Stable expression of insect GABA receptors in insect cell lines
Promoters for efficient expression of *Drosophila* and mosquito *Rdl* GABA receptors in stably transformed mosquito cell lines

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Abstract We are interested in establishing stably transformed insect cell lines efficiently expressing the insect γ-aminobutyric acid (GABA) receptor subunit gene *Resistance to dieldrin* or *Rdl*. In order to facilitate this we utilized a system based on stable transformation of *Aedes albopictus* mosquito cell lines using the *dihydrofolate reductase (dhfr)* gene as a selectable marker. Here we report the production of stable mosquito cell lines carrying high copy numbers of *Rdl* genes from both *Drosophila* and *Aedes aegypti* mosquitoes and the subsequent high efficiency expression of functional GABA gated chloride ion channels. We also used this system to compare the activity of a range of immediate early baculovirus promoters in mosquito cell culture and demonstrate that IE1 promoter constructs work efficiently across insect species. Results are discussed in relation to the potential use of these constructs in the genetic transformation of non-Drosophilid insects.

Key words: GABA receptor; Stable transformation; Cell culture; *Aedes albopictus*; Gene expression; Immediate early baculovirus promoter

1. Introduction

Vertebrate $GABA_A$ receptors are composed of a number of different subunit polypeptides that assemble in the post-synaptic membrane to form GABA gated chloride ion channels [1,2]. In contrast to vertebrate $GABA_A$ receptors *Rdl* expresses efficiently as a homomultimer when *Rdl* mRNA alone is injected into *Xenopus* oocytes [3]. Expression of *Rdl* homomultimers therefore provides a unique opportunity to study GABA receptor structure and function via site directed mutagenesis in the absence of other interferring subunits. Our previous studies have demonstrated that *Rdl* can be efficiently expressed following infection of *Sf*21 cells with a recombinant *Rdl* baculovirus [4]. However, due to the limitations of transient expression following baculovirus infection and associated cell death, we were interested in establishing stably transformed insect cell lines expressing functional *Rdl* GABA receptors. Further, we wished to identify promoters capable of constitutively expressing GABA receptors at levels comparable to those observed following baculovirus infection, but in more readily accessible and stably transformed cell lines.

Stably transformed chinese hamster ovary (CHO) cells expressing α1 and β1 receptor subunits [5] and human kidney cells expressing α1,β2 and γ2 subunits [6] have been established in other studies using vertebrate $GABA_A$ receptors. In insect cells, only low levels of expression of a single vertebrate $GABA_A$ receptor β1 subunit [7] and more recently a single molluscan β subunit [8] have been achieved following the stable transformation of lepidopteran cells. One of the central problems in establishing such stably transformed cell lines is that constitutive overexpression of GABA receptors appears to lead to cell death [5]. In this study we use the *Aedes albopictus* mosquito *dihydrofolate reductase* (*dhfr*) selectable marker system [9], which confers resistance to methotrexate (mtx), to establish stably transformed mosquito cell lines expressing *Drosophila melanogaster* and mosquito *Rdl* GABA receptors. We also compare the levels of constitutive activity of the baculovirus promoters IE-1 and IE-1^{hr5} [10,11] relative to that of the inducible *Drosophila* hsp70 promoter [12] via assay of chloramphenicol acetyl-transferase (CAT) activity. Here we report that both baculovirus promoters function at levels comparable to hsp70 under full heat shock and can functionally express *Rdl* GABA gated chloride ion channels with high efficiency. This demonstrates that the IE-1 baculovirus promoter may be of broader use in insect cell culture expression systems and possibly in insects themselves.

2. Materials and methods

2.1. Cell lines and promoter constructs

The *Aedes albopictus* mosquito cell line (C7-10) used in this study was a phenotypically wild-type (methotrexate-sensitive) clone, C7-10 [13]. Cells were maintained at 28°C in Eagle's medium [14] containing nonessential amino acids, glutamine and 5% heat-inactivated fetal bovine serum (E5-medium).

