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Peter Jang April 13th, 2012

Use of an Inducible Gene System to Study the Dynamics of the Drosophila Dosage Compensation Complex

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Abstract

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In *Drosophila,* males have one transcribing X chromosome while females have two transcribing X chromosomes. The males and females need to transcribe equal amounts of the genes on the X chromosome and achieve dosage compensation. The Male Specific Lethal (MSL) complex is responsible for making this happen. The MSL complex acetylates histones, specifically H4K16, which loosens the chromatin and increases the accessibility of the X chromosome, allowing hypertranscription in males. A mystery lies in how the MSL complex is recruited to active genes and spreads on the X chromosome. Two ideas exist: one, the MSL complex could start at the promoter region and move towards the 3' end of the gene; two, the MSL complex moves in an opportunistic manner to the transcribed region where it modifies the histones between the passages of RNA Polymerase II (Pol II) molecules (Lucchesi, 2009). To figure out this aspect of the MSL complex, an inactive gene that can be activated and that recruits the MSL complex needs to be examined. Previous work by Boehm with *Drosophila melanogaster* used chromatin immunoprecipitation (ChIP) on the inactive, activatable *hsp70 (heat shock protein 70)* gene to examine the movements of different proteins before and after transcription is initiated. A *hsp* gene would be suitable for similar studies with the MSL complex but none exists on the X chromosome. Such a gene is on the XR chromosome of *D. pseudoobscura.* The purpose of this study is to demonstrate that after ensuring its inactive state the *hsp28* gene on the XR chromosome of *D. pseudoobscura* can be activated and is able to recruit the MSL complex, so that it can be used to clarify where the MSL complex is recruited and how it enhances transcription.

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INTRODUCTION

The organization of chromatin:

Deoxyribonucleic acid (DNA) molecules are in all living cells. They are made of two strands in a helix orientation and carry genetic information. The length of DNA in a human cell is about 2 meters. In order to have DNA molecules fit in a cell's nucleus, they must be compacted. This compaction occurs with the assistance of small proteins called histones. Histones are positively charged and DNA molecules are negatively charged, allowing for tight binding. DNA wraps around histone octamers, consisting of two copies of the four core histones H2A, H2B, H3, and H4, like thread wraps around spools. DNA and one histone octamer around which it is wrapped form a structural unit called a nucleosome. These nucleosomes make up chromatin. There must be further compaction to fit the chromatin into the nucleus, so the chromatin coils into a shorter fiber called a solenoid, which is also called the 30 nanometer fiber because of its average width. The solenoid wraps around a central chromosome framework, protein scaffold (Griffiths et al., 1999).

Gene transcription:

Transcription is a process that creates ribonucleic acid (RNA) from the DNA template. It is the messenger RNA (mRNA) that is translated to create proteins. First the pre-initiation complex (PIC) must assemble at the promoter. It is composed of numerous transcription factors, mainly TFIIA, IIB, IID, IIE, IIF, and IIH. TFIIB recruits the RNA polymerase II (RNAPII) to the PIC. TFIIH separates the double-stranded DNA into single-stranded DNA, and the RNA polymerase II becomes an open complex bound to single-stranded DNA. After an open complex

is made, a subunit of TFIIH phosphorylates the carboxyl tail domain (CTD) of RNAPII, specifically at the amino acid serine position five (Ser5) in a heptad repeat. This phosphorylation is an indicator of an early stage in transcription and allows the RNA polymerase to move past the promoter. Some of the transcription factors of the PIC are left behind for the next PIC to be formed; meanwhile, the remaining transcription factors and RNAPII clear the promoter and are now referred to as the transcription elongation complex (TEC). It transcribes 17 to 46 bases and then pauses. Transcription resumes throughout the gene when a positive transcription elongation factor b (P-TEFb) phosphorylates the serine at position 2 (Ser-2) of the RNA polymerase carboxyl tail domain. So Serine 2 phosphorylation (Ser2-P) is an indicator of active transcription elongation (Boehm et al., 2003).

As mentioned above, for transcription to occur, the two DNA strands must be separated to allow the RNA polymerase to transcribe the template strand. The coiled 30 nm fibers and the nucleosomes are barricades that must be dealt with. To temporarily open up the 30nm fiber and remove nucleosomes require the action of chromatin remodeling or modifying complexes (Annunziato, 2008).

Proteins are made from the N terminal end to the C terminal end. In the case of histones, the C terminal end is in the core and the N terminal end extends away from it, allowing the long N terminal tails to be modified. These modifications include phosphorylation, methylation, acetylation, etc., and involve the addition of functional groups by covalent bonds to specific amino acids of the N terminal tails. As mentioned previously, histones are positively charged and DNA molecules are negatively charged. The neutral charge of acetylation and the negative charge of phosphorylation weaken the histone-DNA interaction, loosening the chromatin and

allowing an increase in transcription. These histone modifications are well characterized in *Drosophila melanogaster*.

The phenomenon of dosage compensation:

In *Drosophila*, the ratio of X chromosomes to sets of autosomes determines the sex: 1X:2A flies are males, and 2X:2A are females (Gilbert, 2000). Therefore, 2 doses of X-linked genes are present in females and 1 dose of X in males. On the X chromosome, there are genes that are equally needed by males and females; therefore, both males and females need to have equal levels of X chromosome transcripts. In order to equalize transcription, males upregulate the expression of the X chromosome genes by approximately two fold through a regulatory complex called the MSL (male specific lethal) complex, and produce equal amounts of RNA as females with two X chromosomes. This phenomenon, the equalization of the X chromosome gene expression in males and females, is called dosage compensation. This MSL complex is not present in females, because the 2X:2A ratio in females activates a feminizing gene called *Sex-lethal (Sxl)*. SXL protein prevents the translation of MSL2, an important component of the MSL complex. As a result, another important subunit of the complex called MSL1 is unstable and deteriorates, and the MSL complex is not formed in females.

The MSL, which is present throughout the male X chromosome, is made up of five main proteins (MSL1, MSL2, MSL3, MOF, and MLE) and one of two non-coding RNAs (roX1 and roX2). One of the five main proteins is a histone acetyltransferase (HAT) enzyme called males absent on the first (MOF), which binds nucleosomes, and preferably acetylates histone 4 at lysine 16 (H4K16) (see Hallacli and Akhtar, 2009, for review). This acetylation decondenses the chromatin and helps in the upregulation of transcription.

What remains to be known is the recruitment of the MSL complex to the active genes on the X chromosome and the mechanism of its spreading along these genes. As of now, there are two ideas regarding the movement of the complex. One idea is that the MSL complex starts at the promoter region and goes towards the 3' end of the gene. The other possibility is that the MSL complex goes in an opportunistic manner to the transcribed region where it modifies nucleosomes between the passages of RNAPII molecules (Lucchesi, 2009). To acquire a better understanding, an inactive gene that can be activated and that recruits the MSL complex needs to be examined.

Heat shock genes as a model to study dosage compensation:

A heat shock gene would be a suitable model for this study, because it is not transcribed at ambient culture conditions and can be activated upon the stress of high temperature. Once the gene is activated, all the proteins responsible for transcription will be recruited and play their roles. One of the most commonly used model organisms for the study of dosage compensation is *D. melanogaster.* The problem with utilizing this species is that although it has about nine heat shock genes on autosomes, there is no heat shock gene on the X chromosome (Ashburner and Bonner, 1979). A heat shock gene on the X chromosome that exhibits dosage compensation in males is needed to examine the MSL complex's movement upon activation.

Two such genes lie on the X chromosome of *D. pseudoobscura*. This species has an X chromosome that resulted from a fusion of the ancestral X chromosome and an ancestral autosome that occurred roughly 13 million years ago. Two heat shock genes, *hsp83* and *hsp28,* that are on the *D. melanogaster* chromosome 3L are now located in the 24D proximal region and 40A distal region on the *D. pseudoobscura* X chromosome cytological map. Interestingly, the

once autosomal chromosome became compensated with the expected high level of acetylation on H4K16 (Bone and Kuroda, 1996). These authors also detected components of the MSL complex, such as MLE and MSL-1 on both the left arm (XL, homologous to the *D. melanogaster* X), and the right arm (XR, homologous to the *D. melanogaster* autosomal chromosome 3) of the *D. pseudoobscura* X chromosome. The densities of the MSL complex and H4Ac16 on the fused X chromosome is identical to the original X chromosome. To go along with Kuroda's demonstration that the XR chromosome is dosage compensated, Pierce and Lucchesi had previously demonstrated that specific heat shock puffs on the XR chromosome were compensated (Pierce and Lucchesi, 1980). However, the heat shock transcript level increase of these puffs from a non-heat shock state to heat shock state was not investigated.

