
High efficiency expression of transfected genes in a *Drosophila melanogaster* haploid (1182) cell line

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ABSTRACT

Drosophila tissue culture cells have been important in the study of homologous promoters and more recently in the study of mammalian transcriptional factors such as CTF and SP1 which bind and stimulate transcription from transfected genes. In this paper we show that a *Drosophila melanogaster* haploid cell line (1182-4), not previously used for transfection studies, is capable of taking up and expressing DNA without the use of a facilitating agent such as calcium phosphate. Furthermore expression from a variety of *Drosophila* promoters such as cop*ia*, heatshock and rudimentary as well as a mammalian promoter RSV-LTR, show between 20 and over 100 times more activity in 1182-4 cells than in *D.hydei* DH33 or *D.melanogaster* S3, or D1 cell lines. This cell line should prove to be particularly useful for the analysis of weak promoters and heterologous transcription factors.

INTRODUCTION

The introduction of reporter genes linked to regulatory sequences into cultured cells has proved to be a powerful method for analysing sequences required for the transcriptional regulation of eukaryotic genes. Analysis of promoters by this method requires that the cell line used is capable of taking up and directing expression of adequate levels of the reporter gene.

Transfection studies with different mammalian tissue culture cell lines, using conventional calcium phosphate precipitation procedures to introduce DNA into cells, has demonstrated marked differences in the ability of different cell lines to take up and express plasmid DNA (1). These differences could be attributed to a number of factors such as the efficiency of DNA uptake into the cytoplasm and transfer into the nucleus; efficiency of transcription; and factors effecting mRNA stability, transport, and translation. Thus frequently only expression from strong ubiquitous promoters such as heat shock or retroviral promoters can be analysed in this way.

Methods to increase the level of uptake and expression of transfected DNA in mammalian cells have been tried. These include addition of

polyethylene glycol (2), glycerol (1,3), dimethyl sulfoxide (4), sodium butyrate (5) or chloroquine (6) after a calcium phosphate transfection procedure. Diethylaminoethyl (DEAE)-dextran mediated DNA transfection (7), scrape loading (8) and electroporation (9) have all been used successfully for transient expression of DNA in mammalian cells.

None of these methods has previously been reported to work in *Drosophila* tissue culture cells; though recently 20-hydroxyecdystone has been shown to increase the levels of transient gene expression 4-5 fold in *Drosophila* cells (10). However this method might not be generally useful since ecdysterone has been shown to induce many cellular genes (11) which could effect the expression of transiently transfected DNA.

In this report we describe a haploid *Drosophila melanogaster* cell line (12) that expresses transfected genes between 20 and over 100 times more efficiently than any other *Drosophila* cell lines. We demonstrate how this cell line can be used to study expression from a weak *Drosophila* promoter. The availability of such a cell line will allow the analysis of other genes with weak promoters which in other cell lines may fail to produce assayable levels of activity from a reporter gene.

MATERIALS AND METHODS

Plasmids

The detailed construction of pCV1cat (13) and pRSVcat (14) have been described previously. The prcat5.3 plasmid is the rudimentary gene promoter fused to cat through a short polylinker and inserted into the pBS(+) plasmid. The r promoter consists of a XhoI-SmaI fragment, including the first 17 codons of the protein open reading frame and about 5kb of 5' flanking genomic DNA (15). The r reading frame remains open through 24 codons of a polylinker sequence to the in-frame ATG that is the normal translation initiation codon of cat (16). The cat sequence is a 2.2kb HindIII/BamHI fragment from pRSVcat. The pΔcat plasmid is essentially the prcat5.3 plasmid from which the r promoter sequences have been deleted; thus, pΔcat is a cat insert into the pBS(+) vector without known promoter sequences. Details of the construction of prcat5.3, pΔcat and numerous related plasmids will be described elsewhere (in preparation).