The reporter plasmid pIE1^{hr}CAT/PA was constructed by digesting pMSG-CAT (Pharmacia) with *Sal*I and ligating the 779 bp fragment containing the bacterial CAT gene into the previously described pIE1^{hr}/PA [10] also digested with *Sal*I. The plasmid pIE1-FCAT/PA was constructed by religating pIE1^{hr}CAT/PA that had been digested with *Bam*HI to excise the 495 bp fragment containing the hr5 enhancer. For pIE1^{hr5}2.1.3, the 1.8 kb *Pst*I fragment from cDNA2.1.3 [15] containing the mosquito *Rdl* coding region was ligated into pIE1^{hr5}/PA linearized with *Pst*I. For construction of pIE1^{hr5}14.1, the *Drosophila* cDNA clone 14.1 was digested with *Sal*I and the resulting 2.0 kb fragment containing the *Rdl* coding region was ligated in pIE1hr/PA linearized with *Sal*I. Proper orientation of the introduced genes was determined by restriction analysis (Fig. 1). The plasmids hsp-cat 1 contains the CAT gene regulated by the *Drosophila* hsp70 promoter and has been previously described [12]. The plasmid phsp70pl-14.1 (a kind gift from M. Tomalski) contains the *Drosophila Rdl* 14.1 coding region placed downstream of both the IE1^{hr5} enhancer/promoter and *Drosophila* hps70 promoter and leader sequence, respectively.

For transfection, plasmid DNAs were purified by PEG precipitation and their concentrations measured spectrophotometrically. C7-10

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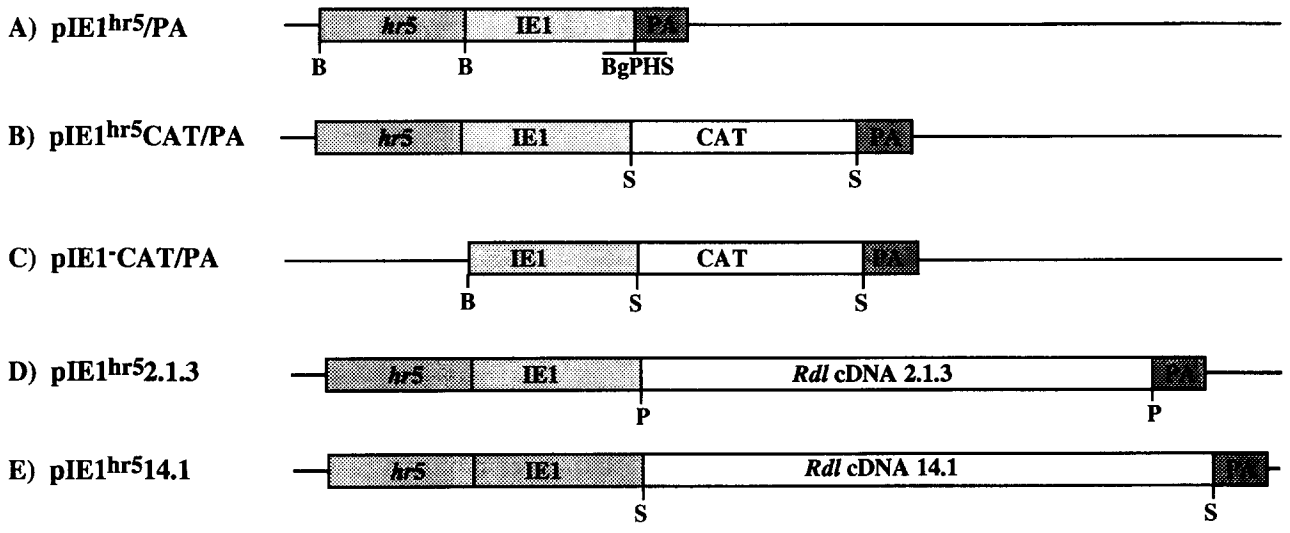

Fig. 1. Diagram showing construction of the IE1 constructs used in this study: (a) pIE1^{hr5}/PA, (b) pIE1^{hr5}CAT/PA, (c) pIE1-CAT/PA, (d) pIE1^{hr5}2.1.3 and (e) pIE1^{hr5}14.1. Note that the polyadenylation signal for all constructs are derived from the native baculovirus.

cells were cotransfected with the selectable vector pFSDHFR9 and hsp-CAT 1, pIE1-CAT/PA, pIE1^{hr5}CAT/PA, pMSG-CAT, phsp70pl-14.1, pIE1^{hr5}2.1.3 or pIE1^{hr}14.1. DNA was added to the cells at a 1:1 molar ratio at a concentration of 3 μg/ml as described previously [16], except that lipofectin (20 μg/ml) (Gibco) was used instead of polybrene. Stably transformed cells were selected in 1 μM mtx [16]. Clonal populations were established and maintained under continuous selection or stored in liquid nitrogen.

