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STABLE TRANSFORMATION OF INSECT CELLS TO COEXPRESS A RAPIDLY SELECTABLE MARKER GENE AND AN INHIBITOR OF APOPTOSIS

JEANNE R. McLACHLIN AND LOIS K. MILLER[1]

Department of Entomology (J. R. M., L. K. M.) and Department of Genetics (L. K. M.), University of Georgia, Athens, Georgia 30602

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SUMMARY

We have constructed several plasmid expression vectors to express foreign genes in stably transformed insect cells. Unlike baculovirus-based expression vectors by which genes of interest are expressed transiently before lysis of the virus-infected cells, genes can be expressed continuously over many passages in a stable cell line. Furthermore, the function of a gene or genes expressed in a stable cell line from an insect-specific promoter that is constitutively expressed can be studied in the absence of virus infection and viral gene expression. In this study, we have expressed a novel, selectable marker gene, *puromycin acetyltransferase*, under the control of the *Drosophila melanogaster hsp70* promoter or under the control of the Ac*M*NPV *ie-1* promoter which is active in *Spodoptera frugiperda* cells in the absence of virus infection. In addition, we have constructed expression vectors which coexpress two genes from separate promoters, the *pac* gene which confers resistance to puromycin and a baculovirus gene which inhibits apoptosis, derived from *Orygia pseudotsugata* nuclear polyhedrosis virus. Both genes were expressed in stable populations of *S. frugiperda* cells in the absence of continuous drug selection.

Key words: Spodoptera frugiperda cells; puromycin acetyltransferase; *Drosophila hsp70* promoter; dominant selectable marker; apoptosis.

INTRODUCTION

The advantages and ease of expressing foreign genes at high levels in insect cells when recombinant baculovirus vectors are used has led to their wide use as a eukaryotic expression system (O'Reilly et al., 1992; Richardson et al., 1995; Shuler et al., 1995). In baculovirus expression vectors (BEV), foreign proteins are most commonly expressed from promoters derived from the *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) which are strongly activated during the very late phase of infection. Maximal expression from very late promoters requires not only the full complement of genes necessary for late gene expression (Todd et al., 1995; Lu and Miller, 1995), but additionally a very late expression factor, VLF-1 (McLachlin and Miller, 1994). Although expression with BEVs can potentially achieve very high levels of recombinant protein production, expression is transient due to the lytic nature of the virus. To study the function of individual genes in the absence of viral infection, investigators have been interested in generating stably transformed insect cell lines which constitutively express introduced genes using promoters known to be active in lepidopteran cell lines. Several genes have been expressed in this manner, including those encoding *Escherichia coli* β-galactosidase (Jarvis et al., 1990; Vulsteke et al., 1993), human tissue plasminogen activator (Jarvis et al., 1990), Ac*M*NPV p35 (Cartier et al., 1994) and a maize auxin-binding protein (Henderson et al., 1995). The selectable marker genes used in all of the previous studies with insect cells have been either the bacterial gene for neomycin resistance (*neo*) or the *hygromycin phosphotransferase* (*hygro*) gene, which confer resistance to G418 sulfate and hygromycin B, respectively.

In this study, we have generated and characterized stable insect cell lines using a novel selectable marker gene, *puromycin acetyltransferase* (*pac*), which confers resistance to puromycin. The *pac* gene has been used successfully as a dominant selectable marker gene in a variety of mammalian cells lines (Vara et al., 1986; Morgenstern et al., 1990; Artelt et al., 1991; Lahoz et al., 1992; Levy et al., 1993; Rastinejad et al., 1993). We have found that selection for puromycin-resistant insect cells that have been transfected with a *pac* expression plasmid is extremely rapid; toxic effects of even low concentrations of puromycin are visible within hours of drug addition. We have cultured such cell lines for over a year and monitored them at regular intervals for continuous expression of the *pac* gene for greater than 100 passages. Additionally, several stable cell lines were produced which coexpressed the *pac* gene and a gene which inhibits apoptosis. These stable lines were derived from transformation of the cells with a single plasmid expressing both genes from separate promoters, placed either in tandem or in the reverse orientation on the same plasmid expression vector. The effects of gene placement will be discussed.

MATERIALS AND METHODS

Cells. Spodoptera frugiperda IPLB-Sf21 (Sf21) (Vaughn et al., 1977) cells were maintained in TC-100 medium (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) 0.26% tryptose broth, 100

[1]To whom correspondence should be addressed at Department of Entomology, 413 Biological Sciences Building, The University of Georgia, Athens, Georgia 30602.