The pHScat plasmid was constructed by replacing the HindIII/BamHI gpt containing fragment of pHSgpt (17) with a HindIII/BamHI cat containing fragment from pRSVcat.

DNA preparation

Plasmids were prepared by alkaline lysis and purified on a CsCl gradient (18).

Drosophila cell lines

The cell lines used in these experiments have been described previously (12,19). These include a *D.hydei* DH33 cell line and *D.melanogaster* cell lines S3, D1, as well as two haploid cell lines 1182-4, 1182-6, which are sub-lines derived from the same primary culture. These latter two cell lines differ considerably in their growth properties. The 1182-4 cell line grows in clumps, sending out processes and appears to depend upon cell-cell contact and therefore does not clone well. The 1182-6 sub-clone resembles more closely a diploid cell line and plates well at low densities. All cells were maintained routinely at 25°C as monolayer cultures in M3 medium (20) and media changed every 5-7 days.

Drosophila cell transfection

Cells were transfected either by a calcium phosphate procedure or by adding DNA directly to the cells in serum-free media.

(i) Calcium Phosphate Transfection method

Duplicate dishes for each assay were plated at a density of approximately $5-10 \times 10^6$ cells in 3 ml complete media in 60 mm petri dishes. To each dish 5 μ g DNA in a 0.5 ml calcium phosphate precipitate was added [Calcium phosphate precipitates were prepared essentially as described (23), except CaCl_2 was added at a final molarity of 0.125M]. After an overnight incubation, the media and precipitate were removed and replaced with 3 ml fresh media. Three days were allowed for the cells to recover and express the transfected DNA. To harvest the cells, media was removed and the cell monolayers were washed with phosphate buffered saline (PBS). Cells from a single plate were then suspended in 1 ml PBS by scraping, spinning (5,000g 2 min) and finally resuspended in 100 μ l 0.25 M Tris pH 7.8. Cells were lysed by three freeze-thaw cycles in liquid N_2 and a 37°C water bath, interspersed by vortexing. Lysates were collected by centrifugation for 5 min and the supernatants transferred to fresh tubes.

(ii) Non-facilitated Transfection

Actively dividing cells were set up at high density ($1-2 \times 10^7$ cells/ml) either in suspension in 0.5 ml media without serum in a 18 ml Falcon tube or as a monolayer culture in 60 mm dishes in 0.5 ml serum free media. To these 5 μ g of DNA was added. The cells in suspension were

gently agitated for 30 min and then replated in 3 ml media plus serum. The cells cultured as a monolayer were gently rocked for 30 min prior to the addition of 3 ml of media plus serum. After an overnight incubation, the cells were treated as above.

Chloramphenicol acetyl transferase assay

Enzyme assays for chloramphenicol acetyl transferase (CAT) activity were performed as described previously (21). The assay mix contained 30 μ l cell supernatant 70 μ l 0.25 M Tris HCl pH 7.8, 50 μ l H₂O, 1 μ Ci [¹⁴C] chloramphenicol (60 mCi/mmol) and 1 μ l 10 mM Acetyl CoA, and was incubated at 37°C for 90 min. The reaction rate was shown to be linear with respect to time and protein concentration and where appropriate cell lysates were diluted to ensure that the activity was within the linear range. Typically 1/10 or 1/100 dilution of the original lysates were used where appropriate. CAT activities are expressed in arbitrary units calculated as the percentage of chloramphenicol converted to the acetylated forms in 90 mins at 37°C for 30 μ l original extract corrected for the concentration of soluble protein (mg/ml). Background radioactivity, which resulted from incubation of mock transfected lysates, was subtracted before calculation of all CAT activities. Protein concentrations were determined using a standard BioRad assay. Calibration curves were constructed using BSA protein standards.