2.2. Analysis of DNA

Genomic DNA was isolated from mosquito cells as previously described [17]. Relative gene copy number was estimated by dot-blot hybridization to genomic DNA in two independent experiments. Dot-blots were probed with either the CAT specific 779 bp *Sal*I fragment from pMSG-CAT or an IE-1^{hr} specific 495 bp fragment from pIE1^{hr}/PA labeled to high specific activity with [α-^{32}P]dCTP (~3000 Ci/mmol; DuPont) by random priming. To show that gene copies were inserted into the genome, genomic DNA was digested with the appropriate restriction enzymes, Southern blotted and hybridized with the IE1^{hr} specific probe.

2.3. CAT assay and electrophysiology

To determine the level of reporter gene expression afforded by each of the different promoters, lysates from three individual clonal derivatives of each of the transformants were assayed for chloramphenicol acetyltransferase (CAT) activity using the CAT Enzyme Assay System (Pomega, Madison, WI). For cells transformed with hsp-cat 1, CAT activity was induced by a 4 h heat shock at 37°C, followed by a 2 h recovery period at 28°C, as described previously [18].

Whole cell patch clamping of cells expressing *Rdl* GABA gated chloride ion channels has been described previously [4]. Briefly, cells were placed on the stage of an inverted phase contrast microscope at room temperature (in a solution consisting of 128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$, 35.5 mM Sucrose, 5 mM HEPES, pH 7.1). Whole-cell patch clamp recordings [19] were made with Sylgard-coated patch electrodes (filled with 140 mM KCl, 10 mM HEPES, 10 mM EGTA, 4 mM MgATP, pH 7.1). Current was recorded with an EPC-7 patch clamp amplifier interfaced to a personal computer running PCLAMP software (Axon Instruments, Foster City, CA). GABA dissolved in bathing solution (50 μM) was injected with pressure pulses provided by a Picospritzer (General Valve Corp., Fairfield, NJ) from patch pipettes positioned with 5 μM of the cell being recorded. Cells were clamped at various holding potentials as indicated in Fig. 4. For patch clamp studies of the cells transformed with phsp70pl-14.1, expression of the *Rdl* GABA receptor was induced by a 4 h heat shock at 37°C, followed by a 2 h recovery period at 28°C.

3. Results and discussion

3.1. Stable transformation of insect cell lines

Following selection with mtx, resistant colonies began to appear after 10–15 days on plates containing cells transfected with pMSG-CAT, pIE1-CAT/PA, and hsp-cat 1, whereas clones transformed with pIE1^{hr5}-CAT/PA, phsp70pl-14.1, pIE1^{hr}2.1.3 and pIE1^{hr}14.1 took up to 30 days to develop. In contrast, no resistant cells were observed in mtx-sensitive C7-10 control cells. Three independent clonal cell lines stably transformed with each of the CAT reporter constructs were selected for further study and a single clone of each of the *Rdl* transformants were maintained for further study. Dot-blot analysis of DNA from the clones transformed with the CAT reporter constructs revealed comparable gene copy number in each of the lines (Fig. 2a) except those containing IE1^{hr}-CAT/PA, which carry at least ten fold fewer copies. In contrast the phsp70pl-14.1 and the pIE1^{hr}2.1.3 have few inserted copies relative to the cells transformed with pIE1^{hr}14.1 (Fig. 2b). Upon over-exposure of the blot shown in Fig. 2b and in reference to the C7-10 control, we estimate that the lines containing phsp70pl-14.1 and pIE1^{hr}2.1.3 show approximately ten gene copies per genome, and that the pIE1^{hr}14.1 has approximately 50 copies (data not shown).