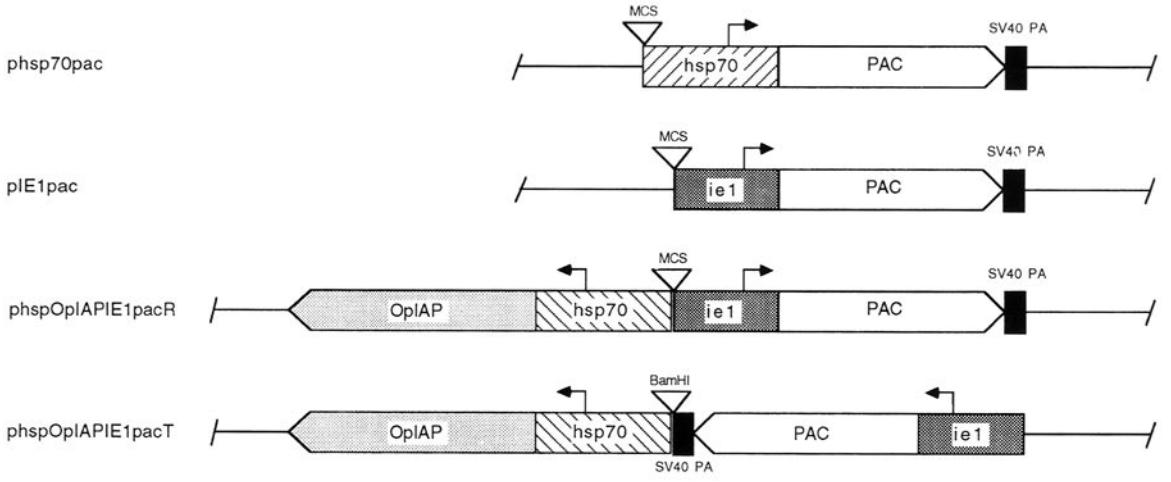

FIG. 1. Diagrams of plasmid constructs used to generate stable cell lines. The Bluescript KS$^-$ sequences are represented by the *single black line*. The direction of the genes is indicated and the transcriptional start sites are indicated by an *arrow*. *Black boxes* show the position of an SV40 polyadenylation signal (SV40 PA); MCS, multiple cloning site.

U/ml penicillin G- Na salt, 0.2 mg streptomycin sulfate per ml, and 0.5 μg amphotericin B (Sigma Chemical Co., St. Louis, MO) per ml. This medium will subsequently be referred to as "complete TC-100."

Plasmid constructs. The plasmid, pBSpacΔp (de la Luna et al., 1988), was digested with the restriction enzymes, *Hin*dIII and *Apa*I to isolate a fragment containing the *pac* sequences and a polyadenylation signal derived from SV40. The pBSpacΔp plasmid is commercially available from Clontech, Inc. (Palo Alto, CA) and is referred to as pPUR. The *pac* fragment was used to make two separate constructs, phsp70pac and pIE1pac (Fig. 1), where the *pac* coding sequence was inserted downstream of the *Drosophila hsp70* promoter or the Ac*M*NPV *ie-1* promoter, respectively. To generate phsp70pac, the plasmid, phsp70PL, which contains the *D. melanogaster hsp70* promoter and leader sequences from approximately −500 to +231 with respect to the start site of transcription (Clem and Miller, 1994) was digested with *Pst*I, filled in with Klenow polymerase and ligated to the *pac* sequence fragment which had been treated with Klenow polymerase. The *pac* fragment described above was cloned into the plasmid pBSIE1G at a *Hin*cII site, downstream of a 560-bp *Cla*I to *Hin*cII fragment of the *ie-1 promoter from* Ac*M*NPV, giving rise to pIE1pac.

The plasmids, phspOpIAPIE1pacR and phspOpIAPIE1pacT (Fig. 1) were constructed by digesting the plasmid, pHSOpIAPNruI (Clem and Miller, 1994), with *Bam*HI and inserting a *Bam*HI fragment of pIE1pac which contained the *pac* coding sequences flanked by the *ie-1* promoter and SV40 polyadenylation signal. The *Op-iap* (inhibitor of apoptosis) gene was originally isolated from *Orgyia pseudotsugata* nuclear polyhedrosis virus (Op-*M*NPV) and retains its native polyadenylation signal (Birnbaum et al., 1994) but is driven by the *Drosophila hsp70* promoter in both phspOpIAPIE1pacR and phspOpIAPIE1pacT. The orientation of the two genes in these double-expression plasmids was determined by restriction endonuclease digestion. The reverse orientation of the two genes was designated R; tandem orientation of the two coding sequences was designated T.