RESULTS

Differential expression of promoters using calcium phosphate transfection

Calcium phosphate precipitation is the only reported procedure that has been used to introduce DNA into *Drosophila* tissue culture cells (22, 23). We therefore used this technique to screen a number of cell lines for their ability to express transfected DNA. We chose a number of promoters which were linked to the bacterial indicator gene encoding chloramphenicol acetyl transferase (cat). These included an inducible *Drosophila* heat shock (HS) promoter (24,26), a long terminal repeat from the *D.melanogaster* retroviral like element copia (CV1) which we had previously shown to be active in a *D.hydei* cell line (17); the Rous Sarcoma Virus (RSV) long terminal repeat which shows very little activity in *Drosophila* cells (17); and as a relatively weak promoter, the *D.melanogaster* rudimentary gene promoter which directs synthesis of a non-abundant mRNA *in vivo* (25). To assay non specific transcription a promoterless cat gene was used which does not contain any obvious eukaryotic promoter sequences.

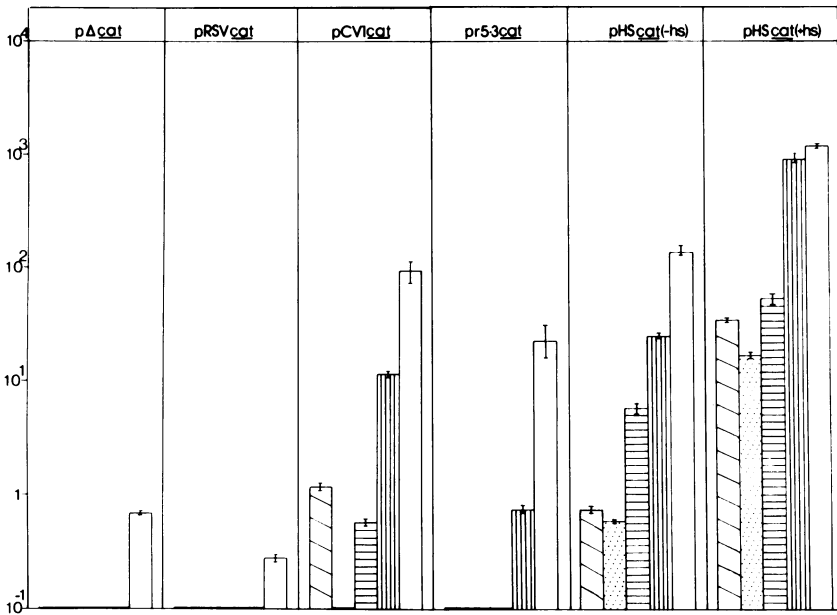


Figure 1

Activity of promoters in various cell lines. The indicated promoters linked to cat were transfected by the calcium phosphate procedure into *D.hydei* DH33, and *D.melanogaster* S₃, D₁, 1182-6 and 1182-4 cell lines. The cells were harvested after 72 hours and assayed for CAT activity as described in Materials and Methods. The histogram shows the average CAT activity (plotted on a log scale and expressed as arbitrary units) from two transfected plates. All data is from one experiment. All cell lines received an equivalent portion of a pooled calcium phosphate precipitate for each plasmid

Fig. 1 shows the relative activity (plotted on a log scale) of these promoters in five different cell lines: a *D.hydei* cell line DH33, and *D.melanogaster* cell lines Schneider S₃, D₁ and two sub-clones of the haploid cell line 1182 (1182-4 and 1182-6). Considerable differences between the haploid cell lines and other cell lines were observed in their ability to express transfected sequences. Activity from promoterless vector (pΔcat) and the RSV based vector gives very low levels of activity (less than 0.1% conversion of chloramphenicol to the acetylated forms in all cell lines except 1182-4). A single copia long terminal repeat gives low but reproducible levels of activity in S₃, D₁ and DH33 cells. However, in the haploid cell line 1182-4 the activity of the copia promoter is 160 times greater than in the best *D.melanogaster* D₁ diploid cell line.

