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Award Number: DAMD17-99-1-9141

TITLE: Investigating the Regulation of Estrogen Receptor-Mediated  
Transcription

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REPORT DATE: June 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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20010928 051

# REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2001	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jun 00 - 31 May 01)	
4. TITLE AND SUBTITLE Investigating the Regulation of Estrogen Receptor-Mediated Transcription			5. FUNDING NUMBERS DAMD17-99-1-9141	
6. AUTHOR(S) Varykina Thackray, B.A. Steven K. Nordeen, Ph.D.				
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words)  The estrogen receptor (ER) regulates the expression of genes involved in the growth, proliferation and differentiation of skeletal, cardiovascular, neural and reproductive tissues. A basic scheme for the mechanism for ER action has been developed, but precise details on the interactions between ER and the cellular signaling and transcription machinery required for receptor-mediated regulation of specific target genes are still lacking. We have developed an estrogen responsive system in the fruit fly, <i>Drosophila melanogaster</i> in order to explore the functional interactions between ER and other cellular proteins. Transgenic flies carrying the human ER alpha and an estrogen responsive green fluorescent protein (GFP) reporter gene were constructed. <i>In vivo</i> expression of the GFP reporter gene was observed when larvae were grown on a food source containing steroidal or nonsteroidal estrogens. The induction of the reporter gene by estrogens was blocked upon treatment with tamoxifen, an estrogen antagonist. The polytene chromosomes of <i>Drosophila</i> larval salivary glands will be used to identify transcription cofactors and complexes recruited to an estrogen responsive promoter <i>in vivo</i> .				
14. SUBJECT TERMS Breast Cancer, Estrogen Receptor, <i>Drosophila</i>			15. NUMBER OF PAGES 13	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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## Introduction

Steroid receptors regulate the expression of many genes involved in human development, metabolism and homeostasis. Ectopic expression of steroid receptors in a genetically amenable organism such as the fruit fly, *Drosophila melanogaster* could provide details on the interactions between ER and the cellular signaling and transcription machinery required for receptor-mediated regulation of specific target genes. There is a significant amount of conservation of cell signaling pathways and the transcriptional apparatus between mammals and fruit flies. Not only does *Drosophila* possess homologues to many mammalian signaling proteins, chromatin remodeling factors, coregulators and basal transcription factors but *Drosophila* also express nuclear receptors. These nuclear receptors include the ecdysone receptor (EcR) which binds the steroid hormone, ecdysone, and *ultraspiracle* (*usp*), the *Drosophila* homologue to the retinoid X receptor. The fact that EcR is used as part of an inducible expression system in mammalian cells and that the glucocorticoid receptor functions in cultured *Drosophila* cells suggests that many insect and mammalian transcription factors are functionally interchangeable. We have developed an estrogen responsive system in the fruit fly, *Drosophila melanogaster* in order to explore the functional interactions between ER and other cellular proteins. The polytene chromosomes of *Drosophila* larval salivary glands will be used to identify transcription cofactors and complexes recruited to an estrogen responsive promoter *in vivo*.

## Annual Summary

Aim One: Develop an estrogen responsive fly in which a reporter gene is regulated by estrogens.

Aim Two: Determine how estrogen receptor agonists and antagonists behave in the transgenic fly compared to mammalian cells.

Published paper summarizes our results for the first two aims. Thackray, V., Young, R., Hooper, J., and Nordeen, S. (2000) Estrogen Agonism and Antagonism on the Human Estrogen Receptor in *Drosophila*. *Endocrinology*, 141(10), 3912-3915

Aim Three: Use polytene chromosomes of *Drosophila* larval salivary glands to directly assess the recruitment of transcription factors by ER to an ERE-containing target gene *in vivo*.

I used standard P element-mediated transformation technology to generate the two transgenic fly lines described below. Schematics of the P element vectors are shown in Figure 1.

