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Tight transcriptional regulation of foreign genes in insect cells using an ecdysone receptor-based inducible system

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Abstract

The use of insect cells has been highly successful for the expression of foreign proteins from baculoviruses or plasmid vectors. Here, we describe a tight transcriptional regulation of foreign genes in insect cells using an ecdysone receptor-based inducible system. The system includes the DEF domains of the spruce budworm (*Choristoneura fumiferana*) EcR (CfEcR) fused to the *Saccharomyces cerevisiae* GAL4 DNA-binding domain and the EF domains of mammalian *Mus musculus* retinoid X receptor (MmRXR) fused to the acidic activation domains (AADs) of the baculovirus transactivators IE1 and IE0. Using a GAL4 response element in reporter constructs, both transient and stable expression in insect lepidopteran cells showed that the chimeric MmRXR and CfEcR only activated the reporter genes in the presence of inducer; no gene expression was detectable in the absence of inducer. Characterization of heterogenous activation domains in insect cells showed that the AADs from *Autographa californica* multiple nucleopolyhedrovirus (MNPV) IE1 and *Orgyia pseudotsugata* MNPV IE0 consistently exhibited higher inducible levels than the archetype AAD from herpesvirus VP16 in insect cells. To confirm the tight regulation of this system the highly toxic protein, diphtheria toxin (DT), was used. In the absence of an inducer no cytotoxic effect was observed in insect cells that had been transiently transformed with DT expressing plasmids. This system will therefore be a very useful tool for biotechnology applications expressing highly toxic proteins in insect cells and for studying the functional genomics of insects and microorganisms that infect them.
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Keywords: Ecdysone receptor; Inducible expression; Stably transformed insect cells; Baculovirus; VP16

The insect steroid hormone 20-hydroxyecdysone (20E) regulates critical developmental stages such as embryogenesis, molting, and metamorphosis [1]. The major biological actions of the hormone are mediated by ecdysone receptor (EcR),[1] a ligand-dependent transcription factor that belongs to the steroid/thyroid hormone receptor superfamily [1,2]. Members of this receptor superfamily share several distinct domains: A/B (transactivation), C (DNA binding and dimerization), D (hinge, dimerization), E (ligand binding, heterodimerization, transcription), and F (ligand binding and transactivation) [2–4]. EcR can form a heterodimer with its natural partner ultraspiracle (USP) or with the USP mammalian homolog, retinoid X receptor (RXR). The formation of a heterodimer is required for functioning of EcR [3–5]. Subsequent to the report that *Drosophila melanogaster* EcR (DmEcR) was able to function as an ecdysone-dependent transactivator in cultured mammalian cells [6], the first ecdysone-inducible gene expression system was developed in mammalian tissue culture and

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[1] *Abbreviations used:* EcR, ecdysone receptor; USP, ultraspiracle; RXR, retinoid X receptor; Cf, *Choristoneura fumiferana*; Mm, *Mus musculus*; Sf, *Spodoptera frugiperda*; Ld, *Lymantria dispar*; EcRE, ecdysone response element; GAL4RE, GAL4 response element; DBD, DNA-binding domain; AAD, acidic activation domain; MNPV, multiple nucleopolyhedrovirus; Op, *Orgyia pseudotsugata*; Ac, *Autographa californica* CAT, chloramphenicol acetyl transferase; DT, diphtheria toxin.

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transgenic mice by using *Dm*EcR and human RXR [7]. Further studies of these receptors have produced a number of EcR-based inducible gene expression systems [8–11]. To date, EcR-based inducible gene expression systems have been well developed for use in gene therapy, functional genomics, and therapeutic protein production. Generally, the acidic activation domain (AAD) of the herpesvirus VP16 protein, a potent transactivator, is used in these EcR-based gene expression systems to replace a native activation domain of EcR to achieve higher levels of transgene expression [6–10].

An essential feature of an inducible gene expression system is that the induction activity would be tightly regulated, with little or no background expression in the absence of a ligand and fast and high induction levels in the presence of a ligand. Although several studies reported the use of EcR-based inducible gene expression in cultured insect cells [12–16], high background expression is always observed when EcR and USP are used to form a heterodimer to activate reporter gene expression [8,10,12,13,15–17]. Studies have shown that EcR and USP can form unstable complexes and then bind to the ecdysone response element (EcRE) in the absence of ligand, resulting a high background of transgenic activity [4,5]. In addition, it is found that USP DNA-binding domain (DBD) exhibits a higher affinity to the ecdysone response element (EcRE) than EcR DBD in the presence or absence of ligand and may act as a specific anchor that locates the heterodimer of EcR and USP onto the EcRE [18,19]. Since endogenous USP and EcR normally exist within insect cells, low level of non-induced gene expression has remained a problem.