Isolation of stable transformed cell lines. Sf21 cells were seeded at a density of 2 × 10^6 cells per 60-mm-diameter dish and allowed to adhere for 1 h. Cells were transfected with 5 μg of plasmid DNA that had been purified on a CsCl density gradient with standard calcium phosphate coprecipitation (O'Reilly et al., 1992). Cells were transfected for 4 h, after which the medium was replaced with complete TC-100 and cultured overnight. The following day, cells were split 1:4 and the medium was substituted with complete TC-100 containing 0, 1, 2, or 4 μg puromycin (Sigma, P-7255) per ml. Control Sf21 cells were mock transfected with pBluescript KS$^+$ and treated in the same manner as cells transfected with the *pac*-expressing plasmids.

Viability in puromycin. To test the drug sensitivity of puromycin-resistant stable cell lines, some cells that had been initially selected in 2.0 μg puromycin per ml were cultured in the presence of higher concentrations of drug. Control Sf21 cells (not expressing the *pac* gene) and *pac*-expressing stable cell lines were plated at a density of 4 × 10^5 cells per 60 mm-diameter plate, in duplicate for each concentration of puromycin tested: 0, 2.0, 6.0, 8.0, and 10.0 μg/ml. Cells were examined daily and viable cells were counted after 72 h of continuous culture in puromycin. A portion of the cells were reseeded at low density (4 × 10^5 cells per 60 mm-diameter plate) and continued growth was monitored in each concentration of puromycin for two additional passages. The percentage of cells surviving after the initial 72 h of culture in all concentrations was scored according to the percentage of cells surviving in the absence of puromycin selection.

Enzyme assay. The activity of puromycin *N*-acetyltransferase (PAC) was assayed from whole cell lysates. Sf21 cells were counted and 2 × 10^6 stably transfected cells were collected for lysis in 100 μl of 0.25 *M* Tris-HCl (pH 8.0) by repeated freeze/thawing. Cell lysates were centrifuged in a microfuge at 4° C for 5 min and the supernatants were transferred to a fresh tube for direct assay or placed at −80° C for storage. PAC activity was assayed according to the method outlined by Lahoz et al. (1991). Briefly, an acetyl CoA mix was made fresh for each set of assays, which for 10 assays contained 50 μl of 1 *M* Tris-HCl pH 8.0, 35 μl 2 m*M* acetyl CoA (Sigma, C-3019), and 15 μl [1-^{14}C]acetyl-coenzyme A which had been reconstituted in 10 m*M* sodium acetate (pH 6.0) at a concentration of 0.05 μCi/μl. Each reaction mixture contained 10 μl of the acetyl CoA mix; 10 μl 10 m*M* ATP; 2.5 μl 100 m*M* $MgCl_2$; 0.3 μl *S*-acetyl CoA synthetase (Sigma, A-5269, equivalent to 0.3 units/reaction); 12.2 μl H_2O; 5 μl 2 m*M* puromycin; and 10 μl cell lysate (equivalent to 2 × 10^5 cells). Reaction mixtures were incubated at 33° C for 1 h, extracted once with 1 ml of ethyl acetate, and dried down for 1 h. The material was resuspended in 20 μl ethyl acetate and spotted onto thin-layer chromatography (TLC) plates (silica gel 60, EM Separations, Gibbstown, NJ). The TLC plates were developed with methanol-ethyl acetate 1:3 (vol/vol) and used to expose X-ray film or quantitated directly with a PhosphorImager 4000 (Molecular Dynamics, Sunnyvale, CA).

Cell viability in the presence of Actinomycin D. Cell lines were plated at a density of 0.85 × 10^6 cells per 35-mm-diameter dish and allowed to adhere for 1 h. We subjected some plates of cells to heat shock by sealing the edges of the dish with parafilm and floating the dish in a 42° C water bath for 30 min. These cells were returned to the 27° C incubator for 4 h before the addition of various concentrations (100 ng to 1 μg) of actinomycin D (Pharmacia, Piscataway, NJ) per ml. Cells were incubated in actinomycin D-containing media for 18–20 h, at which point we gently removed cells from the surface of the plates by spraying with TC-100 and counted them in the presence of 0.04% trypan blue. Viable cell counts were made with a hemocytometer, four grids were counted per sample, and the mean and standard error calculated from replicate plates.