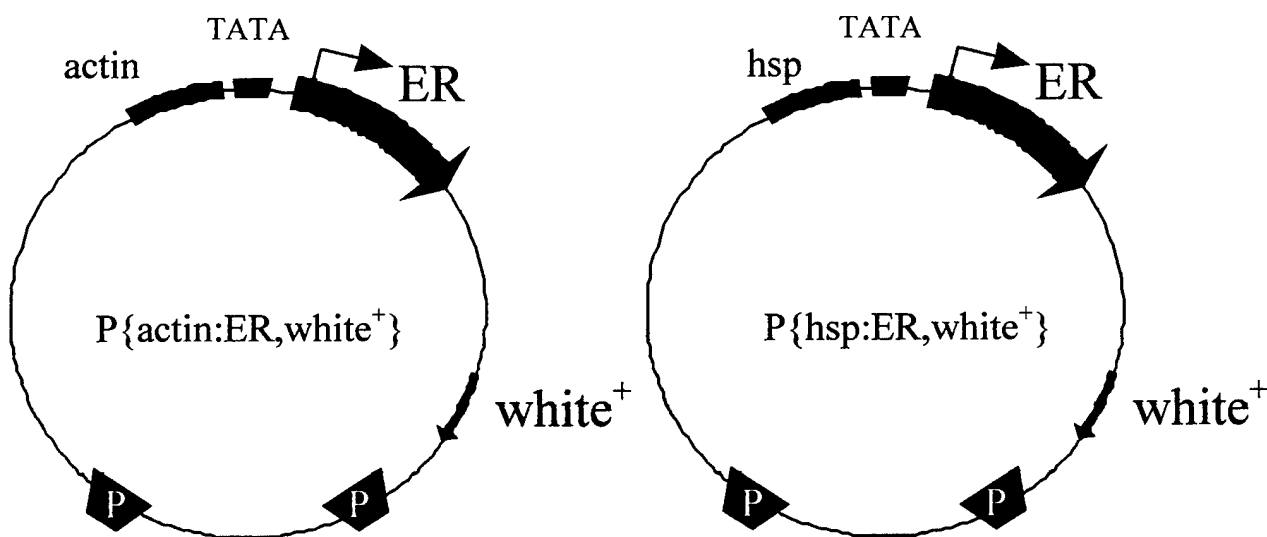


Figure 1. P element transposons for constitutive and heat shock dependent expression of ER.

In order to create a fly containing ER in its salivary glands, we constructed two transgenic fly lines by P-element mediated transposition. The first contained a transposon which allows the expression of ER under the control of the actin promoter. This results in constitutive ER expression throughout the fly body. The eye color marker *white+* is linked to the actin:ER to facilitate initial detection of the transgenic flies as well as later mapping and genetic manipulations. In case, constitutive ER is toxic to the fly, we also constructed a second fly line containing a transposon which allow the expression of ER under the control of the heat shock promoter. The eye color marker *white+* is linked to the hsp:ER to facilitate initial detection of the transgenic flies as well as later mapping and genetic manipulations.

Each construct was microinjected into *Drosophila* embryos (see Figure 2) and the surviving fertile adults were crossed to flies containing a second chromosome balancer. Their progeny were screened using the *white+* marker genes. Only one independent fly line containing the P{actin:ER, white+} transgene was obtained indicating that the constitutive expression of unliganded ER may be toxic to the fly. Interestingly, we obtained many independent lines of the P{hsp:ER, white+} transgene. The frequency of obtaining a transgene with hsp:ER was approximately 5% whereas the frequency of obtaining an actin:ER was much lower.

Injected DNA	# Embryos Injected	# Embryos Hatched	#Larvae Pupated	#Adults Eclosed	#Adults Fertile	#Transgenes
actin:ER	1134	329	144	110	80	1
hsp:ER	180	88	44	43	37	10

Figure 2. Construction of transgenic flies by microinjection of P element transposons.

The transgenic flies carrying the actin:ER and the ERE-GFP reporter gene were crossed and then assessed for estrogen responsiveness. The progeny were raised on media containing estradiol or vehicle control. Third instar larvae were collected and observed using a Zeiss microscope at 200x magnification with a fluorescein isothiocyanate (FITC) filter. We did not observe induction of the GFP reporter gene indicating that the one actin:ER transgene we obtained does not possess a functional ER.