Heat shock genes:

Under normal conditions, generally *hsp* genes are inactive. During environmental stresses, such as elevated temperatures, inactive monomeric heat shock factors (HSF) that are present in the nuclei become active and trimerize, binding to specific DNA sequences, the heat shock sequence elements (HSEs). The HSEs are upstream of the *hsp* genes (Orosz et al., 1996). The HSF is recruited within seconds, showing that adaptation to the stressful environment needs to be rapid for survival. Once HSF binds to the HSE, a change in gene expression occurs, causing rapid transcription of the *hsp* gene (O'Brien and Lis, 1993). The heat shock genes get transcribed and translation of their transcripts results in heat shock proteins (HSP). The HSPs take up chaperone roles that assist in correctly folding other cellular proteins. Under high temperatures, proteins tend to denature and fold incorrectly; therefore, the synthesis of HSP is necessary to protect key proteins and allow the cell to resume its function following the stress period. In the

heat shock response, transcription at previously active regions is greatly reduced and only occurs at a few sites.

Polytene Chromosomes:

Heat shock genes can be visualized on polytene chromosomes (the giant interphase chromosomes found in larval salivary gland nuclei) under the microscope. The salivary gland cells undergo endoreplication, which consists of successive cell cycle's DNA replication phase without the mitotic phase. So there is a high amount of DNA replication without cell division; the chromatids bundle with each other, giving rise to thick, visible chromosomes. Chromatids have chromomeres that are regions with particularly compacted DNA. These chromomeres are tightly associated next to each other and give the appearance of unison dark bands along the chromosomes. Between dark bands are lighter interchromomeric regions, the interbands. These interbands are looser than bands, so they appear lighter. The differences in DNA coiling and proteins associated result in variations of compaction. Among the interbands, numerous nonhistone proteins are present: RNA polymerase II, transcription- and replication- affiliated proteins, histone modifications, and open chromatin related proteins (Zhimulev et al., 2012). The bands contain more histones and less nonhistone proteins than the bands do, since the DNA is compacted (Zhimulev and Koryakov, 2009). These bands can be mapped consistently. For genes to be transcribed rapidly upon heat induction, the chromatin must decondense, which is why the genes will "puff," and increase in size. The heat shock genes' active state is marked by a puffing of the chromosome.

Previous work by others:

Boehm et al. (2003) used the *hsp70* gene in *Drosophila melanogaster* to examine the transcription factor and polymerase recruitment and movement. They administered heat shock and used chromatin immunoprecipitation (ChIP) to observe the presence and levels of total RNAPII, RNAPII phosphorylated at Ser-5 or Ser-2, P-TEFb, and HSF at different time points along the segments of the *hsp70* gene. The temporal and spatial recruitment and movement of these factors were mapped.

In uninduced heat shock genes, the RNAPII transcribes 17 to 46 bases and then pauses (O'Brien and Lis, 1993). Boehm noticed the following patterns after heat shocking and examining the amount of protein present along regions of *hsp70*. After heat shock, RNAPII molecules are found throughout the gene, but there is still more RNAPII at the 5' end of the gene than throughout the open reading frame. The releasing of the polymerase from the 5' start site seems to be the rate-determining step. In the absence of heat shock, high of levels unphosphorylated polymerases are detected at the promoter where the polymerase is paused. Ser-5P is found at the transcription start site in non-heat shock conditions, and after activation more Ser-5P is detected at the start site and moves down the gene. Ser-2P, which is not present in non-heat shock, becomes detectable throughout the gene at equal levels. P-TEFb, which phosphorylates Ser-2P, is not present in non-heat shock, but after heat shock becomes detectable; Ser-2P and P-TEFb accompany each other. HSF is only present on the HSE, not on the open gene (Boehm et al., 2003). These patterns of protein movement in *hsp70* can be a source of reference for the study of dosage compensation that will be done in the future.

By identifying the *hsp* gene through cytological experiments, viewing the recruitment of specific proteins to the gene through polytene immunostaining, assessing dosage compensation of the *hsp* gene, and evaluating the *hsp* gene activity through quantitative real-time RT-PCR, this study can determine whether the *hsp28* gene or *hsp83* gene in *D. pseudoobscura* is a suitable model to examine the sequence of events that precede and occur during the interaction of the MSL complex with a transcribing gene.

Materials and Methods

Drosophila pseudoobscura

The *D. pseudoobscura* were raised at 18°C in vials that contained standard corn meal medium, composed of yeast, cornmeal, and etc. New vials were set up every few days. For optimal quality of salivary glands and polytene chromosomes, one male and two females were placed in vials. This avoids the overcrowding conditions of larvae and smaller, lesser quality salivary glands that arise from placing numerous males and females in a vial. The third instar larvae along the vial walls were utilized.

Orcein solution preparation*:* 100 mL of glacial acetic acid was boiled in a beaker. The beaker was removed from heat. 4 g of synthetic orcein and 100 mL of 85% lactic acid were added to the beaker, and mixed with a magnetic stirring rod for 2 hours. The solution was filtered with No. 4 Whatman filter paper over a funnel and collected in a 500 mL flask.

Orcein polytene chromosome staining*:* The salivary glands were dissected from the larvae in 45% acetic acid using forceps. The fatty tissue, head, intestine, and other body parts were removed from the salivary glands. The glands were left in 45% acetic acid for 2 minutes. One drop of orcein was placed on the middle of a siliconized microscope slide. To siliconize the microscope slides, Sigmacote solution was applied on a kimwipe and wiped over the microscope slide. The salivary glands were transferred from the acetic acid to the orcein, and stained for 5 minutes. 20 uL of 1:2:3 solution (lactic acid: water: acetic acid) was placed onto the salivary glands in the one drop of orcein. A 22 mm coverslip was placed over the glands and solution by utilizing forceps to prevent air bubbles. No. 4 Whatman filter paper was folded over the slide to

absorb excess solution and the area where the salivary glands were was tapped with the pencil tip. The breakage of the cells was checked under a light microscope. The chromosomes were spread by tapping with a pencil eraser. The chromosomes were flattened by pressing down on the slide with the thumb. Nail polish was applied around the edges of the coverslip to preserve the slide. The slides were viewed and pictures were taken with the Olympus BX51 System Microscope of Dr. Tao's lab.

Administration of heat shock: Male and female larvae were separated on a dissection wells plate in S2 medium. Males were distinguished from females by the presence of male gonads that appear as transparent holes.

For 2.5 minutes heat shock: Male and female salivary glands were dissected in 330 uL of S2 medium. The salivary glands were transferred into separate tubes. 330 uL of 42°C S2 medium were added to the tubes for heat shock. After 2.5 minutes, 330 uL of 4°C S2 medium were added to the tubes to bring the environment to room temperature and stop further heat shock.

For 20 minutes heat shock: Male and female larvae were placed in separate 1.5 mL tubes that contained moist kimwipe. The tubes were placed in a 37°C water bath for 20 minutes.

Administration of cold shock*:* For dissection under ice, the dissection plate was placed on top of a plastic box containing ice. Male larvae were separated, dissected, and orcein stained in this condition.

For 30 minutes cold shock, the male larvae were placed in a 1.5 mL tube and put into ice for 30 minutes. Dissection and orcein staining followed.

Polytene chromosome immunostaining

Polytene slide preparation: Male third instar larvae were dissected in 0.7% NaCl (solution 1). Dissection in 0.7% NaCl allowed better spreading of the chromosomes than the dissection in Ringer's solution did. This is because Ringer solution is a high salt concentration. Different salt concentrations result in different degrees of compaction. Drops of solution 2 (100 uL of 16% paraformaldehyde, 450uL of acetic acid, and 450uL of water) were placed on a microscope slide. The salivary glands were transferred to solution 2 for 30 seconds. Most of solution 2 was removed with a Kimwipe. Drops of solution 3 (450 uL of acetic acid and 550 uL of H_2O) were added to the salivary glands and left for 10 minutes. A coverslip was placed on top of the salivary glands, using forceps to prevent air bubbles. The nuclei were broken up with the tapping of a pencil point and verified under a light microscope with 10x lens. The chromosomes were spread with the eraser. The general location of the polytene chromosomes was circled on the other side of the microscope slide with a diamond blade. The slide was dipped into liquid nitrogen until the fizzing stopped. The coverslip was removed using a blade.

