INVESTIGATING THE MOLECULAR CONTROL OF ECDYSONE RESPONSE GENE, *E74*,
IN THE *DROSOPHILA* OVARY.

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I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree.

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Date: 5-04-17
ABSTRACT

Oogenesis is the process by which an egg develops from precursor cells in the ovary. This process has been widely studied; however, many of the molecular mechanisms that regulate oocyte development and growth remain unclear. The Drosophila melanogaster ovary is an exceptional model system for studying the mechanisms of oogenesis. As in humans, germ cells are surrounded by somatic cells which aid proper oocyte development and maturation. Steroid hormones largely drive this process, and in Drosophila, the predominant steroid hormone is ecdysone, similar to human estrogen. Ecdysone binds to a heterodimeric receptor which then functions as a transcription factor to promote gene expression. Other factors, including additional transcription factors and chromatin remodeling factors, likely refine this response. Ecdysone signaling is necessary for oogenesis via the regulation of many target genes. One target, Ecdysone-induced protein at 74EF (E74), is required for oogenesis and is highly expressed in ovarian germ cells; however, regulation of E74 expression in the ovary has not been well-studied. To investigate how E74 expression is regulated in the ovary, we used enhancer mapping to identify regions of the E74 locus critical for germline expression. Twenty-eight fly lines carrying pieces of the E74 gene locus fused to a minimal promoter and Gal4 were crossed with flies containing UAS-lacZ responder transgene. We identified two 200-bp regions within a large intron of the E74 locus that are sufficient to drive expression of a reporter. Together, these regions fully recapitulate the endogenous E74 expression pattern. We then identified several factors, including the chromatin binding factor Trl, as putative regulators of E74 expression at those sites. Trl expression partially overlaps with that of E74, suggesting that Trl may be an important modifier of ecdysone signaling in oogenesis. Future studies will characterize the roles of Trl in this process.
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INTRODUCTION

Oogenesis is the process by which an egg develops in the ovary (Belles and Piulachs 2014). Oogenesis occurs in all organisms that sexually reproduce although specific mechanisms vary between species. In all species, oocytes arise from primordial germ cells during development which differentiate to form oocytes that become encapsulated by somatic cells(follicle cells) to aid further oocyte growth (Spradling 1993). The process of oogenesis has been widely studied in many different organisms; however, there is still more information to be gained about this elaborate process. Many species produce large quantities of eggs through oogenesis while others only produce a few. For instance, female humans only have a limited number of eggs that are produced during their lifetime. Whereas, fish produce a lot of eggs daily through oogenesis that will later be fertilized typically externally. In mammalian oogenesis, the oocyte develops from a primordial germ cell which becomes surrounded by a layer of somatic cells known as follicle cells (Grive and Freiman 2015). This new follicle progresses through multiple stages of growth where the oocyte undergoes meiosis, acquires nutrients, and makes macro-molecule precursors that will help support the embryo (Grive and Freiman 2015). Even though the germline stem cells have still yet to be found during mammalian oogenesis, invaluable information has been gained about the development of the follicle stages and oocyte (Grive and Freiman 2015). An essential part of this process is the use of hormone signaling during growth and maturation from the follicle cells that surround the primordial germ cell, the oocyte (Grive and Freimand 2015).

*Drosophila melanogaster*, the fruit fly, has many similarities with mammalian oogenesis which makes it an ideal model organism in which to study the developmental processes of oogenesis. Oogenesis in the fruit fly has the same progression from primary follicle cell to a
mature oocyte. In *Drosophila*, the anterior tip is referred to as the gerarium and it houses the germline stem cells in their niche which subsequently divides to produce a daughter cell, cystoblast (Figure 1) (Spradling 1993, Ting Xie 2013 review). The cystoblast continues to divide into 2-cell cysts, 4-cell cysts, 8-cell cyst, 16-cysts until the cyst is encapsulated by follicle cells, producing the first follicle (Spradling 1993, Wu et al 2008). At this point in the process, mammalian oogenesis and *Drosophila* oogenesis are comparable. The fruit fly’s ovary is set up in such a way that studying the process of oogenesis gives us a snapshot into the processes occurring during oocyte development. Each ovary is comprised of 10-16 ovarioles, the gerarium to oocyte ladder –like progression, which allows scientists to see what is occurring in different cell types at different stages allowing for a more complete picture (Figure 1) (Wu et al 2008). The fruit fly is good model organism to use because it has underlying mechanisms and genes that are conserved in humans as well as in other organisms (Jennings 2011). It has been shown that 75% of known human disease causing genes also have corresponding matches in the *Drosophila* genome, providing a strong reason for the use of fruit flies in medical and health related research (Jennings 2011). Fruit flies are also relatively inexpensive to maintain in a lab when compared to other model organisms such as mice and fish. They have a short generation time allowing experiments involving fruit flies to occur in a relatively short amount of time. There is a multitude of research that has already been conducted using fruit flies meaning there is a wealth of information to access and to aid in ongoing research.
Figure 1: *Drosophila melanogaster* Oogenesis