Our experiments have demonstrated that human steroid receptors can function in *Drosophila melanogaster* and suggest that *Drosophila* genetics could be applied to dissecting the mechanisms of receptor action. We plan to use the polytene chromosomes of *Drosophila* larval salivary glands to visualize the recruitment of factors to an estrogen-responsive promoter *in vivo*. One advantage of a genetic strategy is that it would not be biased by preconceptions about the mechanism of steroid receptor action.

## Key Research Accomplishments

- Developed an estrogen responsive system in the fruit fly, *Drosophila melanogaster* in order to use the polytene chromosomes of *Drosophila* larval salivary glands to directly assess the recruitment of transcription factors by ER to an ERE-containing target gene *in vivo*.
  - Constructed P element vectors carrying the human ER alpha gene linked to an actin promoter so ER will be expressed in a ubiquitous manner or linked to a heat shock promoter so ER will be expressed upon heat shock of the flies.
  - Constructed transgenic fly lines carrying the P{hsp:ER, white+} and P{actin:ER, white+} transgenes.
  - Crossed the P{actin:ER, white+} transgene with the P{ERE:GFP, rosy+} transgene and did not observe estrogen responsiveness indicating that the P{actin:ER, white+} fly line does not contain a functional ER.



## **Reportable Outcomes**

### **Publications:**

Thackray, V., Young, R., Hooper, J., and Nordeen, S. (2000) Estrogen Agonism and Antagonism on the Human Estrogen Receptor in *Drosophila*. *Endocrinology*, 141(10), 3912-3915

## Estrogen Agonist and Antagonist Action on the Human Estrogen Receptor in *Drosophila*

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**Abstract.** The estrogen receptor (ER) regulates the expression of genes involved in the growth, proliferation and differentiation of skeletal, cardiovascular, neural and reproductive tissues. A basic scheme for the mechanism for ER action has been developed, but precise details on the interactions between ER and the cellular signaling and transcription machinery required for receptor-mediated regulation of specific target genes are still lacking. We have developed a genetic approach to explore the functional interactions of ER. In this work, we describe the development of an estrogen responsive system in the fruit fly, *Drosophila melanogaster*. Transgenic flies carrying the human ER alpha and an estrogen responsive green fluorescent protein (GFP) reporter gene were constructed. *In vivo* expression of the GFP reporter gene was observed when larvae were grown on a food source containing steroidal or nonsteroidal estrogens. The induction of the reporter gene by estrogens was blocked upon treatment with tamoxifen, an estrogen antagonist. However, we failed to recapitulate ligand-independent activation of the receptor *in vivo* or in cultured *Drosophila* cells. An estrogen responsive *Drosophila* system could be used to identify and characterize the complex functional interactions between ER and the other components of the cellular transcriptional apparatus.

Steroid receptors regulate the expression of many genes involved in human development, metabolism and homeostasis. Genetic analyses of steroid receptors expressed in yeast have led to a greater comprehension of steroid receptor function. In particular, yeast and mammalian genes that influence steroid receptor action have been identified using classical genetics and yeast two-hybrid screens [1-3]. However, yeast have limitations for the analysis of steroid receptor action. For example, the behavior of some ligands in yeast does not reflect their properties in mammalian systems. The widely used antiestrogen, tamoxifen, behaves as an agonist in yeast whereas it acts as an antagonist or partial agonist in mammalian cells [4].

Compared to yeast, there is significantly more conservation of cell signaling pathways and the transcriptional apparatus between mammals and the genetically amenable fruit fly, *Drosophila melanogaster*. Not only does *Drosophila* possess homologues to many mammalian signaling proteins, chromatin remodeling factors, coregulators and basal transcription factors but *Drosophila* also express nuclear receptors. These nuclear receptors include the ecdysone receptor (EcR) which binds the steroid hormone, ecdysone, and *ultraspiracle* (*usp*), the *Drosophila* homologue to the retinoid X receptor [5, 6]. The fact that EcR is used as part of an inducible expression system in mammalian cells and that the glucocorticoid receptor functions in cultured *Drosophila* cells suggests that many insect and mammalian transcription factors are functionally interchangeable [7, 8]. In this paper, we describe the construction and characterization of an estrogen responsive system in *Drosophila*. We show that

the behavior of the human ER alpha, in response to both steroidal and nonsteroidal ligands, mimics ER action in mammalian cells. The application of genetic approaches in *Drosophila* could help to determine how steroid receptors regulate gene expression. Furthermore, insects could be used to monitor levels of environmental estrogens.