Note: For long term storage, the slides were placed in a slide rack submerged in a staining dish filled with 67% glycerol, 33% PBS solution at -20°C.
For use right away, the slides were placed in a slide rack submerged in a staining dish filled with 1xPBS.

Polytene slide immunostaining: The slides were washed with water through submergence in a staining dish with distilled water for 2 minutes. The slides were moved into a staining dish with 0.1% Triton X 1xPBS (100 mL of 10xPBS, 900 mL of H_2O, and 1 mL of 10% Triton X-100). The slides were tilted and placed on a paper towel. 0.3 g of Bovine serum albumin (BSA) stored

in 4°C and 10 mL of PBST were mixed in a 15 mL Falcon Conical Centrifuge tube on a vortexer until all the BSA was completely dissolved. 30 uL of 3% BSA was applied, and reapplied if necessary, to the circled area on the slide for 10 minutes. The slides were dipped in PBST and placed back onto the paper towel. The primary antibody solution was made in a 2 mL tube (1 mL of PBST, 1 mL of 3% BSA, 10 uL of MSL antibody (1:200 antibody: solution ratio)). 100 uL of the primary antibody was added to the circled area of the slide for 15 minutes. The slides were washed in PBST for 5 minutes and the coverslips disassociated from the slide in the PBST. The secondary antibody solution was made in a 2 mL tube (1mL of PBST, 1 mL of 3% BSA, 2 uL of secondary antibody (1:1000 antibody: solution ratio)). The prepared secondary antibody solution was kept in the dark. Slides were placed on paper toweling and 100 uL of secondary antibody solution was applied for 20 minutes. A coverslip was placed over the area and the slides were kept in the dark by placing a box over the slides. The slides were washed in PBST for 5 minutes and kept in the dark by placing a box over the staining dish. The slides were tilted and placed on the paper towels to remove excess solution. A drop of 4',6-diamidino-2-phenylindole (DAPI) was applied to the area and a coverslip was placed. The slides were viewed under the Zeiss Axiophot fluorescent microscope and the confocal microscope.

Antibodies: The primary antibodies utilized were the MSL2 antibody raised in rabbit, Ser-2P antibody raised in mouse, and HSF antibody raised in rabbit. The secondary antibodies utilized were anti-rabbit RRX and anti-mouse FIT-C.

Immuno-FISH

The protocol formulated by Grimaud, Bantignies, and Cavalli was used (Grimaud et al., 2005).

In situ hybridization:

Probe labeling: (the preparation of the probes described in this section was performed by Dr. Satish Kallappagoudar). The gene sequences for *hsp28* and *hsp83* were found by searching for their gene regions in *D. melanogaster* on flybase.org. The gene sequences were plugged into the Basic Local Alignment Search Tool (BLAST), which searches the desired fly species genome, in this case *D. pseudoobscura,* and finds the closest sequence. These *hsp* gene sequences on the *D. pseudoobscura* were then ordered from a primer creating company called Integrated DNA Technologies (IDT). Once obtaining the primers, DIG-dUTP probes were made by using the DIG Probe Synthesis Kit.

Hsp28 sequence:
GCAAGGATGGCTTCCAGGTCTGCATGGACGTGTCGCAGTTCAAGCCCAACGAGCTGACTGTGAAAGTG
GTGGACAAGACCGTCGTGGTCGAGGGAAAGCACGAGGAGCGCGAGGACGGGCACGGCATGATTCAGC
GCCACTTTGTGCGCAAGTACACGCTGCCAAAGGACTTTGATCCCAACGAGGTCGTGTCCACTGTCTCCT
CGGACGGCGTGCTCACCCTGAAGGCGCCCCCGCCGCCCAGCAAGGAGCAGCCCAAGCAGGAGCGCAT
CGTTCAGATCCAACAAACCGGTCCTGCGCACTTGAGCGTC

Hsp 83 sequence:
CCCAAGATCGAAGATGTCGGCGAGGATGAGGATGCCGACAAGAAGGACAAGGATGGCAAGAAGAAG
AAGACCATTAAGGAGAAGTACACCGAAGACGAGGAGCTGAACAAGACCAAGCCAATTTGGACCCGCA
ACCCCGATGATATCTCCCAGGAGGAGTACGGCGAGTTCTACAAGTCCTTGACCAACGACTGGGAGGAT
CATCTGTGTGTGAAGCACTTCTCAGTCGAGGGTCAGCTGGAGTTCCGCGCCCTCCTCTTTATCCCCCGT
CGCACTCCCTTCGATCTCTTCGAGAACCAGAAGAAGCGCAACAATATTAAGCTGTACGTGCGCCGTGT
GTTCATCATGGACAACTGCGAGGATCTCATTCCCGAGTACTTGAACTTCATCAAGGGAGTGGTCGACT
CTGAGGATCTGCCTCTGAACATCTCTCGTGAGATGTTGCAGCAGAACAAGGTTCTGAAGGTGATCCGC
AAGAATTTGGTGAAGAAGACCATGGAGCTGATCGAGGAGCTTACCGAGGACAAGGAGAACTACAAGA
AATTCTACGAACAGTTCAGCAAGAACTTGAAATTGGGTGTCCACGAGGACAGCAACAATCGCGCCAA
GCTCGCCGATTTCCTGCGCTTCCACACATCTGCCTCTGGCGATGATTTCTGCTCTTTGTCGGACTACGTG
TCCCGCATGAAGGAGAATCAGAAGCACGTCTACTTCATCACTGGCGAATCCAAGGACCAGGTCAGCA
ACTCTGCCTTCGTTGAGCGTGTGAAGGCCCGCGGCTTCGAGGTTGTCTACATGACCGAGCCCATTGATG
AGTATGTCATCCAGCACTTGAAGGAGTACAAGGGCAAGCAGCTGGTGTCCGTCACCAAAGAAGGTCT
GGAGCTGCCCGAGGATGAAGCCGAGAAGAAGAAGCGTGAGGAAGATAAGGCCAAGTTCGAGGGTCT
CTGCAAGCTGATGAAGTCTATCTTGGACAGCAAAGTGGAGAAGGTGGTCGTCTCCAACCGTCTCGTGG

ACTCGCCCTGCTGCATCGTCACCTCGCAGTTCGGCTGGTCCGCCAACATGGAGCGTATCATGAAGGCC
CAGGCTCTGCGCGACACCGCCACCATGGGCTACATGGCTGGCAAGAAGCAGTTGGAAATCAATCCCG
ACCATCCCATTGTTGAGGCCCTGCGCCAGAAGGCCGATGCCGACAAGAACGACAAGGCTGTCAAGGA
TCTGGTCATCCTTCTGTTCGAGACCTCGCTGCTGTCTTCCGGATTCTCTCTGGACAGCCCCCAGGTTCAT
GCCAGTCGCATTTACCGCATGATCAAGCTGGGTCTTGGCATCGATGAGGATGAGCCCATGACCACCGA
GGACGCCCACAGTGGTGGAGATGCCCCCGGTCTGGTCGAGGACACCGAGGATGCCTCCCACATGGAG
GAAGTCGATTAA

Extraction of polytene chromosomes: Polytene slides were made, following the "polytene chromosome preparation" step mentioned above.

DNA hybridization on polytene chromosomes: The slides were put in a slide container filled with 2xSSC at room temperature, then into a 65.0°C water bath for 45 minutes. The slides were put in containers filled with 70% Ethanol for 10 minutes and then 95% ethanol for 10 minutes. Then the slides were put in containers filled with 0.07M NaOH for 10 minutes and then 2xSSC for 7 minutes. The slides were once again put in containers filled with 70% Ethanol for 10 minutes and then 95% ethanol for 10 minutes. The DIG-dUTP probe was denatured by incubating at 95°C for 5 minutes then placed in ice. Then the probe was moved to 37°C. 12uL of the probe was placed on the slide. Using forceps, coverslips were laid on the slides. The slides were placed in a Genemachines' Hybridization Chamber (water was added to the sides of the slides to humidify) and left overnight at 37°C. The coverslips were removed and slides were put in containers filled with 2xSSC for 15 minutes at 42°C and 5 minutes at room temperature. The slides were placed in a container filled 1xPBS for 15 minutes. The slides were put in blocking solution for 1 hour at room temperature. 20uL of primary antibodies diluted in blocking solution was added to the slides, coverslips were placed on the slides, the slides were placed in hybridization chambers, and left overnight at 37.0°C. The slides were placed in PBS for 15 minutes at room temperature, in PBS with 300mM NaCl, 0.2% NP40, 0.2% Tween20 while

being on a shaker for 15 minutes, then transferred to PBS with 400mM NaCl, 0.2% NP40, 0.2% Tween20 while being on a shaker for 15 minutes, and in PBS for 5 minutes.