Each female fly has two ovaries containing 10-16 ovarioles (circled in red) (A). These ovarioles consist of a germarium and up to fourteen follicles ending with an oocyte that is ready for fertilization (B). Stages 3-8 cells are referred to as the mid-oogenesis region throughout this project. The germaium of the ovariole houses the germline stem cells (GSCs) (tan), in the anterior tip, which asymmetrically divide to produce a daughter cell, the cystoblast (C). The cystoblast is the cell divides to produce 2, 4, 8, and 16-cell cysts (light gray). These cysts are surrounded by somatic, follicle, cells (purple) to form the first stage follicle.
As in humans, oogenesis in *Drosophila* is regulated by hormone signaling (Belles and Piulachs 2014). Oogenesis requires hormone signaling in order for cell divisions and maintenance as well as for the processes to continue properly. A steroid is any organic molecule that has four rings and is stems from cholesterol (Britannica 2016). Steroid hormones are hormones made in the adrenal cortex, testes, or the ovaries that meet all criteria to be classified as steroids (Britannica 2016). In particular, steroid hormones are critical mediators of oogenesis (Belles and Piulachs 2014). In *Drosophila*, the predominant steroid hormone is ecdysone, which plays important roles in oogenesis, including maintenance and stem cell function, cyst growth and survival, and progression throughout oogenesis (Belles and Piulachs 2014). Ecdysone is homologous in structure and function to estrogen and progesterone in humans (Belles and Piulachs 2014). Ecdysone cannot have an affect without its receptor, EcR, and ultraspiracle, usp, which form a heterodimer (Yao et al 1992). After ecdysone binds to its receptor in the nucleus, ECR/USP act as a transcription factor to activate downstream targets (Kvon et al. 2014).

One such ecdysone receptor target gene is *Ecdysone-induced protein at 74EF* (*E74*), known to be required for oogenesis at multiple steps of oocyte development, stem cells and the mid-oogenesis cysts (Ables et al. 2012, Buszczak et al. 1999). The *E74* locus encodes two isoforms: *E74A* and *E74B* (Brody 2014) (Figure 2). The A isoform has eight exons and the B isoform has four exons; the two isoforms have three overlapping exons (Brody 2014). *E74* has also been shown to be crucial during metamorphosis; primarily *E74A* is thought to regulate the late genes during metamorphosis (Fletcher et al. 1995). This shows that *E74* has a variety of functions and is not limited to oogenesis making it essential for survival. *E74* can self-regulate its transcription through multiple binding sites along the gene (Fletcher et al. 1997).
It is unknown if ECR/USP is the only transcription factor that regulates E74 or if works in conjunction with other transcription factors to refine the expression of this gene to different ovarian cell types. Transcription factors are molecules that bind to regulatory regions on the gene to repress or enhance the gene that is being transcribed. Transcription factors bind DNA at specific regions to aid in recruitment of RNA polymerase. The goal of this study is to map the location(s) of cis-regulatory regions involved in the regulation of E74 in the ovary. Once, identified we will investigate what other transcription factors may also bind in the regulatory region. I postulate that transcription factor Trl binds specific sites in the E74 regulatory region and confer ovarian cell activity. My work will help us understand gene regulation in oogenesis.

**METHODS**

*Fly Husbandry and Setting Test Crosses*

*Drosophila* stocks of all of the Vienna Tile, Putative Brain (GMR), and control lines (nosGal4 and yw) were maintained at 22-25°C in vials with Nutri-Fly MF medium (Vienna Drosophila Resource Center (VDRC) and Bloomington Drosophila Stock Center) There were a total of 16 Vienna Tile (VT) and 12 GMR fly lines set with the germline permissive reporter (UASp). The test crosses consisted of female virgins from the UAS lines and males from the Vienna Tile, GMR, or control lines (Table 1).