Table 1
CAT activity from cells stabily transformed with the constitutive immediate early baculovirus promoters IE1-CAT and IE1^{hr5}-CAT compared to induced activity from the *Drosophila* heat shock promoter hsp70-CAT

CAT reporter	CAT activity (*n*)
Commercial control	293 ± 43 (4)
pIE1^{hr5}CAT/PA	250 ± 12 (3)
pIE1-CAT/PA	70 ± 14 (3)
hsp-CAT 1	98 ± 27 (3)
pMSG-CAT	3 ± 0.1 (3)

Results are shown as CAT activity in cpm/μg of total cell lysate (± S.E., $n = 3$ individual cell lines). MSG-CAT was included as a negative control and a commercial CAT control was included to establish maximum CAT expression.

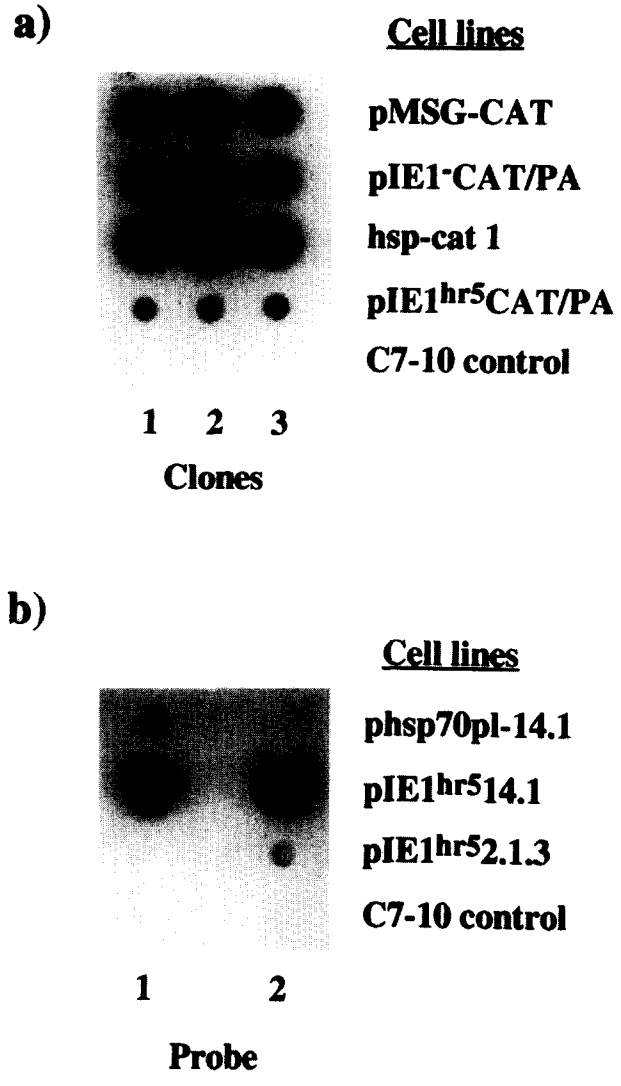

Fig. 2. (a) Dot blots indicating relative copy number of the inserted reporter constructs MSG-CAT, IE1-CAT, hspCAT 1 and IE1^{hr}. Three clonal derivatives of each (1-3) were examined. (b) Dot blots indicating relative inserted copy number of IE1^{hr}514.1 and hsp70pl-14.1 constructs. Where 14.1 represents a cDNA of the *Drosophila Rdl* GABA receptor subunit gene.

Examination of Southern blots of genomic DNA from the cells transformed with *Rdl* GABA receptor constructs suggests that copies are stably inserted into the genome. DNA digested with enzymes that do not cut within the transformed vectors migrate in the high molecular weight range (Lanes 1, 3, 5 of Fig. 3). When digested with an enzyme that restricts at a single site, much of the introduced DNA migrates at the size corresponding to that of the linear plasmid suggesting the integration head to tail concatomeres. The DNA migrating at a range of sizes probably correspond to random points of insertion (Lanes 2, 4, 6 of Fig. 3).