Mouillet et al. [20] reported that the A/B domains of *Drosophila* EcRB1 and of EcRB2 when fused to the *Saccharomyces cerevisiae* GAL4 DBD were sufficient to activate transcription of a reporter gene under the transcriptional control of the GAL4 response element (GAL4RE) in yeast and mammalian cells. Palli et al. [8] developed a two-hybrid EcR-based inducible system in mammalian cells that exhibits low background levels of reporter gene expression in the absence of ligand when GAL4 DBD is fused to the DEF domains of CfEcR and a reporter gene construct contains the GAL4RE. Hu et al. [13] recently reported ecdysone dose-dependent reporter gene expression but in an EcR-deficient insect cell line when using a reporter containing the GAL4RE and DmEcR containing the GAL4 DBD partnered with the DmUSP. Data from these recent studies suggested that using a modified promoter containing the GAL4RE and a chimeric EcR containing the GAL4 DBD may exclude the binding of endogenous USP and EcR in insect cells and thus achieve a very low background level of transgene expression. In addition, it has been shown that there is a high level of amino acid sequence conservation between mammalian retinoid X receptor (RXR) and USP DBDs; RXR and USP can substitute for each other in DNA binding when partnered with EcR [3,4,21]. RXR and USP support ecdysone-responsive transactivation equally in transfection assays in mammalian cell lines [21]. Previous data have also demonstrated that heterodimerization of EcR and RXR is much more ligand-dependent than EcR–USP dimerization [3–5].

In this study, we have used these concepts to develop an EcR-based inducible gene expression system in insect cells with no detectable background expression. The system utilizes the DEF domains of the spruce budworm (*Choristoneura fumiferana*) EcR (CfEcR) fused to the GAL4 DNA-binding domain and the EF domains of *Mus musculus* RXR (MmRXR) fused to the AADs of the baculovirus transactivators IE1 and IE0. Using a GAL4 response element in reporter constructs, both transient and stable expression in insect lepidopteran cells showed that the chimeric MmRXR and CfEcR only activated the reporter genes in the presence of inducer; no gene expression was detectable in the absence of inducer. The use of the highly toxic protein, diphtheria toxin (DT) confirmed the tight regulation of this system.

Materials and methods

Plasmids

(i) EcRE-CAT and GAL4RE-CAT (Fig. 1A) have been previously described [12]. 6× GALRE-TI-DT was a kind gift from Dr. David Potter (Rheogene LLC). GAL4RE-GFP (Fig. 1A) was constructed by insertion of the green fluorescent protein (GFP) ORF as a *Bam*HI fragment into a *Bam*HI partially digested pGAL4RE-CAT. pHyg-GAL4RE-CAT (Fig. 4A) was constructed as follows. Using pCM54 [22] as a template a *Nhe*I–*Kpn*I fragment containing the hygromycin resistance gene ORF was amplified with the upper primer 405 (5′ GAT CGC TAG CGG CAA TGA GAT ATG AAA 3′) and the lower primer 406 (5′ CAG ATC CCG GTA CCC ATC TAC TCT ATT C 3′) and was used to replace the Zeocin resistance gene of p2ZeoKS [23]. Using p2Zeoks as a template an *Nhe*I–*Kpn*I fragment containing the OpMNPV *ie2* promoter-EM7 promoter and the OpMNPV *ie2* poly(A) was amplified with upper primer 403 (5′ GCA GGA CTG ACC GGT ACC GAC CAA C 3′) and lower primer 404 (5′ CCA TGC TAG CCC TCC TAT AGT GAG TCG T 3′). Ligation of the two fragments produced p2Hygks. A 1173 bp *Hin*dIII–*Sac*II fragment from pGAL4RE-CAT was cloned into *Hin*dIII–*Sac*II partially digested p2Hygks to give pHyg-GAL4RE-CAT.

(ii) CfEcR-AcIE1 (Fig. 1B) has been previously described [12]. GAL4-EcR:DEF (Fig. 1B) was

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