Each cross we generated to contain a fragment from the gene attached to Gal4 and the germline permissive upstream activating sequence (UASp) attached to a reporter enzyme, lacZ (Figure 4). The offspring of the cross or F1 generation contained all of the elements needed to perform the experiment.
Figure 2: E74 gene locus

The A isoform is the longer of the two isoforms and has eight exons whereas the B isoform is shorter and only has four exons. The exons are indicated in purple.
Figure 3: *E74* mRNA expression pattern

This ovariole sematic shows a depiction of the *E74* mRNA expression pattern illustrated in Buszczak et al. 1999. In purple, mRNA expression is only in germline cells and mainly concentrated during mid-oogenesis which a previously indicated is follicle stages ~3-8. There is also mRNA expression in 8 and 16-cell cysts in the germarium. It is important to note that there is not any expression in the surrounding somatic cells or follicle cells which are indicated in light purple or gray.
**Figure 4: Experimental Design**

Crosses were generated using male flies containing fragments of the gene, Vienna Tile and GMR flies, attached to a transcription factor *Gal4*. Female flies contain a germline permissive reporter, *UASp-lacZ*. Through these crosses the offspring or F1 generation has all of these genes. When *Gal4* is activated it binds to an Upstream Activating Sequence (*UAS*). *UASp* is attached to an enzyme reporter, *lacZ*. If there is a transcription factor binding site in the hormone response element (HRE) then when the substrate, X-gal, is added to the enzyme it will result in a blue color change.
Table 1: Cross results and stock numbers

Represents all genetic crosses set with male flies from the Vienna Tile lines, GMR lines, or controls and female virgins from the germline permissive reporter (UASp) fly lines. Positive results are indicated by the X in the box. Ovaries with positive expression located only in the oviduct were not counted as a positive result. In the table, the Vienna Tiles and GMR lines are organized as they appear going left to right (5’ to 3’) on the E74 gene locus.

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X-gal staining, and light microscopy

Crosses were placed in the incubator. Ten to twelve days after egg laying (eclosion), flies were collected and placed in new vials with yeast paste, a nutrient food given to them in order to make them larger and easier to dissect, or molasses vials, which lack substantial nutrients, in order to visualize differences in expression patterns due to diet 3 days prior to the ovaries being dissected. Molasses vials are comprised of Grandma’s Gold standard molasses, agar, MilliQ water, and 2.5% tegosept, a preservative that prevents fungus from forming (Drummond-Barbosa and Spradling 2001).

Ovaries were dissected in Grace’s dissecting medium, teased apart, and moved to a pre-coated bovine serum albumin (BSA) tube and placed on ice. Ovaries were fixed in 0.5% glutaraldehyde in Grace’s medium (Sigma) for 8 minutes at room temperature on the nutator, a three dimensional rotating gyrator (Ables et al 2015). During the fixation step, the staining solution was prepared without x-gal and placed covered in foil at 37°C to pre-warm. The staining solution minus x-gal (GALS kit; Sigma) consisted of 5 µL of Reagent A: 200 mM MgCl₂, 5 µL Reagent B: 400 mM K₃Fe(CN)₆, 5 µL Reagent C: 400 mM K₄Fe(CN)₆, 150 µL 1% triton-X-100 in PBS (phosphate-buffered saline), and 310 µL 1 X PBS for each sample. After fixation, ovaries were washed three times for 10 minutes each with 0.1% PBS-Triton-X-100 (PBT) on the nutator at room temperature. During washes, 25µL of x-gal was added per sample to the pre-warmed staining solution, and mixed by inversion. Following removal of the last wash, 500µL of the staining solution was added to each tube. Samples were wrapped in foil and incubated in the x-gal staining solution overnight on the nutator at 4°C. Samples were then rinsed with PBT three times for 10 minutes. The last wash was removed and ~4 drops of
mounting media were placed in the tubes to maintain the samples until they could be mounted on slides for imaging (Ables et al 2015).

Ovaries were mounted on glass slides and imaged using the Olympus BX41 light microscope along with CellSense computer program. Ovaries were scored for where and in what cells the gene is expressed for each of the ovaries collected from the test crosses. Expression was determined from the color change that occurs, white or blue, if there is a transcription factor binding site when X-gal is present. This data was compared to the full E74 mRNA expression patterns in the ovary.