3.2. Comparison of different baculovirus promoters

The IE-1 promoter from *Autographa californica* polyhedrosis virus (Ac*M*NPV) has been shown to have high levels of constitutive activity in *Sf*21 cell lines [11]. This activity has been further enhanced by the addition of the hr5 enhancer element [10]. In these experiments cell lysates were assayed for CAT activity to determine the relative efficiency of the different promoter constructs. Cells transformed with pIE1-FCAT/PA produced levels of expression comparable to cells transformed with the hsp-cat 1 construct under full heat shock conditions (Table 1). The activity of IE1 was enhanced approximately three fold by the addition of the hr5 enhancer element, despite the lower copy number of inserted genes.

As the pIE1-CAT/PA and IE1^{hr5}/PA-CAT constructs showed high levels of reporter activity in mosquito cells, we engineered the GABA receptor *Rdl* cDNAs from both the mosquito and *Drosophila* onto these promoters and compared levels of functional *Rdl* expression with those observed in a similar construct containing the *Drosophila* heat shock promoter, hsp70. GABA application to voltage clamped *Rdl* transformed cells increased the membrane current (Fig. 4, left). Whereas control cells showed no response. Current increased linearly with voltage and reversed near 0 mV, as expected for the activation of chloride selective channels in symmetrical chloride solutions (Fig. 4, right). Large electrophysiological responses were recorded from cells transformed with the *Drosophila* GABA receptor constructs phsp70pl-14.1, IE1^{hr}14.1 and IE1^{hr}2.1.3 (Fig. 3a–c). Current vs. voltage plots (Fig. 4, right) reverse at or near zero as expected for recordings of chloride currents in symmetrical chloride ion concentrations. These GABA responses from *Rdl* homomultimers reported here are significantly larger (nA rather than pA) than those observed following stable expression of β $GABA_A$ receptor subunits in insect cell lines [7]. It should be noted that control experiments on non-heat shocked hsp14.1 cells revealed that heat shock was not necessary to achieve high levels of functional GABA receptor expression. The loss of heat induction implies that the hr5 enhancer and IE1 promoter upstream of the hsp70 promoter may contribute to the high level of expression from cells transformed with this construct. Only minimal electrophysiological

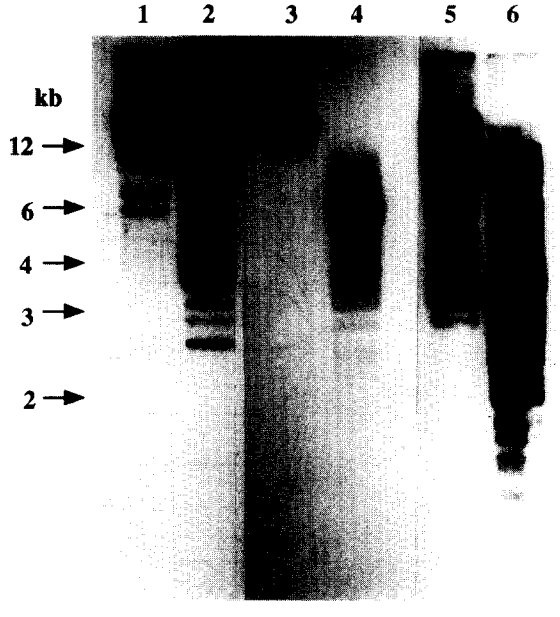

Fig. 3. Southern blot showing that inserts have been incorporated into the genome of the cells (see text for discussion). Legend: hsp14.1 cut with *Sac*I and (lanes 1 and 2 respectively) *Bsm*I, IE1^{hr5}14.1 cut with *Afl*II and *Xho*I (lanes 3 and 4) and IE1^{hr5}2.1.3 cut with *Afl*II and *Xho*I (lanes 5 and 6). Note that lanes 3 and 4 (from the same blot) have been exposed for a shorter period of time and that the panel is therefore a composite.