Immunodetection:

FISH signal: 20uL of FITC-anti-dUTP antibody was applied to the slides. Coverslips were placed on the slides. The slides were placed in the hybridization chambers and incubated for 1 hour at room temperature in the dark. From this step on, the slides must be left in the dark and this was accomplished by covering with a box. The slides were moved into a container with PBS for 5 minutes and the coverslips were removed.

Immunostaining signal: 20uL of anti-rabbit secondary antibody was applied to the slides. Coverslips were placed on the slides. The slides were placed in the hybridization chambers and incubated for 45 minutes at room temperature. The slides were placed in the container filled with PBS for 5 minutes. The slides were placed in PBS for 15 minutes at room temperature, in PBS with 300mM NaCl, 0.2% NP40, 0.2% Tween20 while being on a shaker for 15 minutes, in PBS with 400mM NaCl, 0.2% NP40, 0.2% Tween20 while being on a shaker for 15 minutes, and in PBS for 5 minutes.

Viewing: One drop of DAPI was applied to the slides for 10 minutes at room temperature. Coverslips were placed on the slides. The slides were viewed under the Zeiss Axiophot fluorescent microscope and confocal microscope.

Quantitative Real-Time RT-PCR with whole larvae

TRIzol RNA isolation of whole larvae*:* Four 1.5 mL test tubes contained one of the following samples: 30minute HS male, 30 minute HS female, NHS male, and NHS female. 300 uL of TRIzol was added to the test tube. Each sample contained 10 larvae. Using a rotor power homogenizer, the larvae were homogenized to break up the tissue. Once the larvae were broken apart, 700 uL of TRIzol were added to the tube, shaken, and left at room temperature for 5 minutes. This was done for each sample. 200 uL of chloroform was added. The tubes were shaken for 15 seconds and left at room temperature for 3 minutes. The samples were centrifuged at 16,100 x g at 4C for 15 minutes; this result in the separation of phases. In a new 1.5 mL tube for each sample, 2 uL of DNase was added. The 500 uL of the aqueous layer was transferred to this new 1.5 mL tube for each sample. The tubes were incubated at 37°C for 30 minutes. 500 uL of isopropanol was added to the aqueous layer. The tube was shaken for 15 seconds and left in room temperature for 10 minutes. The samples were centrifuged at 16,100 x g at 4C for 15 minutes to pellet the RNA. The supernatant was removed and 1 mL of 75% (DEPC-H_2O) ethanol was added. The samples were vortexed for 5 seconds to wash the pellet then centrifuged at 16,1000 x g at 4C for 5 minutes to re-pellet RNA. The supernatant was carefully removed and the pellet was air-dried at room temperature for 10 minutes; the pellet should not be overdried. The pellet was dissolved in 100 uL of DEPC-H2O by pipetting and incubation at 42°C for 10 minutes.

The RNA was quantified by utilizing the NanoDrop2000 spectrophotometer. 1 uL of the sample was loaded onto the pedestal. Three measurements were done and averaged to determine the RNA concentration for each sample. Once the RNA was quantified, the equation M1V1=M2V2 was utilized to dilute and create a 5ng/uL stock.

RT-PCR:

Primers used:

Hsp83F3:GATGAGTATGTCATCCAGCACT

Hsp83R3:AAGATAGACTTCATCAGCTTGCA

Hsp28F2:TGCCAAAGGACTTTGATCCC

Hsp28R2:GACGCTCAAGTGCGCAG

Dpsehsp70BbF:ACAAGCGAAGAGAACACATCT

Dpsehsp70BbR:GCTGCTTCTACTGTCGCAG

DpseRp49F:TGCGTCGTCGCTTCAAGG

DpseRp49R:CGACGATTTCCTTGCGCTTC

Selection of primers: **(the selection of the probes described in this section was performed by Dr. Satish Kallappagoudar).** To determine that the primers are specific, a trial RT-PCR reaction was set up with *D. pseudoobscura* RNA from flies. The product was visualized on a gel and only primers that gave a specific band were taken. Melting curve analysis also told of whether the primers were specific or not.

Calculations for the Mastermix solution: Eight samples needed to be loaded for four primers, and duplicated. 64 wells (8 samples x 4 primers x 2 duplicates) of the 96 Bio-Rad white PCR well-plate were loaded. Each well requires 10 ul: 7 uL of 2x buffer in H_2O, 1 uL of RNA, 1 uL of forward primer, and 1 uL of reverse primer. To make up for any pipetting errors and shortage of liquid, the solution volume per well was increased to 13uL.

Solution preparation: The Bio-Rad iScript One Step RT PCR kit with SYBR Green stored in -20°C was used. The 2x SYBR Green buffer was thawed at room temperature, and then stored on ice. The Reverse Transcriptase was not thawed and placed on ice. The forward and reverse primers were thawed at room temperature. In a 1.5 mL tube labeled "mastermix," 416 uL of the 2x SYBR Green buffer, 207.4 uL of H2O, and 16.64 uL of reverse transcriptase were added (1 uL of reverse transcriptase for every 50 uL of total volume). The mastermix tube was vortexed to mix the contents. The mastermix solution was divided into eight 1.5 mL tubes; tubes were labeled as male, female, 30 minute heat shock male, 30 minute heat shock female, 1 uL of wildtype (wt) *D. pseudoobscura*, 10 uL of wildtype (wt), 100 uL of wildtype (wt), and 1000 uL of wildtype (wt). 8 uL of RNA was added into the eight tubes containing the mastermix, and vortexed: this creates a mastermix solution for each RNA sample.

Loading the well-plate: A Bio-Rad white 96-wells plate was placed on the table. 1 uL of the forward primers was added to all the wells. 1 uL of the reverse primers was added to all the wells. A metal 4°C cold plate was fitted under the well plate. 8 uL of the mastermix with RNA solution was added to its prospective wells. The table below illustrates this.

	Male RNA		Female RNA		30 min. HS male RNA		30 min. HS female RNA	
Hsp83 primer								
Hsp28 primer								
Hsp70 primer								
Rp49 primer								
	1 uL of wt RNA		10 uL of wt RNA		100 uL of wt RNA		1000 uL of wt RNA	
Hsp83 primer								
Hsp28 primer								
Hsp70 primer								
Rp49 primer								

Once all the wells were filled with the necessary reagents, a plastic cover was sealed over the well plate. The well plate was spun down for a few seconds at 600 rpm to amass the liquid at the bottom of the wells. The well plate was loaded into the CFx96 Real-Time PCR Detection System and the Bio-Rad CFX Manager program on the computer was opened. The reaction protocol was entered.

Reaction protocol:

1. 30 minutes at 50°C
2. 5 minutes at 95°C
3. 30 seconds at 95°C
4. 20 seconds at 50°C
5. Read plate
6. Go to step 3 and repeat 39 more times
7. 5 minutes at 72°C
8. 10 seconds at 95°C
9. 5 second intervals from 65.0°C to 95.0°C at 0.5°C increase increments and plate read

1ng/uL, 10 ng/uL, 100 ng/uL, and 1000 ng/uL of RNA from *D. pseudoobscura* were used as standards. A standard curve was made for each primer.

Results

Identification of *hsp28* and *hsp83*

In order to cytologically identify the *hsp* genes in *D. pseudoobscura,* polytene chromosomes of non-heat shocked and of 30 minute heat shocked larvae were prepared and stained with orcein. The XR chromosome was differentiated from the other chromosomes using the cytological maps made by Dobzhansky and Tan (1936). The non-heat shocked polytene chromosome shows the *hsp28* gene in the 40A band region being in an uninduced state (Figure 1A). The *hsp28* gene that lies on the distal portion of the chromosome can be easily identified by the puffing that occurs (Figure 1B).