RESULTS AND DISCUSSION

Growth of puromycin-resistant cell lines. The plasmid constructs used to generate the stable cell lines described in this paper are

illustrated in Fig. 1. Two plasmids express only the *pac* gene from either the *hsp70* promoter or the Ac*M*NPV *ie-1* promoter, phsp70pac and pIE1pac, respectively. Two other stable cell lines were produced which expressed both the *Op-iap* gene and the *pac* gene from separate promoters in either a reverse (phspOpIAPIE1pacR) or tandem (phspOpIAPIE1pacT) arrangement with regard to the orientation of the two genes and promoters. In the double-expression constructs, the *Op-iap* gene was driven by the *hsp70* promoter and the *pac* gene was driven by the *ie-1* promoter. The *hsp70* promoter has been shown to function constitutively in Sf21 cells by transient transfection, even in the absence of heat shock, and the levels of activity can be increased significantly in Sf21 cells with a 30-min heat shock regimen at 42° C (Clem and Miller, 1994).

One of the advantages to using the *pac* gene as a selectable marker is the rapid selection of drug resistant cells. Other selectable marker genes have been used successfully to produce stable transformed insect cell lines such as the bacterial gene for neomycin resistance (*neo*) (Jarvis et al., 1990) and the *hygromycin phosphotransferase* (*hygro*) gene which confers resistance to hygromycin B (Vulsteke et al., 1993). A drawback to using the *neo* gene is the length of time for the complete selection process, which is approximately 1 mo. for selection in G418 (Jarvis and Guarino, 1995). Nonresistant cells will continue to divide for a short time at lethal doses of G418, whereas puromycin acts within hours. Another factor to consider is the observation that Sf9 cells grown in media containing antibiotics (amphotericin B and gentamycin) show significant background resistance to G418 which necessitates starting with cells that have never been grown in the presence of antibiotics (Jarvis and Guarino, 1995). We have not observed this phenomenon with Sf21 cells when puromycin selection was performed on nontransformed cells routinely cultured in media containing a combination of penicillin G, streptomycin sulfate, and amphotericin B. There were no surviving Sf21 cells, even in low concentrations of puromycin (1 μg/ml) when the cells were not expressing the *pac* gene. Another advantage of using the *pac* gene in place of the *neo* gene as a selectable marker is cost. Puromycin is significantly less costly than G418 sulfate used for selection of cells expressing the *neo* gene, and the concentration of drug suitable for selection of resistant cells is 1000-fold less with puromycin.

The growth characteristics of all the stable puromycin-resistant Sf21 cell lines were monitored for greater than 100 passages. This represents continuous culture for over 1 yr, and in the case of the Sf21/hsp70pac cell line, cells were cultured for an 18-mo. period following the initial selection process to generate a stable cell line. All puromycin-resistant cell lines were cultured in the absence of puromycin, and every 10–12 passages a portion of the cell population was plated in 2.0 μg puromycin per ml to test for continued drug resistance. None of the cell lines showed any cell loss when exposed to puromycin selection at regular intervals during the extended time of continuous culture, indicating stable expression of the *pac* gene. There were no obvious morphological or growth rate differences observed by simple visual inspection in any of the cell lines generated during the entire period of culturing compared to a control population of Sf21 cells. Thus, it should be noted that continuous selection in puromycin was not a requirement for stable expression of the *pac* gene in any of the plasmid constructs tested.

Expression of puromycin N-*acetyltransferase.* There were no differences in growth or stability of expression with either the *hsp70* promoter or the *ie-1* promoter driving *pac*, nor were there any growth differences observed in the cell lines generated with expression plasmids carrying both the *pac* and the *iap* genes on the same construct. Even though all cell lines were initially selected in 2 μg puromycin per ml, we wanted to determine if there was any variation in the expression levels of the *pac* gene. Several cell lines were exposed to concentrations of puromycin ranging from 2.0 to 10 μg/ml (Table 1). There was no cell loss in the lines expressing the *pac* gene from the *ie-1* promoter and only marginal loss of cells in the cell line expressing *pac* from the *hsp70* promoter in the higher concentrations of puromycin (6.0–10 μg/ml). It is not known whether this represents lower overall levels of *pac* expression from the *hsp70* promoter or a generally higher copy number of the *pac*-expressing sequences in some of the stable cell lines. It should be noted that at no time during the selection process or testing in higher concentrations of puromycin were the transformed Sf21 cells subjected to heat shock. Thus, induction of the heat shock promoter is not necessary to achieve adequate levels of PAC expression in Sf21 cells for selection of a drug-resistant cell line.