This large increase in activity in the 1182-4 cell line is not limited to the copia LTR, since it is also apparent that the rudimentary promoter in the vector prcat5.3 is much more active in 1182-4 cells than in D1 cells.

Transfected *Drosophila* heat shock genes have been shown to be regulated in other *Drosophila* cells (17,26), and we find that this is also true for the 1182-4 and 1182-6 cell lines (Fig. 1). Interestingly, the heat-shocked 1182-6 cells expressed the heat shock promoter at levels comparable to heat-shocked 1182-4 cells, the only experimental case in which the 1182-4 cells failed to express 10- to 30-fold more activity than the 1182-6 cell line. A possible explanation is that a positively regulating cellular transcription factor (27) is limiting at these high levels of expression. In addition deletion analysis of the *Drosophila* rudimentary gene transfected into the 1182-4 cell line showed an upstream sequence required for efficient expression of this gene (manuscript in preparation). This suggests that correctly regulated transcription is occurring and that the increased CAT activity in the 1182 cell lines is unlikely to be due to non-specific transcriptional initiation from vector sequences.

Transfection in the absence of Calcium phosphate

It is possible that the increased levels of expression observed in the 1182-4 and 1182-6 cell lines is a consequence of their ability to take up DNA as a calcium phosphate precipitate more efficiently than the other cell lines. Therefore cells were transfected by the simple addition of DNA to the media without a facilitating agent. Surprisingly, both D1 cells and 1182-4 cells are capable of the regulated expression of the pHScat (data not shown) and also expressed pCV1 cat when the DNA is added to the medium in the absence of calcium phosphate (Fig. 2). The levels of expression measured when DNA is added to the cells, in the absence of calcium phosphate and serum, is generally comparable to those achieved with a conventional calcium phosphate precipitate. There is however, an inverse dependence upon cell density (unpublished observations), and whether cells are in suspension or growing as a monolayer at the time the DNA is added (Fig. 2).

Optimization of Transfection Conditions

Several parameters were investigated in an attempt to further optimize the calcium phosphate and non-facilitated DNA transfection protocols. The highest transfection frequencies with naked DNA were obtained with cells in suspension at high density $1-2 \times 10^7/\text{ml}$ in the absence of serum in the media. In contrast serum in the media in the calcium phosphate experiments

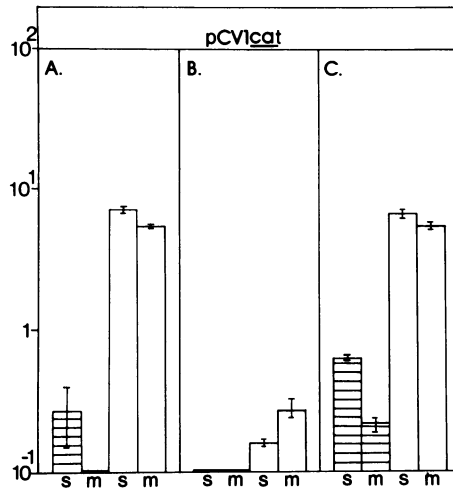


Figure 2

Non facilitated transfection of D1 and 1182-4 *Drosophila* cells. Cells, either in suspension (s) or as a monolayer (m), were transfected with pCV1cat as described in Materials and Methods, either by adding DNA to cells in serum free media (A), in serum free media plus 10% PEG 1500 (B), or as a calcium phosphate precipitate (C). The CAT activity was measured as described in Materials and Methods and expressed in arbitrary units plotted on a log scale.

appeared to be essential since absence of serum caused the almost complete disintegration of the cells after the addition of the calcium phosphate precipitate.

The effect of PEG as a facilitating agent in the transfection of DNA was also studied but, did not appear to increase expression of transfected DNA (Figure 2).

DNA uptake by 1182-4 and D1 cells

The difference in the activity of vectors in 1182-4 and D1 cells transfected with either naked DNA or as a calcium phosphate precipitate could be due to differences in DNA uptake or replication.

