heat shock" response is elicited by various forms of stress, including anoxia (21), and that certain harvesting conditions stimulated hsp-cat expression. Whether the basal level measured under the conditions of Fig. 3 represents true constitutive activity, as suggested by others (24, 25), or slight stimulation by suboptimal conditions is not known.

In the experiment of Fig. 3 CAT activity increased linearly during 90 min of recovery from a 20-min heat shock and reached a value 30 times above the basal level. This accumulation of CAT activity under the control of the hsp-cat hybrid gene allows an inference about translational control under these conditions. Heat shock regulates metabolism at both the transcriptional and translational level: not only are heat shock genes activated but heat shock mRNAs are preferentially translated while "normal" mRNAs are suppressed (21, 26, 27). During recovery from a 30-min heat shock, synthesis of normal proteins

is not detected for 120 min and resumes gradually thereafter (28). If the hsp-cat mRNA behaved like a normal mRNA it would not be expected to support linear accumulation of CAT during the first 90 min of recovery. Therefore, the results shown in Fig. 3 suggest that the hybrid mRNA exhibits the translational control properties of a heat shock message, implying that the
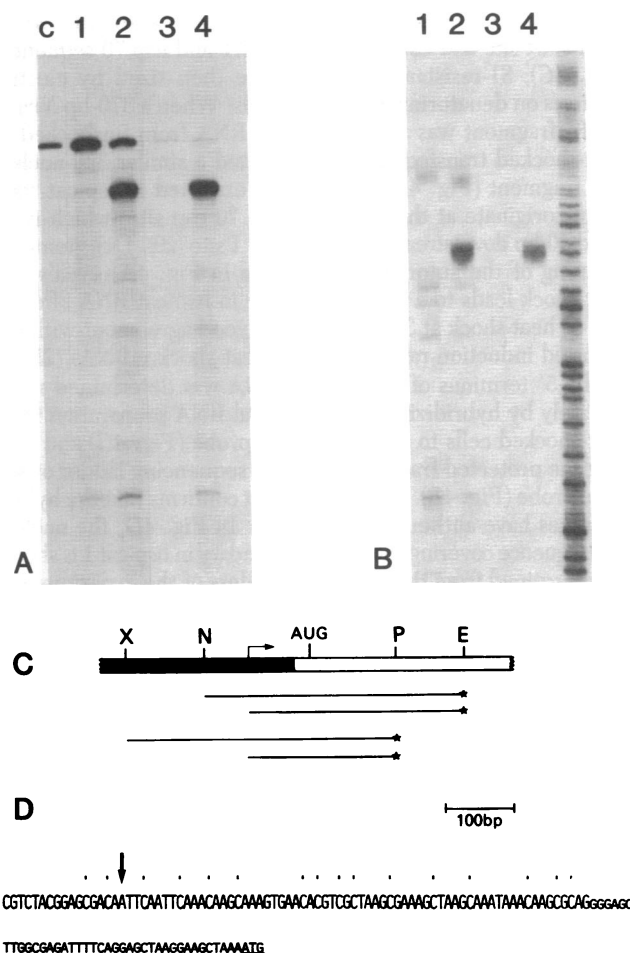
FIG. 4. S1 analysis of hsp-cat RNA from transfected cells. (*A*) Twenty micrograms of total RNA from untransfected Schneider cells (lane c), transfected cells (lanes 1 and 3), and transfected and heat-shocked cells (lanes 2 and 4) was hybridized to a 5′-end-labeled *Eco*RI–*Nru* I fragment prepared from hsp-cat 1 DNA (see *C*). The hybridization reactions were incubated with 500 units of S1 (lanes 1 and 2) or 1,500 units of S1 (lanes 3 and 4) for 40 min at 25°C. S1-resistant hybrids were sized on a 5% acrylamide/7 M urea gel. The RNA was extracted 48 hr after transfection of Schneider II cells with hsp-cat 1 DNA. When indicated, cells were heat shocked for 40 min at 37°C before RNA extraction. (*B*) The same RNA preparations from hsp-cat 1 transfected cells were hybridized to a 5′-end-labeled *Pvu* II–*Xho* I fragment from hsp-cat 1 (see below), and the hybrids were treated with two different S1 concentrations as in *A*. The protected DNA fragments were separated on a 6% acrylamide/7 M urea gel, next to a G-specific sequencing ladder obtained by partial chemical cleavage of the probe (20). (*C*) A map of the region is given and the S1 probes prepared from hsp-cat 1 DNA are indicated. *Drosophila* hsp 70 sequences are shown as solid bars and an arrow indicates the hsp 70 RNA start site (29). The AUG of the CAT protein and the restriction sites defining the two probes are indicated (X, *Xho* I; N, *Nru* I; P, *Pvu* II; E, *Eco*RI). The 5′ label is indicated by asterisks, and the length of the protected S1 fragment is shown below each probe. (*D*) The nucleotide sequence of the hsp/cat junction is shown. *Drosophila* hsp sequences are in uppercase letters; bacterial sequences are in lowercase letters. The AUG of the CAT mRNA is underlined, and an arrow indicates the 5′ terminus of hsp 70 transcripts. Dots above the sequence indicate the C residues complementary to the G ladder shown in *B*.
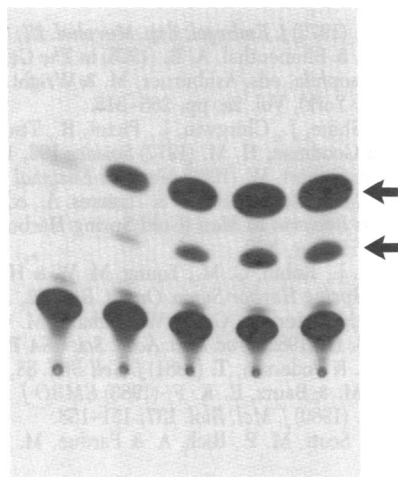