The *hsp83* gene was located in the same manner, through polytene chromosome staining of non-heat shocked and of heat shocked larvae. This gene is located on the proximal end of the XR chromosome. The non-heat shocked *hsp83* lies in the 24D band region (Figure 2A). The location of *hsp83* becomes apparent by the puffing that occurs after heat shock (Figure 2B).

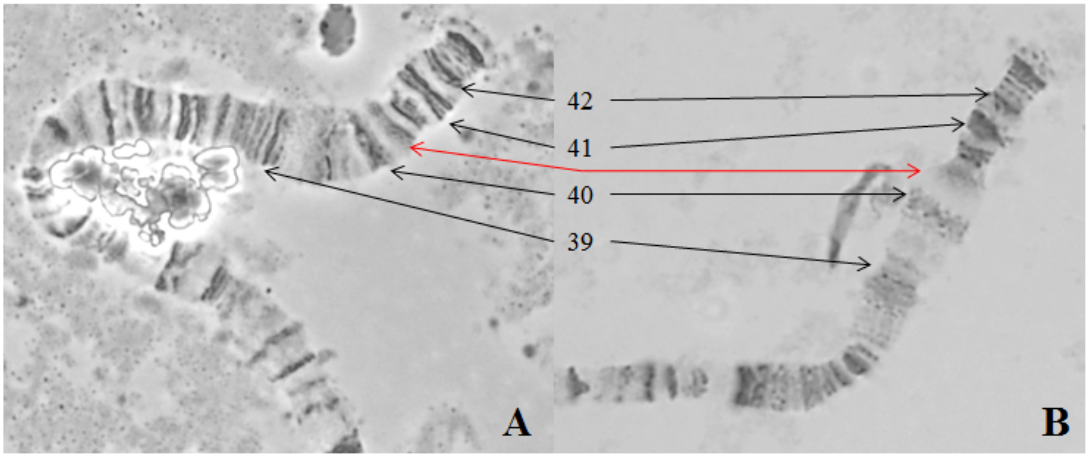

Figure 1 Chromosome orcein staining of the distal XR chromosome of non-heat shocked and heat-shocked larvae. Polytene chromosome squashes stained with orcein from *D. pseudoobscura* third instar male instar larvae that were non-heat shocked (A) and heat shocked (B). The black arrows denote the band regions. The heat shock puff is at the 40A region of the XR chromosome, denoted by the red arrows. This is where the *hsp28* gene lies.

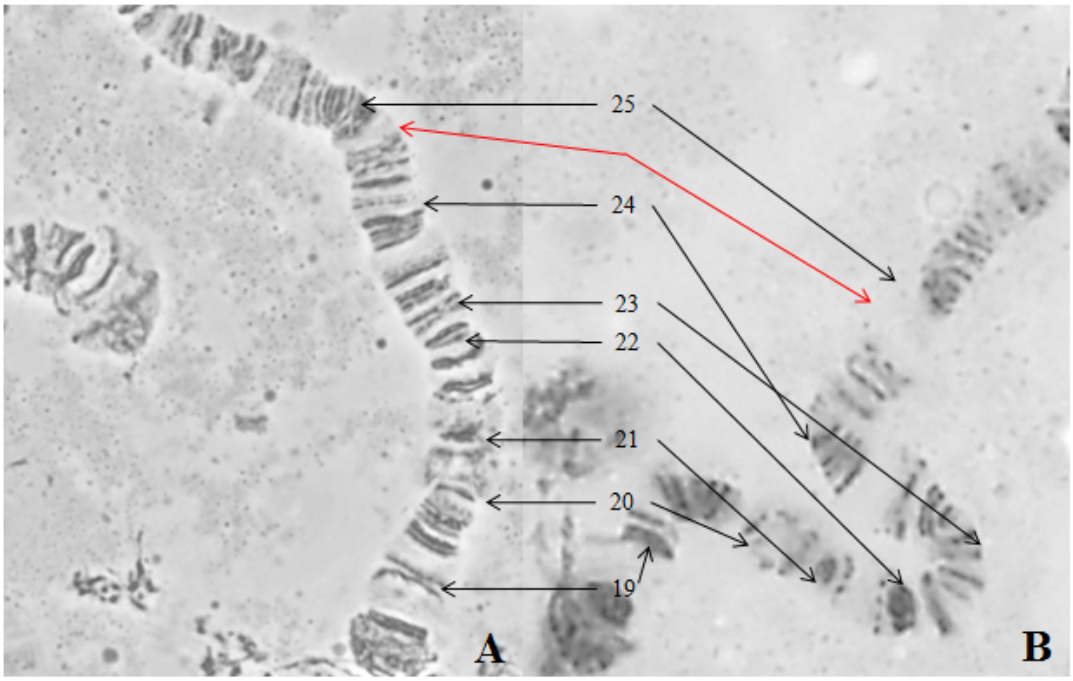

Figure 2 Chromosome orcein staining of the proximal XR chromosome of non-heat shocked and heat-shocked larvae. Polytene chromosome squashes stained with orcein from *D. pseudoobscura* third instar male instar larvae that were non-heat shocked (A) and heat shocked (B). The black arrows denote the band regions. The heat shock puff is at the 24D region of the XR chromosome, denoted by the reds arrow. This is where the *hsp83* gene lies.

The locations of *hsp28* and *hsp83* genes were additionally verified through fluorescence in situ hybridization (FISH). Polytene slides were made from non-heat shocked larvae and fluorescent probes complementary to the DNA sequences of the *hsp28* and *hsp83 genes* were utilized. The location of *hsp28* was marked by the green fluorescent band (Figure 3B). Then the same polytene chromosome slide was stained with orcein, so the bands could be labeled (3A). *Hsp28* lies in the 40A region.

The location of *hsp83* was marked by the green fluorescent band (Figure 4B). Thereafter, Figure 4B was compared to Figure 4A, which is an orcein stained polytene chromosome (Figure 4A). With this comparison, the location of *hsp83* being at the 24D band region was additionally supported.

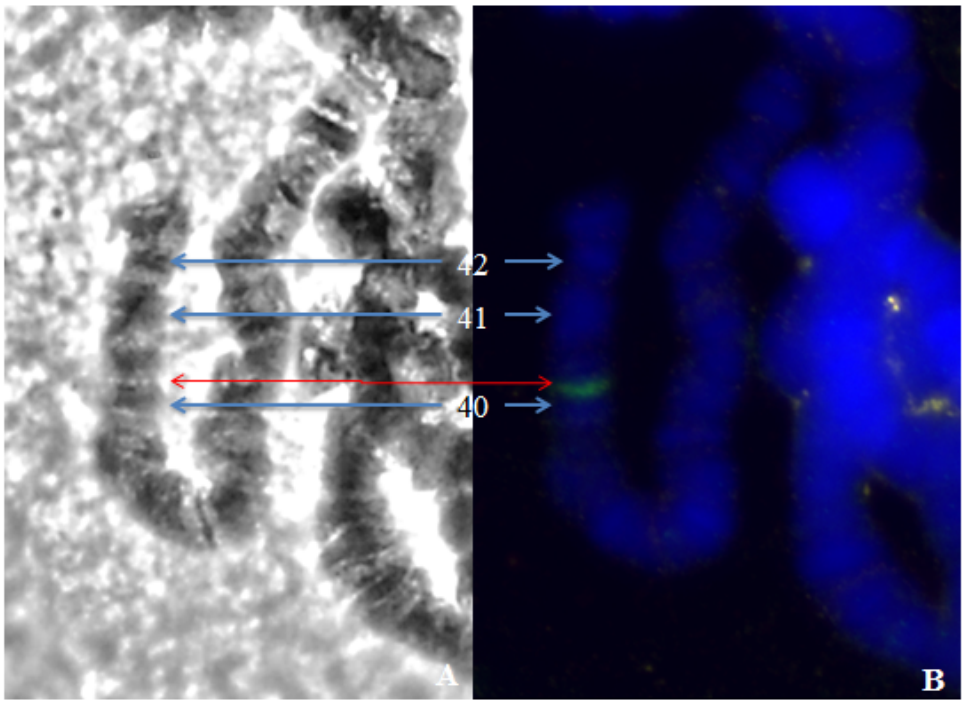

Figure 3 FISH of *hsp28*. Polytene squashes were made from non-heat shocked larvae. The distal XR chromosome is shown in orcein staining (A) and in fluorescence in situ hybridization (B). The blue staining in (B) is DAPI, which stains DNA. The blue arrows denote the band regions. The fluorescent green probe, denoted by the red arrows, is localized to *hsp28*, which is in the 40A region.