### Materials and Methods

**Reagents.** 17- $\beta$ -estradiol ( $E_2$ ), 17- $\alpha$ -ethynylestradiol (et $E_2$ ), diethylstilbesterol (DES), 20-hydroxyecdysone (20-HE), 4-hydroxytamoxifen (4OHT) (Sigma, St. Louis, MO) and ICI 162,780 (ICI) (Tocris Cookson, Ballwin, MO) were obtained from commercial sources.

**Plasmids.** For these studies, two human ER alpha expression vectors were constructed; one in which ER expression was driven by the *Drosophila* actin promoter pP{CaSpeR-actin:ER, *white*<sup>+</sup>} and the other by the eye-specific glass multiple repeat upstream of the basal hsp 70 promoter pP{GMR:ER, *rosy*<sup>+</sup>}. Two estrogen responsive reporter plasmids were also constructed; pERE<sub>4</sub>:lacZ and pP{ERE<sub>4</sub>:GFP, *rosy*<sup>+</sup>}. pEGFP-C1 was obtained from Stratagene (Palo Alto, CA). The ERE<sub>4</sub> consists of four tandem copies of the consensus estrogen response element (ERE) in the *Xenopus laevis* vitellogenin promoter [9]. Additional details about the construction of these plasmids are available from the authors upon request.

**Cell culture and transient transfections.** *Drosophila* Schneider line 2 (S2) cells were cultured in Schneider's *Drosophila* medium (Gibco BRL Life Technologies, Rockville, MD) with 10% fetal bovine serum. 24 hours prior to transfection, the S2 cells were diluted to 0.8x10<sup>6</sup> cells/ml in media containing 10% charcoal-stripped fetal

Received 06/08/00.

bovine serum. The cells were transiently transfected using a calcium phosphate precipitation method [10]. The cells were treated with hormone or vehicle control 24 hours after transfection and harvested 48 hours after transfection.  $\beta$ -galactosidase and luciferase assays were conducted as described previously [11]. All transfections were performed in duplicate and each experiment was repeated 2-4 times with similar results.

**Transgenic *Drosophila*.** We used standard P-element mediated transformation to generate transgenic fruit flies carrying ER or the GFP reporter gene [12]. Double transgenic flies carrying ER and the GFP reporter gene on the third chromosome were obtained by recombination and recognized by GFP expression following growth on food containing  $10 \mu\text{M}$   $\text{E}_2$ .

**Analysis of GFP expression in *Drosophila*.** Estrogen responsiveness was assessed in the eye-antennal imaginal discs of third-instar larvae. P{GMR:ER, *rosy*<sup>+</sup>::P{ERE:GFP, *rosy*<sup>+</sup>} *rosy*<sup>506</sup> flies were raised on media containing hormone or vehicle control. Third instar larvae were collected and dissected to obtain eye-antennal imaginal discs. The imaginal discs were fixed at room temperature for 20 minutes in 3% formaldehyde in 3% sucrose, 1 x PBS pH 7.5. Differential interference contrast and fluorescence photographs were taken using a Zeiss microscope at 200x magnification with Nomarski and fluorescein isothiocyanate (FITC) filters.

### Results and Discussion

Since ecdysone and its receptor play key roles in *Drosophila* development, we wanted to test whether there was any interaction between the ER and EcR pathways. This was a potential concern since the ER homodimer and the EcR/*usp* heterodimer can recognize repeats of the same hexameric sequence although with a different orientation and spacing [13]. The induction of an estrogen-responsive reporter gene and an ecdysone-responsive reporter gene was examined in cultured *Drosophila* cells after treatment with estrogen or ecdysone (Fig. 1). In the absence of hormone, no reporter gene activity was observed.  $100 \text{ nM}$   $\text{E}_2$  induced the ERE<sub>4</sub>:lacZ reporter gene but not the EcRE<sub>7</sub>:lacZ reporter gene while  $1 \mu\text{M}$  20-HE induced the EcRE<sub>7</sub>:lacZ reporter gene but not the ERE<sub>4</sub>:lacZ reporter gene. This experiment indicated that insect cells possess the necessary cellular machinery to activate transcription of an estrogen-dependent reporter gene via the human ER. These results also suggested that an ER transgene is unlikely to interfere with normal ecdysone-regulated developmental pathways and that EcR would not activate an ERE-driven transgene in flies.