TABLE 1

VIABILITY OF PAC-EXPRESSING CELL LINES IN PUROMYCIN

Concentration of Puromycin (μg/ml)	Growth of Cell Line[a]			
	Sf21	Sf21 + hsp70pac	Sf21 + ie1pac	Sf21 + hsOpIAPIE1pacT
0	+++[b]	+++	+++	+++
2.0	–[c]	+++	+++	+++
6.0	–	++	+++	+++
8.0	–	++	+++	+++
10.0	–	++	+++	+++

[a]Cell lines were established as described in Materials and Methods. All cells were plated at a density of 4×10^5 cells per 60-mm-diameter plate and allowed to grow to confluence in medium containing the concentration of puromycin indicated.

[b]Cells were counted after 72 h, scored according to the percentage surviving and compared to counts of cells grown in the absence of puromycin. Scale (+++) = > 99%, (++) = 60–80%, (–) = 0% of cells growing in medium without puromycin.

[c]All control Sf21 cells were not viable after 48 h in concentrations of 2 μg puromycin per ml or higher.

The levels of puromycin *N*-acetyltransferase can be assayed quite rapidly by a simple method similar to one designed to measure chloramphenicol acetyltransferase (CAT) activity (Gorman et al., 1982). The assay uses ^{14}C-labeled acetyl coenzyme A to monitor the acetylation of puromycin. The reaction products were counted directly following extraction in organic solvent or visualized following TLC separation (Fig. 2). There was virtually no background level of PAC activity in control Sf21 cells lacking the *pac* gene when 10 μl of cell lysate (equivalent to 2×10^5 cells) was assayed. Various amounts of cell lysate were assayed for the cell lines expressing the *pac* gene, ranging from 10 to 0.2 μl of cell lysate. PAC activity was monitored in different cell lines at several different times during the entire culture period and no differences were observed within a given cell line with the number of passages (data not shown). The cell line expressing *pac* from the *ie-1* promoter showed consistently higher levels of PAC activity than the cell line with the *hsp70* promoter driving *pac* (compare hsp70pac to IE1pac). We quantified the level of acetylation of puromycin using a PhosphorImager 4000 (Molecular Dynamics, Inc.). The amount of PAC activity at the lowest dilutions (0.2–0.1 μl) were approximately 20-fold higher for IE1pac compared

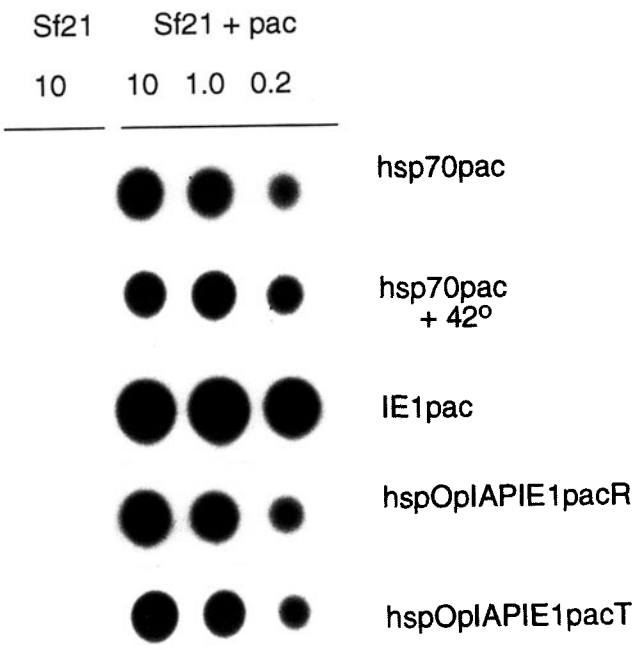

FIG. 2. Expression of puromycin *N*-acetyltransferase in stable cell lines. PAC activity was measured in cell lysates according to the protocol outlined in Materials and Methods. The amount of cell lysate assayed is indicated: 10, 1.0, and 0.2 μl is equivalent to 2×10^5, 2×10^4, and 3×10^3 cells, respectively. The protein content for each sample of 2×10^4 cells was measured by a Bradford assay and the values ranged from 3.2 to 4.1 μg. As a negative control, 10 μl of *Spodoptera frugiperda* cell lysate equal to 2×10^5 cells (Sf21/10) was assayed for PAC activity. The effect of heat shock on the hsp70 promoter was measured by incubating the hsp70pac cell line at 42° C for 30 min (hsp70pac + 42°).