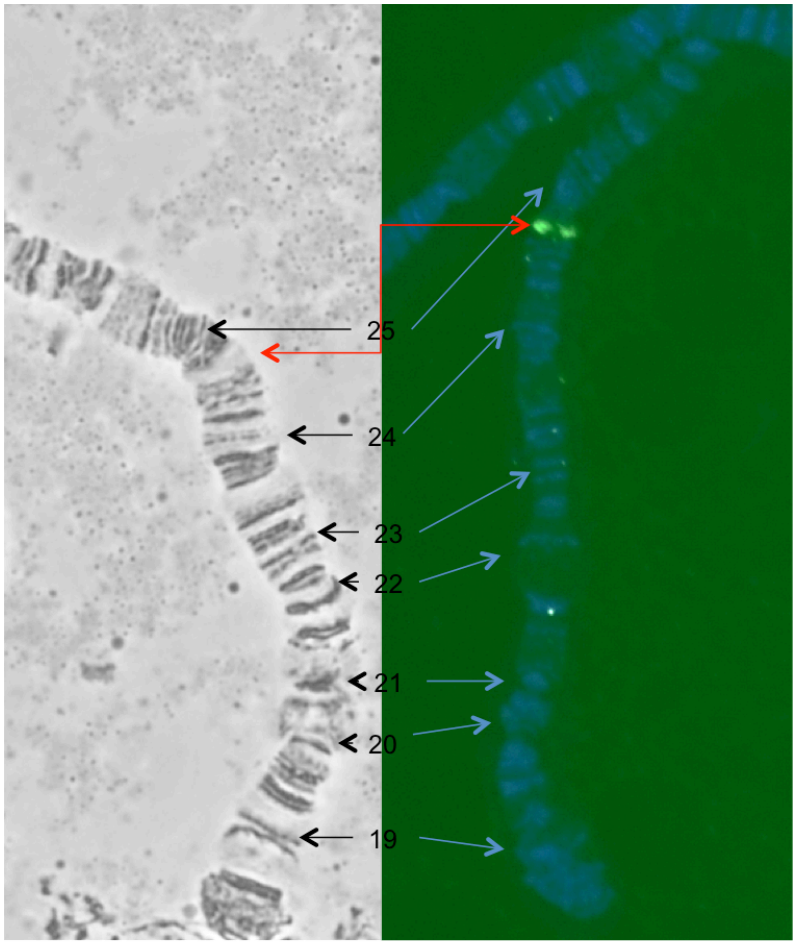

Figure 4 FISH of *hsp83*. Polytene squashes were made from non-heat shocked larvae. The proximal XR chromosome is shown in orcein staining (A) and in FISH(B). The blue staining in (B) is DAPI, which stains DNA. The black and blue arrows denote the band regions. The fluorescent green probe, denoted by the red arrows, is localized to *hsp83,* which is in the 24D region.

Detection of the MSL Complex

In order to detect the MSL complex's presence in immunostaining and for future use to see the MSL complex's movement, an antibody against a major component of the MSL complex is required. The lab carries antibodies against *D. melanogaster's* MSL1, MSL2, MSL3, MLE, MOF, and H4K16ac. Male *D. pseudoobscura* polytene chromosome slides were made and stained for the proteins mentioned. Of the stainings, antibodies against *D. melanogaster*'s MSL2

and MLE worked. In Figure 5, the red MSL2 staining was only present on the X chromosome and not on the autosomes, which had only blue DAPI DNA staining. MLE too was only stained on the X chromosome and not on the autosomes, but to a lesser extent than with anti-MSL2 (Figure 6). These results indicate that there is conservation between the MSL2 and MLE proteins of *D. melanogaster* and *D. pseudoobscura.* When comparing the amino acids between *D. melanogaster* and *D. pseudoobscura* for each protein MSL2 and MLE, there was over 50% conservation. MSL2 antibody was later used for the detection of the MSL complex at the *hsp* gene.

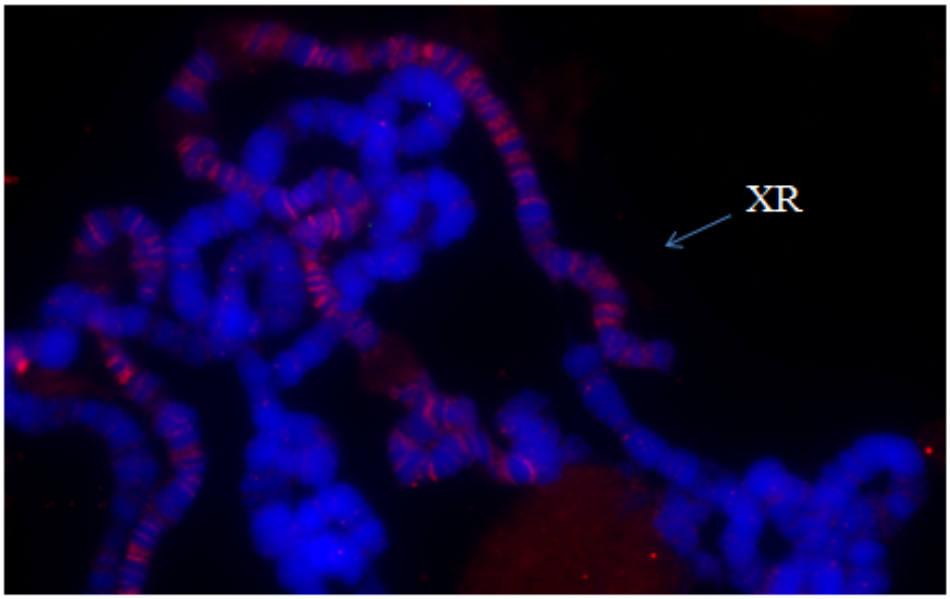

Figure 5 Conservation of MSL2 between *D. melanogaster* and *D. pseudoobscura*. Polytene chromosomes of *D. pseudoobscura* were stained with a MSL2 antibody for *D. melanogaster.* The blue DAPI staining is DNA and the red staining is MSL2. The MSL2 staining occurs only on the X chromosome and not on the autosomes. The arrow denotes the XR chromosome.

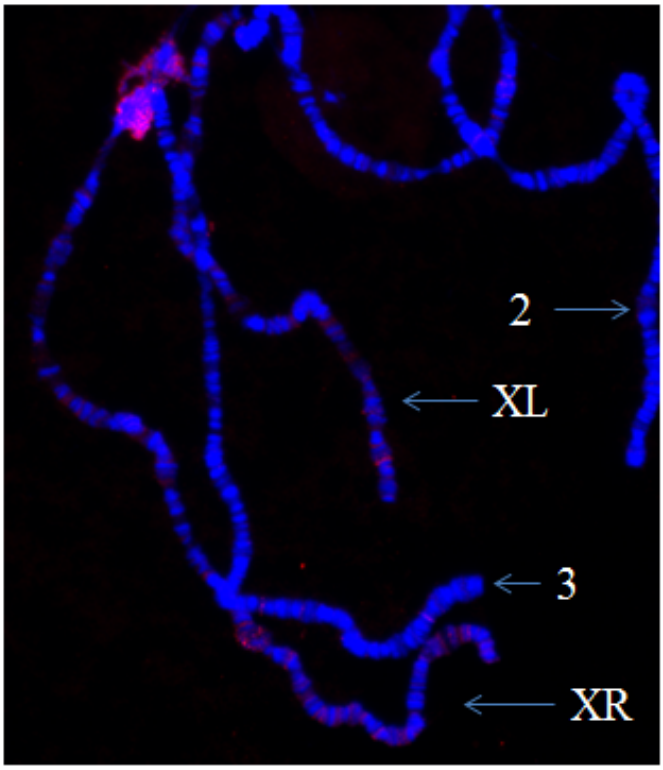

Figure 6 Conservation of MLE between *D. melanogaster* and *D. pseudoobscura*. Polytene chromosomes of *D. pseudoobscura* were stained with a MLE antibody for *D. melanogaster.* The blue DAPI staining is DNA and the red staining is MLE. The MLE staining occurs only on the X chromosome and not on the autosomes. The arrows denote the individual chromosomes.