In order to create an estrogen-responsive *Drosophila*, we constructed two transgenic fly lines by P-element mediated transposition. The first contained a transposon which directed the expression of ER under the control of the glass multiple repeat. The multimerized glass element promotes transgene expression specifically in the developing

*Drosophila* eye [14]. The second line carried a GFP transgene driven by the same estrogen-responsive promoter used in the cell culture studies shown in Fig. 1. Double transgenic flies carrying both transgenes were obtained by crossing the single transgenic flies and screening for a recombination event that placed both P-element insertions on a single chromosome.

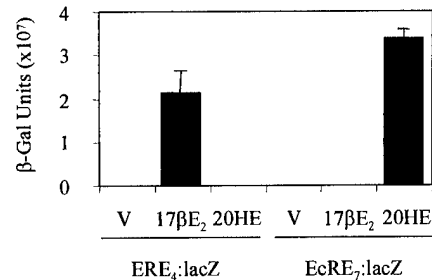


Figure 1. ER and EcR specificity in *Drosophila* cells. S2 cells containing endogenous EcR were transfected with 0.5  $\mu\text{g}$  of pP{CaSpeR-actin:ER, *white*<sup>+</sup>} and 0.5  $\mu\text{g}$  of the ERE<sub>4</sub>:lacZ or the EcRE<sub>7</sub>:lacZ reporter gene. The cells were treated with vehicle (V),  $10^{-7} \text{ M}$  17 $\beta$ E<sub>2</sub> or  $10^{-6} \text{ M}$  20-HE. The data represent the mean and standard deviation of 3 experiments.

The double transgenic flies carrying ER and the ERE-GFP reporter gene were assessed for estrogen responsiveness (Fig. 2A). GFP was expressed in a hormone-responsive fashion in eye imaginal discs from third instar larvae (Fig. 2B). No reporter gene expression was observed in the absence of hormone or at  $0.1 \mu\text{M}$  *etE*<sub>2</sub>. Expression was evident at  $1 \mu\text{M}$  *etE*<sub>2</sub> and increased at  $10 \mu\text{M}$  *etE*<sub>2</sub>. *E*<sub>2</sub> or the nonsteroidal estrogen, DES, induced GFP expression to levels comparable to *etE*<sub>2</sub>. By itself, the partial agonist/antagonist, 4OHT, failed to exhibit detectable agonist activity but effectively abolished GFP expression induced by feeding on  $10 \mu\text{M}$  *etE*<sub>2</sub>. However, no inhibition of the response to  $10 \mu\text{M}$  or  $1 \mu\text{M}$  *etE*<sub>2</sub> was seen with  $10 \mu\text{M}$  ICI, possibly because we could not use a sufficient excess of antagonist compared to agonist (data not shown). In general, the *in vivo* agonist and antagonist behavior recapitulated that seen in mammalian systems. It is noteworthy that the transgenic flies exhibited a proper response to estrogen agonists and antagonists given the apparent lack of a *Drosophila* homologue to the p160 coactivator family of proteins and the weak sequence similarity of the *Drosophila* protein, SMRTER to the mammalian N-CoR/SMRT homologues [15]. It is possible that as yet unidentified proteins in *Drosophila* are functionally equivalent to the vertebrate p160 coactivators and corepressors.