**Computational Analysis**

In order to identify putative transcription factor binding sites, each DNA sequence corresponding to a positive ovarian expression pattern was run through an online computational program called MEME (Multiple Em for Motif Elicitation) (Bailey and Elkan 1994). MEME searches through a library of DNA motifs looking for putative factors binding there. Transcription factors have some variability to where they can bind which is referred to as wobble and this is illustrated in the MEME analysis with larger and smaller letters. A large letter indicates a conserved base in the sequence while a small letter indicates variability. After the motifs were identified from MEME were obtained, they were run through TomTom to compare them with other known motifs across other Drosophila species to see whether the motif of interest is conserved (Gupta et al. 2007). If the motif is conserved across Drosophila species then function of the gene or genes in the area of interest has a crucial, conserved role.
RESULTS

This study will map the location(s) of cis-regulatory regions sufficient to drive the expression of *E74* in the ovary, and will investigate what other transcription factors may also bind at these sites. An important publicly available resource being utilized in this experiment is the Vienna Tile and GMR transgenic fly lines. The Vienna Drosophila RNAi Center (VDRC) created the Vienna Tiles (VT) library and Pfeiffer et al created the GMR fly library (Kvon et al. 2014, Pfeiffer et al. 2008). Both Vienna Tile and GMR fly lines contain a transcriptional reporter (UAS), a *GAL4* reporter gene, a small DNA fragment, and a promoter which directly correspond to small overlapping sections of the *E74* gene (Kvon et al. 2014, Pfeiffer et al 2008). Each of the Vienna Tiles and GMR lines were crossed with the germline permissive reporter, *UASp*. The results from these crosses can be seen in Figure 5.

A ~125 bp Enhancer is Sufficient to Drive Mid-oogenesis Expression in Germ cells

On the 5' end of the *E74* gene locus in the A isoform a Vienna Tile and a GMR line, VT032351 and GMR36C03, were positive for blue color change. These two positive tiles as well as the overlapping surrounding negative tiles, VT032350 and VT032352, help to narrow the possible transcription factor binding site to ~125bp (Figure 6). The expression patterns in both of the positive tile lines show that it is localized to germ cells in the mid-oogenesis stages (Figure 7A, 7B). Using the zoom function on FlyBase with the gene locus and tiles turned on was used to estimate the size of the transcription factor binding site. The mid-oogenesis localization seen in Figure 7 partially overlaps with the *E74* mRNA expression pattern which was expressed in mid-oogenesis as well as 16-cell cyst in the germaria (Figure 3) (Buszczak et al 1999).

To better identify the stages at which the two putative enhancers are able to drive expression, we used immunofluorescence against β-galactosidase (Figure 10). The first two
immunofluorescence images, A and B, are the controls. The positive control, B, has expression of β-gal (red) throughout the germline including in the gerarium. Whereas, the negative control, A, has expression of β-gal it is not as strong and occurs mostly in later oogenesis. C and D were both positive for X-gal expression in mid-oogenesis and overlap with one another. The immunofluorescence images for C and D confirm the area of localization to the mid-oogenesis as both have β-gal (red) in this region. Together with the x-gal results, C and D allow us to confidently conclude that this enhancer region is sufficient for driving expression in mid-oogenesis in germ cells.
Figure 5: Results from all of the crosses

The results obtained from the crosses between the Vienna Tile and GMR lines with *UASp*. Tiles that are red indicate a negative result from the x-gal staining, and tiles that are blue indicate a positive result for germline expression.
Figure 6: 125bp enhancer region size determination

The ~125bp enhancer region as determined through overlapping positive and negative tiles. This region is indicated by the dotted box on the $E74$ gene locus above. Tiles that are shaded in blue were positive for blue color change in germ cells and tiles that are shaded in red were negative for color change. These two positive tiles fall on the 5’ end of the $E74$ A isoform.
An intronic enhancer element is sufficient to drive expression in germ cells at mid-oogenesis.

Blue expression appears in about stages 3-8 of ovarioles for these germline enhancers.

Expression is limited to germline cells and as depicted in the diagram the surrounding follicle cells are not positive for blue color when using the germline permissive reporter (UASp).
A 200 bp Enhancer Drives Expression in 8-cell cysts, 16-cell cysts, and Mid-oogenesis

Near the N terminus of the B isoform but still located on the A isoform is the location of the third germline localizing enhancer, VT032337. This tile also overlaps with another tile that was not positive for color change, VT032336, which helps narrow down the possible transcription factor binding site to about 200 bp (Figure 8). Expression was localized starting at about 8-cell or 16-cell cyst and showed expression throughout mid-oogenesis (Figure 9A). The zoom function on FlyBase was used to estimate the size of this region using the overlap of the positive and negative tiles as guide. This expression pattern most closely resembles that of E74 mRNA expression identified by Buszczak (Figure 3) because there is expression of X-gal in 16-cell cysts as well as mid-oogenesis (Buszczak et al 1999).