FIG. 3. Time course of CAT synthesis after heat shock. Schneider II cells transfected with hsp-cat 1 DNA were heat shocked 48 hr after transfection at 37°C for 20 min and returned to 25°C. Samples were removed after 5, 45, 90, and 180 min, and CAT activity was measured on equal aliquots of cell extract (lanes 2–5, left to right). The far left lane shows the CAT activity of similarly transfected cells not exposed to heat shock. Arrows indicate reaction products (see legend to Fig. 1). Reaction product accumulated linearly during 90 min of the recovery period and then reached a plateau.

terminal 65 nucleotides of the 5'-untranslated portion of the hsp 70 mRNA may be sufficient to secure selective translation of the hybrid RNA during heat shock.

Analysis of 5' Termini of hsp-cat Transcripts. To analyze whether the hsp-cat hybrid RNA directing the synthesis of CAT in cultured cells has the same 5' terminus as normal hsp 70 RNA, total RNA from hsp-cat 1 transfected cells was hybridized to different 5'-end-labeled probes spanning the authentic hsp 70 RNA start site and containing both CAT and hsp 70 sequences (Fig. 4C); S1-resistant products were then sized by electrophoresis on denaturing acrylamide gels. When a 370-bp *Nru* I–*Eco*RI fragment was used as probe, RNA from unshocked or heat-shocked transfected cells protected a similar 320-nucleotide fragment (Fig. 4A). This size is expected if hsp-cat transcripts originate at the authentic hsp 70 cap site, which is located 50 bp downstream from the *Nru* I site (29). Densitometric scanning of the autoradiogram shown in Fig. 4A reveals that heat shock leads to a 58-fold increase in hsp-cat RNA after 40 min of heat shock at 37°C, a value in good agreement with the reported induction ratio of normal heat shock mRNAs (21).

The 5' terminus of the hsp-cat RNA was determined more precisely by hybridizing the same total RNA preparation from heat-shocked cells to a *Xho* I–*Pvu* II probe (Fig. 4C) and running the protected fragment next to a sequencing ladder of the same probe (Fig. 4B). This experiment confirms that the hybrid messages have authentic 5' hsp ends. In Fig. 4D, the nucleotide sequence covering the hsp-cat boundary in hsp-cat 1 is shown as determined from the additional ladders of the experiment of Fig. 4B. The sequence confirms the structure of hsp-cat 1 deduced from its construction.

Conclusion. These experiments demonstrate that calcium phosphate coprecipitation can be used effectively to achieve DNA-mediated gene transfer into cultured cells from *Drosophila*. Two cell lines from different species and with different *in vitro* growth habits were used successfully, suggesting that the procedure should be applicable to many cell lines from *Drosophila* and perhaps insects in general.

We have used the CAT marker system as a convenient way to measure expression of different promoters introduced into *Drosophila* cells. One heterologous promoter tested, the LTR from RSV, did function in Schneider cells but less strongly than the homologous hsp promoter. This may be a function of evolutionary distance because the RSV LTR has been shown to be a particularly effective promoter in mammalian and avian cells (23). The LTR from the repetitive element *copia* was active in this system but also was less effective than the hsp promoter, which, in its heat shock induced form, proved to be the strongest promoter tested.

The regulation of transcription of the hsp 70 gene of *Drosophila* has been studied extensively in heterologous systems, leading to the conclusion that a short upstream region is responsible for both promoter action itself and induction by heat shock (30). The present report shows that regulation is also seen after introduction of the hsp 70 promoter into homologous cells. In our experiments, as in those of some other workers (24, 25), a basal level of hsp 70 expression was observed; heat shock led to stimulation of mRNA production up to 58-fold and CAT accumulation up to 27-fold. The significance of the basal level of expression is difficult to assess because it cannot easily be excluded that any particular cell population has not experienced some stress before assay.

Finally, these experiments provide preliminary information on the region of the hsp 70 mRNA that is required for its selective translation during heat shock. It appears that the 65 5'-terminal nucleotides may be sufficient to allow selective translation. The homologous transformation-expression system described here should help in the further analysis of this question.

Note Added in Proof. DNA-mediated transformation of *Drosophila* cells has been reported recently by Bourouis and Jarry (31).

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