One shortcoming of the present system is that we did not observe GFP expression in the adult eye although expression of a UAS-GFP reporter gene was detectable in the adult eye of flies in which eye-specific GAL4

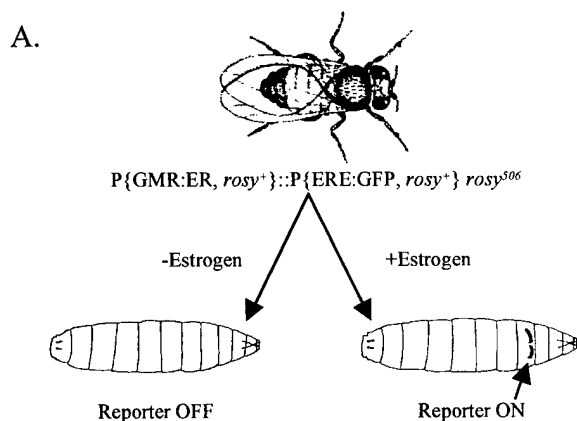


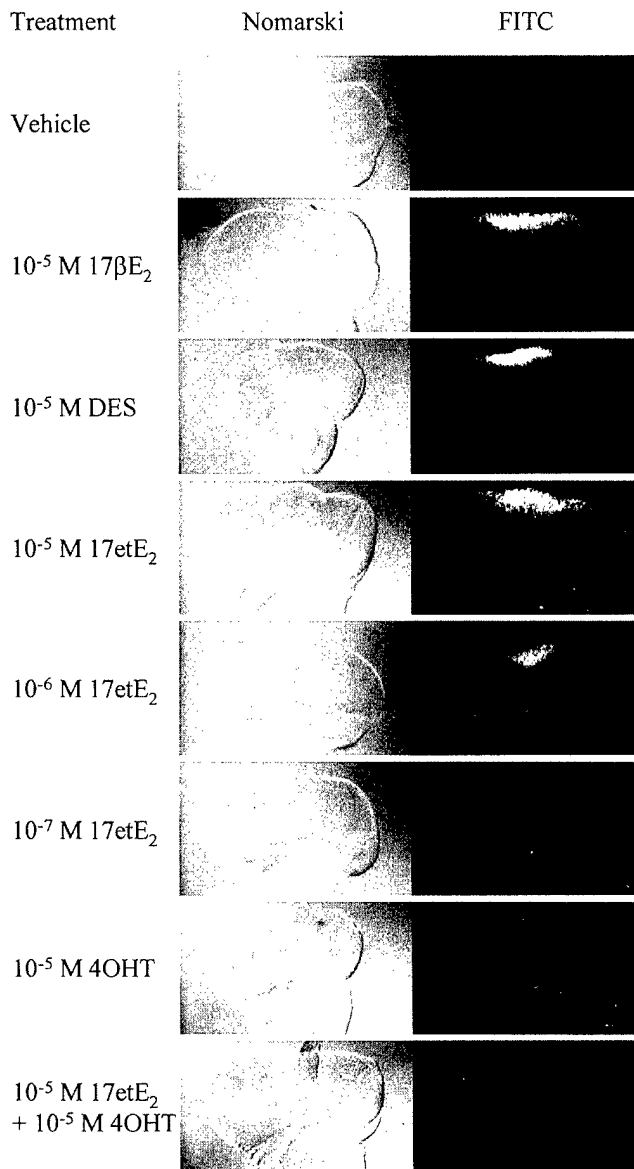
Figure 2. Estrogen responsive reporter system in *Drosophila*. A. Double transgenic flies carrying ER and the ERE-GFP reporter gene were raised on media containing vehicle or estrogen. GFP expression was assessed in the eye imaginal discs of third-instar larvae. B. Differential interference contrast and fluorescence photographs were taken at 200x magnification with Nomarski and FITC filters. Representative photographs from 2-4 experiments are shown.

expression is controlled by the glass multiple repeat (data not shown). In order to address this issue, we are constructing a transgenic fly line that expresses ER under the control of the heat shock 70 promoter. This promoter will allow the receptor to be expressed in a heat shock inducible manner during different developmental stages.