In order to ask whether the increase in expression of CAT activity in the haploid 1182-4 cell line was due to replication of the input DNA, total DNA was extracted from 1182-4 and D1 cells after a calcium phosphate transfection with pHScat plasmid, and analysed for replication by using *Sau3A* (which cuts DNA methylated in *E.coli*) or *MboI* (which cuts only non-methylated DNA and therefore DNA replicated in eukaryotic cells) and Southern blot analysis. Figure 3A shows that the input plasmid DNA from D1

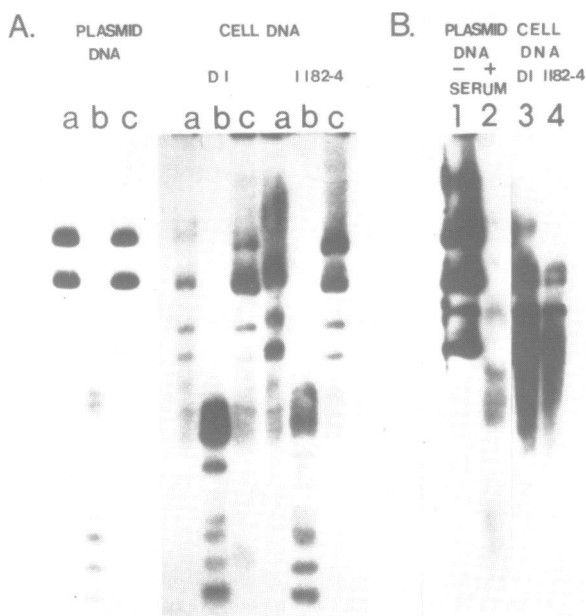


Figure 3

Southern analysis of cells transfected with pHScat plasmid DNA. (A) Equivalent numbers of 1182-4 and D1 cells were transfected with pHScat using the calcium phosphate procedure described in Materials and Methods. Total DNA was then extracted 72 hr later. Genomic DNA was either (a) undigested, (b) digested with *Sau3A*, or, (c) digested with *Mbo1*, fragments were separated on an agarose gel and then transferred to Hybond N (Amersham). (B) Non facilitated transfection of D1 and 1182-4 cells. Plasmid DNA, pHScat (5 μ g) was added to 1182-4 or D1 cells in suspension in serum free media, and incubated for 30 minutes at 23°C as described in Materials and Methods. Cells were then plated as a monolayer, the media changed and cells incubated for 24 hr at 23°C. As controls, 5 μ g of plasmid DNA was incubated for 24 hr in 1.0 ml of medium with, or without, 10% foetal calf serum. After 24 hr an aliquot (0.05ml) of media from the control (1) in the absence of serum, or (2) in the presence of serum was removed and electrophoresed undigested on an agarose gel. Total DNA was extracted from D1 (3), and 1182-4 (4) cells and also electrophoresed. All hybridizations were performed with random primed ³²P labelled pHScat.

and 1182 cells was not cut by *Mbo1* indicating that differential replication of the plasmid DNA cannot account for the increase in CAT activity in the 1182-4 cells. In addition there appears to be no degradation of plasmid DNA in D1 cells which could account for the relatively lower levels of CAT activity in the cells as compared with 1182-4 cells.

Figure 3B shows that plasmid DNA remains intact in serum free media during a 24 hr incubation (lane 1), whereas in the presence of serum the

DNA is almost completely degraded (lane 2). The intact plasmid in lanes 3 and 4 were extracted from D1 cells (lane 3) and 1182-4 cells (lane 4) 24 hr after a non-facilitated transfection (Materials and Methods). These data indicate that the plasmid is likely to be inside the cells and thus protected from exogenous nucleases. This result suggests that the high efficiency expression of transfected genes in the 1182-4 haploid cell line compared to D1 cell line is not the result of differential uptake of DNA by these cells.