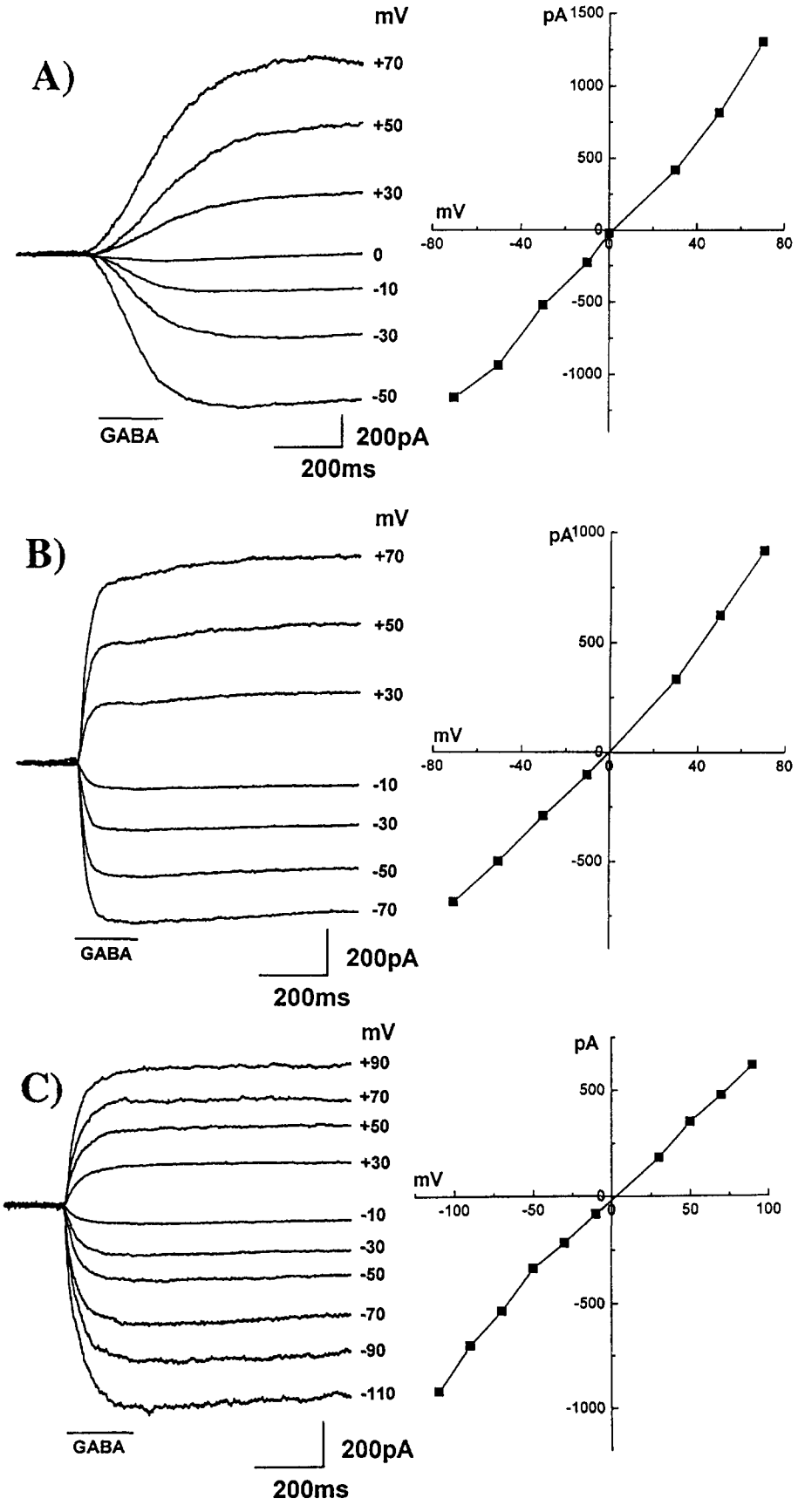

Fig. 4. GABA activated currents from mosquito (*Aedes albopictus*) cell lines stably transformed with constructs (a) hsp70pl-14.1, (b) IE1^{hr5}14.1 and (c) IE1^{hr5}2.1.3. Note that GABA receptor cDNA 14.1 is from *Drosophila melanogaster* and that cDNA 2.1.3 is from the mosquito *Aedes aegypti*. Potentials (left) range from −110 mV to 90 mV. Plots of peak current vs. voltage (right) exhibit linear behavior, reversing at or near zero.

responses (<20 pA) were recorded from cells transformed with the constructs lacking the hr5 enhancer.