In addition, estrogens and estrogen antagonists were effective at doses well above those expected given the affinities of the ligands for ER. This was likely due to the fact that the ligands were taken up by ingestion from the larval medium and therefore the actual tissue concentrations of ligands were significantly lower than the concentration in the medium. In this case, lower doses of estrogen and estrogen antagonists would be expected to be effective in cultured *Drosophila* cells. This prediction was confirmed as shown in Fig. 3. Estrogen responses could be observed at subnanomolar levels of  $E_2$ ,  $etE_2$ , and DES, and were maximal ( $>10^3$  over basal) at 100nM (Fig. 3A). In cultured cells, both 4OHT and ICI antagonized induction, mediated by 10 nM  $E_2$  in a dose-dependent fashion (Fig. 3B). Since ICI antagonizes  $E_2$  in S2 cells, the lack of inhibition we observed *in vivo* could be due to differential tissue uptake or metabolism of different ligands.

These experiments demonstrate that human steroid receptors can function in *Drosophila melanogaster* and suggest that *Drosophila* genetics could be applied to dissecting the mechanisms of receptor action. For example, an estrogen responsive system in *Drosophila* could be used to identify and analyze *Drosophila* cofactors that influence estrogen responsiveness. One advantage of a genetic

B.



strategy is that it would not be biased by preconceptions about the mechanism of steroid receptor action. In addition, the polytene chromosomes of *Drosophila* larval salivary glands could be used to visualize the recruitment of factors to an estrogen-responsive promoter *in vivo* [16].

Although ER transgenic flies imitated the mammalian response to estrogen agonists and antagonists, there was one feature of estrogen action in mammalian cells that we were unable to recapitulate. We did not observe ligand-independent activation of ER when transgenic flies carrying ER and the ERE<sub>4</sub>-GFP were crossed with transgenic flies carrying a constitutively active *Drosophila* EGF receptor or mouse catalytic subunit of protein kinase A [17, 18]. Similarly, there was no activation of the ERE<sub>4</sub>-lacZ reporter

gene in S2 cells transfected with ER following 8-bromo cAMP treatment (data not shown). While the failure to observe ligand-independent ER activation did not rule out the possibility that ER could be activated in insect systems under appropriate conditions, it suggests the possibility of using complementation of the insect system to identify signaling molecules involved in ligand-independent activation.

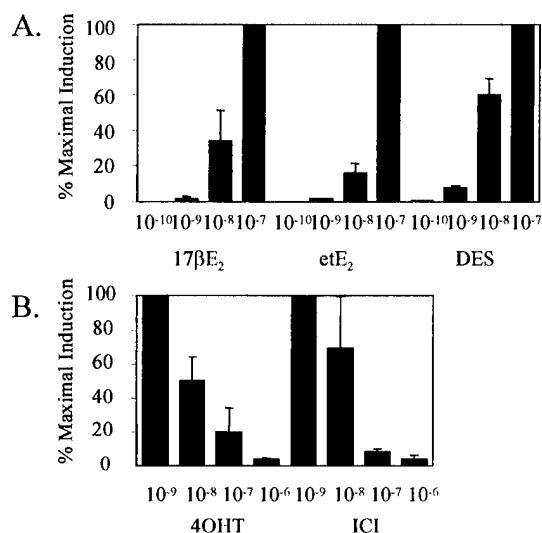


Figure 3. Dose dependent estrogen responsiveness and antagonism in cultured *Drosophila* cells. S2 cells were transiently transfected with 0.5  $\mu$ g of pP{CaSpeR-actin:ER, *white*<sup>+</sup>} and 5  $\mu$ g of the ERE<sub>3</sub>-TATA-luciferase reporter gene. A. The cells were treated with 10<sup>-10</sup> to 10<sup>-7</sup> M 17βE<sub>2</sub>, etE<sub>2</sub> or DES. B. The cells were treated with 10<sup>-8</sup> M 17βE<sub>2</sub> and 10<sup>-9</sup> to 10<sup>-6</sup> M 4OHT or ICI. The data represent the mean and standard error of 2-4 experiments.

#### Acknowledgements

We thank G. Rubin, K. Wharton, J. Heilig, C. Thummel, D. Hogness and D. McDonnell for plasmids and S. Kelley for critical reading of the manuscript. These studies were supported by a Predoctoral Traineeship from the U.S. Army Breast Cancer Research Program (VGT) and a grant from the Cancer League of Colorado (JEH).

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