DISCUSSION

Analysis of weak promoters in *Drosophila* cells has in the past proved difficult because of the low levels of expression of transfected gene fusions. Few methods of increasing the uptake and expression of transfected genes have been attempted in *Drosophila* cells, although a 4-5 fold increase in assayable activity was observed by the addition of ecdysterone (10). Though we are aware that transient transfection efficiency can vary from one experiment to another, we show here that a haploid cell line consistently gives at least a 20-fold increase in assayable CAT activity compared with other cell lines. This effect is not promoter specific and as far as we can tell the normal regulation of a gene is maintained.

This effect was not attributable to replication, nor preferential uptake of the input DNA by the haploid 1182-4 cell line, nor was it shown to be due to degradation of transfected DNA by the D1 *D.melanogaster* cell line (Fig. 3). Furthermore, we have also excluded the possibility that the effect is due to different expression times for the DNA in the different cell lines by assaying CAT activity from cells harvested 24, 48, 72 hrs after transfection. There was an increase in CAT activity over time with little difference in CAT activity between the 48 and 72 hr time points in all cell lines (data not shown). We have also tested each of the cell lines for deacetylase activity (28). No difference in CAT activity was observed between heated and non-heated extracts from any of the cell lines tested (data not shown).

The finding that DNA taken up from the medium by the 1182-4 cell line in the absence of serum can give levels of CAT activity equivalent to that for DNA added by the more conventional calcium phosphate precipitate method was surprising. Calcium is therefore unlikely to be the factor responsible

for the observed increase in assayable CAT activity in the haploid 1182-4 cells compared with the other cell lines.

It has been suggested that the main barrier between gene transfer and expression is the movement of DNA from the cytoplasm to the nucleus and the processing of the DNA in the nucleus (29). Thus the breakdown of the nuclear membrane at metaphase and reformation during telophase may be a prerequisite for high efficiency expression of input DNA. In *Drosophila* 20-hydroxyecdysterone is known to arrest *Drosophila* cells in G2 just prior to mitosis (30). So the ability of this hormone to stimulate transient gene expression might be due to an increase in the number of cells able to efficiently transport DNA into their nucleus following removal of the hormone block (10). The 1182-4 cell line is not only haploid but has been reported to have no centrioles (Dubec, personal communication) which may effect the organisation of the microtubules in the mitotic spindle. How this affects the cell cycle, entry into mitosis and nuclear membrane breakdown is not known, but might give a clue as to 1182-4 cells high efficiency expression of input DNA. Recently microtubule-dependent cell cycle regulation has been implicated in the G2 phase of cells from *Hydra* (31).

The 1182 cell line should be useful for analysis of genes with weak promoters which to date have given very little or no activity in other cell lines. We have previously transfected *D.melanogaster* D1 and *D.hydei* DH33 cell lines with human metallothionein cat and human cfos-cat genes and obtained no assayable CAT activity. Transfection of these constructs into 1182-4 cells not only gave assayable CAT activity but in the case of the metallothionein promoter the gene was shown to be copper inducible (unpublished observation).

The ability to obtain high levels of regulated gene expression in *Drosophila* cells is an important factor in identifying and analyzing transcription factors from other organisms. For example Santoro *et al.* (32) have recently shown using a Schneider cell line that *Drosophila* does not express a protein which activates transcription by binding to the promoter sequence CCAAT. However, transfected genes containing CCAAT sequences can be activated *in trans* by co-transfection with a cDNA encoding the CTF protein. The high levels of expression obtainable in 1182-4 cells may allow the detailed characterization of this and other transcription activating proteins (33). Furthermore the observation that DNA can enter a *Drosophila* cell and be expressed in the absence of any facilitating agent

will be useful in interpreting experiments aimed at understanding calcium sensitive processes such as the role of second messengers in gene activation, and avoids the problems associated with transcriptional stimulation by a calcium phosphate precipitate (34).

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