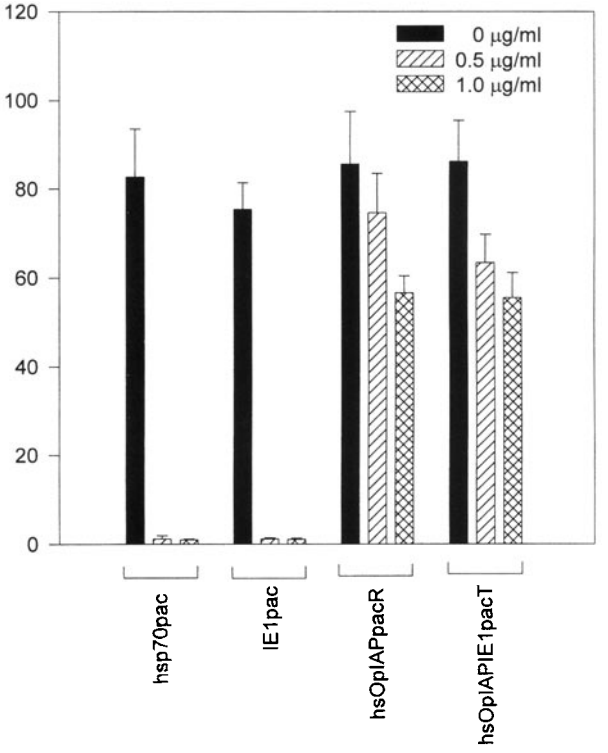

FIG. 3. Testing of expression of *Op-iap* in stable cell lines. Cell viability was tested in cell lines exposed to actinomycin D at two concentrations, 0.5 and 1.0 μg/ml for 18–20 h after an initial heat-shock treatment of 42° C for 30 min. Cell counts were made in the presence of trypan blue and the mean number of cells ± SEM from three replicates is indicated for each concentration of drug for the cell lines tested.

to hsp70pac, and heat shock treatment of the cells expressing hsp70pac increased activity only twofold (data not shown). The *Drosophila hsp70* promoter has been found to be active in a variety of lepidopteran insect cell lines (Morris and Miller, 1992). Additionally, the *hsp70* promoter can be induced in transfected Sf21 cells as previously shown by immunoblot analysis (Clem and Miller, 1994). Although the levels of PAC activity were somewhat lower in the cell lines coexpressing OpIAP and PAC, the orientation of the genes within the single expression plasmid did not have an effect on *pac* expression. This is of particular interest for the phspOpIAPIE1pacR construct, indicating that there is little or no promoter interference when the hsp70 promoter is placed in close proximity to the *ie-1* promoter.

Expression of Op-IAP. We tested individual cell lines for expression of the *Op-iap* gene by examining for the ability of the gene to protect from actinomycin D-induced apoptosis (Fig. 3). The hsp70pac and IE1pac cell lines which only express the selectable marker gene from either the *hsp70* or *ie-1* promoter were included as negative controls. In the absence of Op-IAP, the level of apoptosis was close to 100%. The control cells exhibited extensive cell blebbing, characteristic of apoptosis, and there were no surviving cells in 1.0 μg actinomycin D per ml as measured by trypan blue exclusion. On the other hand, virtually all of the cells expressing Op-IAP did not undergo apoptosis. At the higher concentration of actinomycin D (1.0 μg/ml) the number of viable cells decreased, which may indicate that the levels of Op-IAP were not sufficient in some of the cells to protect from actinomycin D-induced apoptosis. This could be a reflection of the overall lower levels of expression observed with the *pac* gene under the control of the *Drosophila hsp70* promoter compared to levels of expression from the AcMNPV *ie-1* promoter in Sf21 cells.

We conclude that the *puromycin acetyltransferase* gene is an excellent choice as a selectable-marker gene for the generation of stable, transformed insect cells and that two genes can be expressed from two separate promoters on the same expression plasmid, regardless of orientation. The use of this type of double-expression construct has proven valuable from the standpoint that the majority of selected cells within the population will also express the second gene of interest.

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