For the *hsp* gene to be a usable model, the MSL complex must not be present in the uninduced state. A combination of immunostaining to detect MSL2, an indicator of the MSL complex, and FISH to detect the *hsp28* location was carried out. Non-heat shocked male polytene chromosomes were stained with DAPI to detect DNA (Figure 7A), hybridized with *hsp28* fluorescent probe (Figure 7B), and stained for MSL2 (Figure 7C). Figure 7D is an overlap of all the images. The teal hash marks in all four pictures mark the same location, which is right

next to where the *hsp28* fluorescent probe is localized. At this location is the *hsp28* gene and upon careful observation of Figure 7C's MSL2 immunostaining, MSL2 was not stained. So the MSL complex was not present at the *hsp28* gene in the uninduced state.

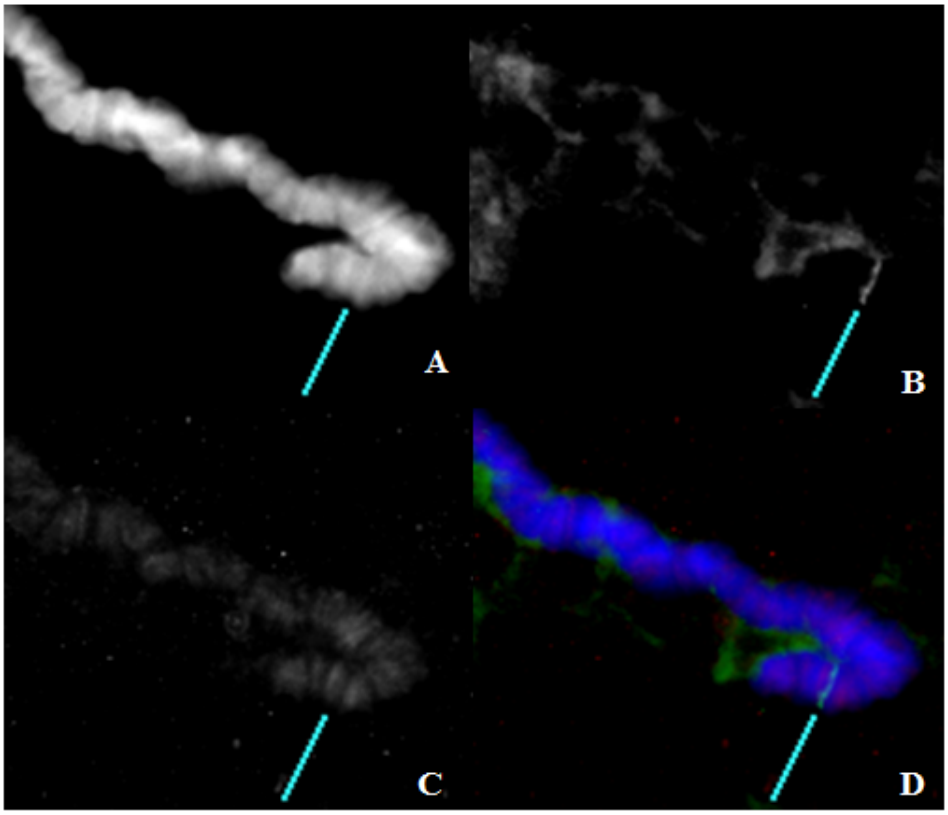

Figure 7 Immuno-FISH of MSL2 protein and *hsp28* gene. Polytene chromosomes from non-heat shocked larvae were stained with DAPI (A), *hsp28* probe (B), and MSL2 (C). (D) is an overlap of all the images. The teal hash marks denote the area the *hsp28* probe localized.

To verify that the *D. melanogaster* HSF antibody (kindly provided by Dr. John T. Lis) works in *D. pseudoobscura,* immunostaining for HSF and FISH of the *hsp28* gene were carried out. Male heat-shocked polytene chromosomes were stained with DAPI (Figure 8A), hybridized with *hsp28* fluorescent probe (Figure 8B), and stained with HSF antibody (Figure 8C). Figure 8D is an overlap of all the images: blue is DAPI and red is HSF antibody. The green bars mark the

location right next to *hsp28* where HSF is clearly active and present. This result shows that HSF is conserved between *D. melanogaster* and *D. pseudoobscura* and that the HSF antibody is usable for the future experiments.

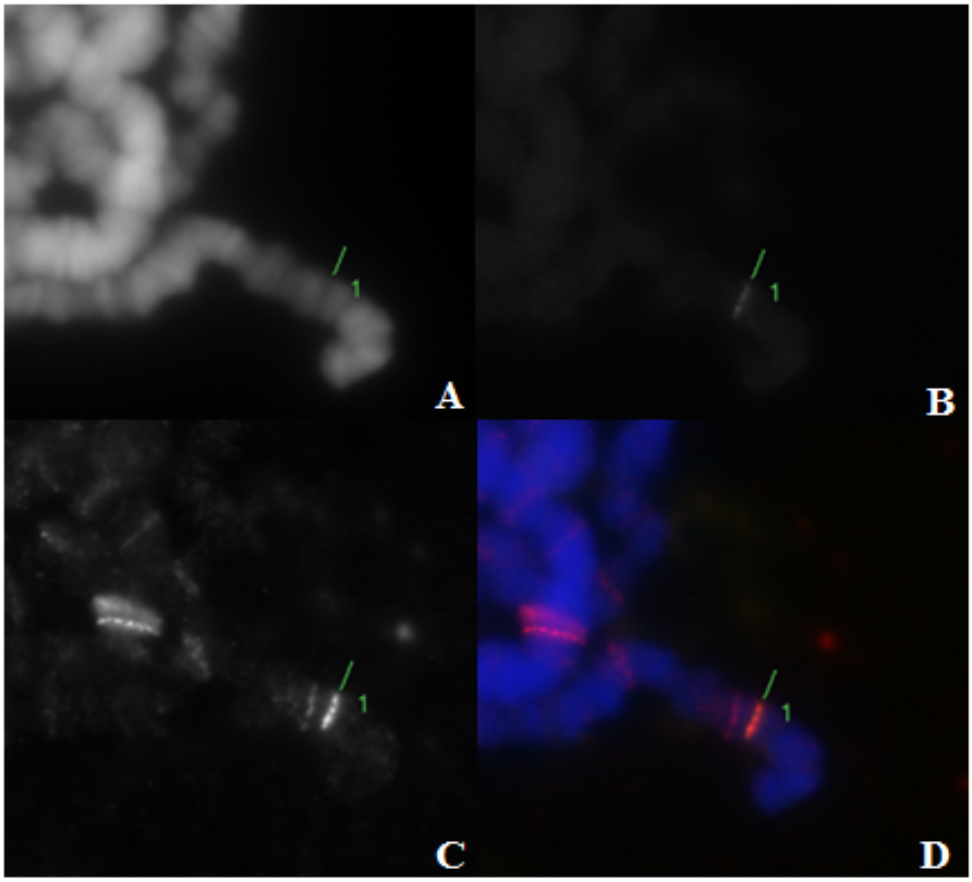

Figure 8 Immuno-FISH of HSF protein and *hsp28* gene. Polytene chromosomes from heat shocked larvae were stained with DAPI (A), *hsp28* probe (B), and HSF antibody (C). (D) is an overlap of all the images. The green hash marks denote the area the *hsp28* probe localized.

Visualizing the sequence of events through the activation of *hsp28*

D. pseudoobscura salivary glands of male non-heat shocked and heat shocked at time intervals of 2.5 minutes and 20 minutes were dissected, and the polytene chromosomes were stained for the following proteins by utilizing antibodies against them: Ser-2 phosphorylation of RNAPII (elongating polymerase), MSL2 (presence of the MSL complex) and HSF. In non-heat shocked polytene chromosomes, MSL2, marked by the red areas, (Figure 9A) Ser-2P, marked by the green areas (Figure 9B) are present in interbands and puffs (Figure 9C, is an overlap of the two images; yellow coloration arises from the overlap of the red and green fluorescence signals) and appear to be absent at the hsp28 locus. The arrow denotes the 40A region where *hsp28* resides.

2.5 min heat shocked polytene chromosomes were as above (Figures10A and 10B). Figure 10C is an overlap of the two images. The arrow denotes the 40A region where *hsp28* resides, which has puffed. MSL2 was still present throughout the interbands, as well as Ser-2P. Upon closer examination, it seems that more Ser-2P is localized in the puffed regions of 40A and the interband preceding 40A.