Immunofluorescence was used to determine specific cell types in which expression occurred for the 200bp enhancer (Figure 10E, F). E is the cross that showed X-gal expression whereas F did not. Panel E shows β-gal expression (red) in mid-oogenesis. Some of the expression is more concentrated in later mid-oogenesis stages but there is expression in the earlier mid-oogenesis stages. Panel F clearly shows expression throughout, from the germarium to the later stage follicle cells, which resembles the positive control (Figure 10B). This leads to the conclusion that there is not a transcription factor binding site in this Vienna Tile contributing to differences in expression; these conclusions correspond to the results obtained from the X-gal experiment. Together E and F along with the X-gal experiment results illustrate that the enhancer region identified drives expression in 8-cell cysts, 16-cell cysts, and mid-oogenesis.
Figure 8: 200bp enhancer region size determination

Enhancer VT032337 located on the A isoform near the B isoform’s N terminus. The overlap between the VT032337 and VT032336 lines allowed the transcription factor binding site to be narrowed down to about 200 bp region indicated by the dotted box. The blue shaded boxes are the tiles that were positive for blue color change and were localized to germline cells. The boxes that are shaded red were negative for color change.
**Figure 9: 200 bp enhancer region most closely resembles E74 mRNA expression pattern**

This 200 bp enhancer is sufficient to drive expression in 8-cell cysts, 16-cell cysts, and mid-oogenesis. Blue expression occurs in 8-cell cysts, 16-cell cysts, and in follicle cell stages 1-9. Expression is limited to germline cells and as depicted in the diagram the surrounding follicle cells are not positive for blue color when using the germline permissive reporter (UASp).
Figure 10: Immunofluorescence confirms X-gal results

β-gal staining affirms germ cell at mid-oogenesis expression. A is the wildtype, control. B is the positive control which should have β-gal expression throughout in the germ cells. C, D, and E were all previously shown to have expression of E74 during mid-oogenesis. F overlaps with E in the enhancer region that is nearest to the 5’ end of the E74B locus, and looks very similar to that of the positive control.
DISCUSSION

The objective of this study was to characterize the cis-regulatory regions sufficient to drive the expression of \textit{E74} in the germline of the ovary, and to investigate what other transcription factors could be binding at these sites. I have identified two 200 bp enhancer regions that are sufficient for driving expression of \textit{E74} in the germline in the ovary. All three tiles that were positive during x-gal staining and $\beta$-galactosidase immunofluorescence staining closely resembled the \textit{E74} mRNA expression pattern previously shown (Buszczak et al 1999). However, \textit{VT032337} most closely resembled the mRNA expression pattern because it not only contains expression in mid-oogenesis but also is expressed in 16-cell cyst in the germarium (Buszczak et al 1999). The other two tiles that were positive did not have any expression in the germarium. In Figure 11, it seems to show that Enhancer A might be a repressor while Enhancer B is likely to be an activator. This is deduced by the lack of a peak when ecdysone is present for Enhancer A, a repressor, and the appearance of peaks with and without ecdysone for Enhancer B, an activator. This repression and activation function could be what allows \textit{E74A} to self-regulate during metamorphosis. Another important item of note from Figure 11 is that both enhancer regions have a portion of a \textit{Trl} binding site within them making it a possible transcription factor that binds at these sites (arrows). \textit{Trl} also known as GAGA factor has been shown to have many functions including gene expression regulation, nucleosome conformation, and assists during mitosis (Lomaev et al. 2017). \textit{Trl} can function to activate or silence gene expression so in one or both of the enhancer regions found \textit{Trl} could be functioning as a silencer or an activator. In order to determine if this is the case \textit{Trl} mutants will need to be set to see what happens when \textit{Trl} is partially or wholly removed.
Figure 11: Both enhancer regions contain Trl binding sites

Known ecdysone binding sites and Trl locations using STARR-seq computational data overlaid with the two enhancer regions determined from this study. Enhancer A which includes VT032351 and GMR36C03 contains a Trl binding site (orange arrows), and it does contain a peak when no ecdysone is present but in the presence of ecdysone there is not a peak (indicated by the asterisks). Enhancer B which only includes VT032337 has an ecdysone binding site (yellow arrow), has portions of multiple Trl binding sites (green arrows), and most interestingly has peaks with and without ecdysone indicated by the asterisks.
LITERATURE CITED