Previous studies assaying the transient expression of different promoters have not adequately assessed the likelihood of the detrimental effects of prolonged overexpression of introduced genes. In this study we therefore developed stably transformed mosquito cell lines expressing either a CAT reporter or the GABA receptor gene *Rdl*, in order to quantify the levels of constitutive expression of two very different proteins and to assess the effect of their continuous overexpression on the resulting cell lines. Interestingly, the levels of functional GABA receptor expression seen were similar despite the wide variation in the number of inserted gene copies of each of the different constructs (Fig. 2b). These data suggest that inserted gene copy number is not a rate limiting factor in the expression of GABA receptors in these cells and that the promoters used are sufficiently efficient as to be able to produce large GABA gated chloride ion currents from a limited number of inserted gene copies. For each of the cell lines expressing recombinant GABA receptors, the cells had normal cell morphology and grew at a normal rate. However, cells over-expressing CAT under the hr5 enhancer showed slow growth rates and distorted morphology, suggesting that the high level of CAT produced by this enhancer construct may have been detrimental to normal cell growth.

3.3. Advantages of mosquito expression system

In previous studies we have expressed *Rdl* in baculovirus infected cells [4]. Although such a system produces high levels of functional GABA receptors, such expression is transient and by necessity the cells die following infection. The production of the stably transformed insect cell lines described here therefore has a number of distinct advantages in the study of this important GABA receptor subunit. (1) Insect GABA receptors are expressed in *insect* cell lines. This stands in contrast to studies of vertebrate $GABA_A$ receptor subunits expressed in insect cell lines or *Xenopus* oocytes, both reflecting expression in cell systems derived from widely divergent hosts. (2) Stably transformed cell lines represent a permanent, easily stored and highly accessible source of receptors for functional expression and biochemical studies. Thus, the cell lines described here were easily recovered from storage in liquid nitrogen. (3) For functional analysis the complications associated with baculovirus infection and cell death are avoided. Further, high level expression of both CAT and *Rdl* did not appear to have detrimental effects on cell line viability, except when the hr5 enhancer element was used to drive CAT. All the cell lines discussed here have been maintained through at least 35 passages, with no apparent changes relative to uninfected control cells. Although we do note that we were unable to select for cell lines containing high $IE1^{hr}$/PA copy number and we therefore cannot exclude the possibility that high levels of expression from this particular construct were detrimental.

The presence of the hr5 enhancer was required to generate large electrophysiological responses from our transformed cells. Since there has been some speculation regarding the mechanism by which enhancers activate transcription, the single cell measurements obtained from these experiments may be useful for studying enhancer function. Data from recent studies have provided evidence suggesting that enhancers function by increasing the probability but not the level of gene expression (reviewed in [20]). Data from our experiments suggest that the hr5 enhancer functions by increasing the level of gene expression.

Here we have demonstrated functional expression of both mosquito and *Drosophila Rdl* GABA receptor genes in *A. albopictus* C7-10 cells. These findings have important implications for the potential use both of *Rdl* as a selectable marker for the genetic transformation of non-drosophilid insects and for the use of the IE1 promoters in different insect hosts. In relation to *Rdl*, here we have shown that the *Drosophila* cDNA can be functionally expressed in a mosquito cell line. This therefore indicates that at least the open reading frame of the gene (coding for the GABA gated chloride ion channel) may function across insect species. We are therefore currently investigating if *Rdl* will need to be expressed under its native promoter in each insect species in turn, of if heterologous promoters will restore adequate expression across species.

The IE1 promoters tested in this study may also be useful in the expression of mosquito genes themselves following their future introduction by genetic transformation. Previous studies have largely focused on the use of inducible promoters in insect cell lines. These include the heat shock promoter hsp70 [9,12] and the metalothionene promoter [21], which are inducible by heat shock and copper or cadmium, respectively. In contrast few promoters identified to date provide high levels of constitutive activity, except the actin promoter [22] and the incidental constitutive activity shown by a *Drosophila* hsp70 construct [23]. The demonstration that the IE1 constructs studied here function across species is therefore an important first step in determining their applicability to use in mosquitoes themselves.

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