In the 20 min heat shocked polytene chromosomes the 40A region has puffed. In contrast to the 2.5 min HS, it seems that MSL2 is mostly present in the 40A interband and interband next to it. MSL2 and Ser-2P have heaviest fluorescence intensity localized in the puffed regions of 40A and the interband preceding 40A. Therefore, as heat shock is induced for a longer period of time, more of the MSL complex and transcription elongation are localized specifically in the puffed regions.

The next series of figures depict HSF immunostaining of non-heat shocked chromosomes (Figure 12), 2.5 minutes heat shock (Figure 13), and 20 minutes heat shock (Figure 14). The red staining in the lower portion of Figure 13 shows HSF localized at an autosomal *hsp*. The blue arrow points to the 40A region and the red staining shows the localization of HSF. Oddly, in the non-heat shocked chromosomes, HSF was present in the 40A region; as expected, HSF was present in the 40A region of heat shocked chromosomes. Interestingly, in many of these chromosomes the region preceding 40A has a faint red band, showing that HSF is present there as well. This may be explained by the fact that HSF was found to bind to other HSEs of non-heat shock genes (Gonsalves et al., 2011).

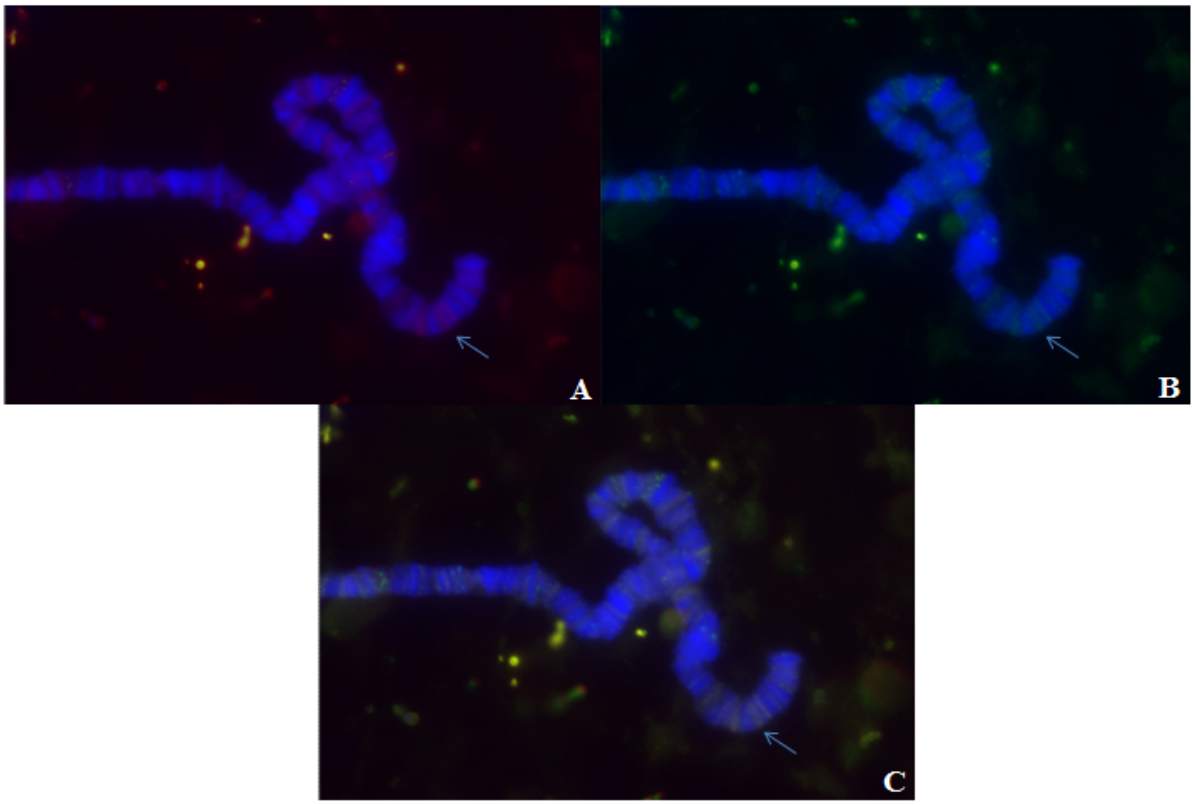

Figure 9 MSL2 and Ser-2P immunostaining of non-heat shocked. Polytene chromosome squashes of non-heat shocked larvae were stained for MSL2 (A) and Ser-2P (B). (C) is an overlap of MSL-2 and Ser-2P. The blue DAPI stained DNA. The blue arrows denote the approximate 40A region.

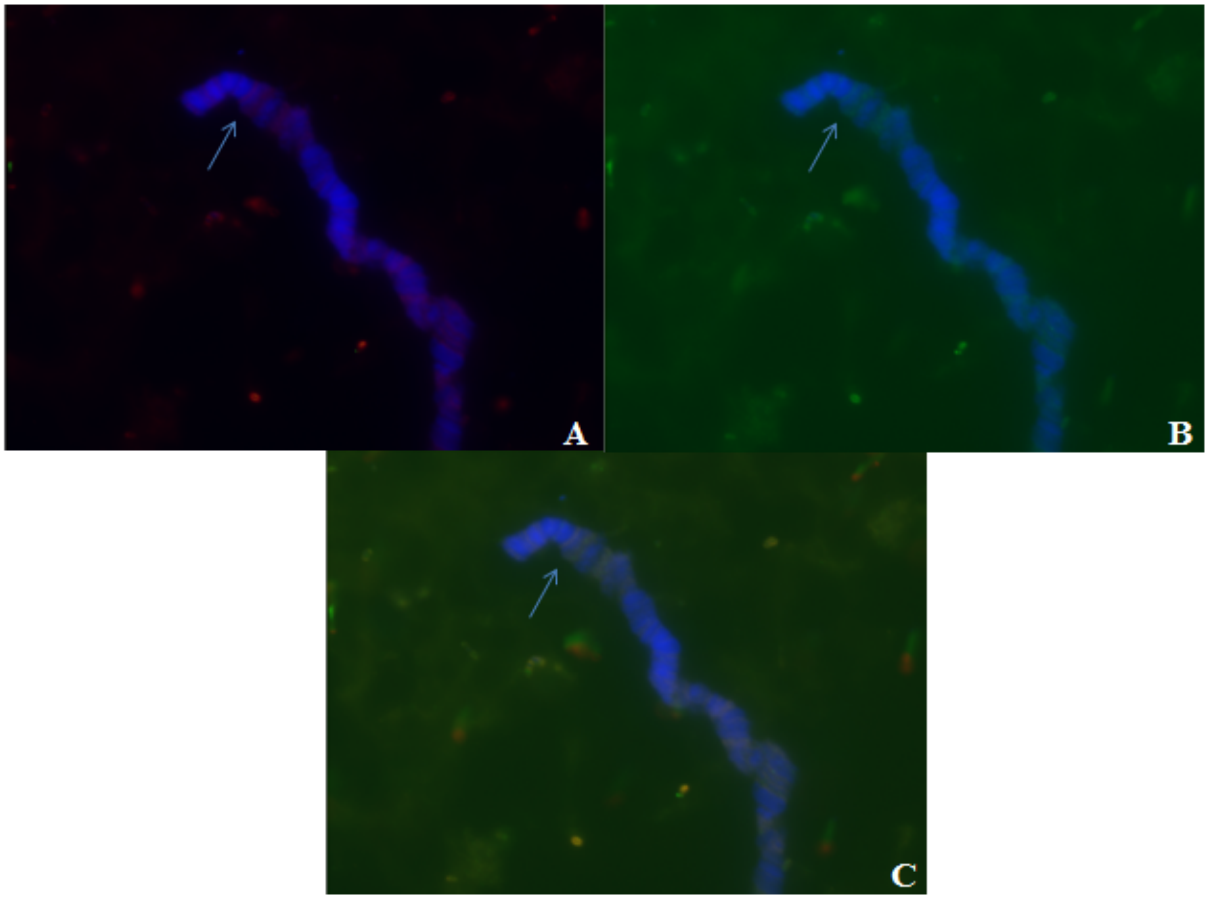

Figure 10 MSL2 and Ser-2P immunostaining of 2.5 minute HS. Polytene chromosome squashes of 2.5 minutes heat shocked larvae were stained for MSL2 (A) and Ser-2P (B). (C) is an overlap of MSL-2 and Ser-2P. The blue DAPI stained DNA. The blue arrows denote the approximate 40A region.

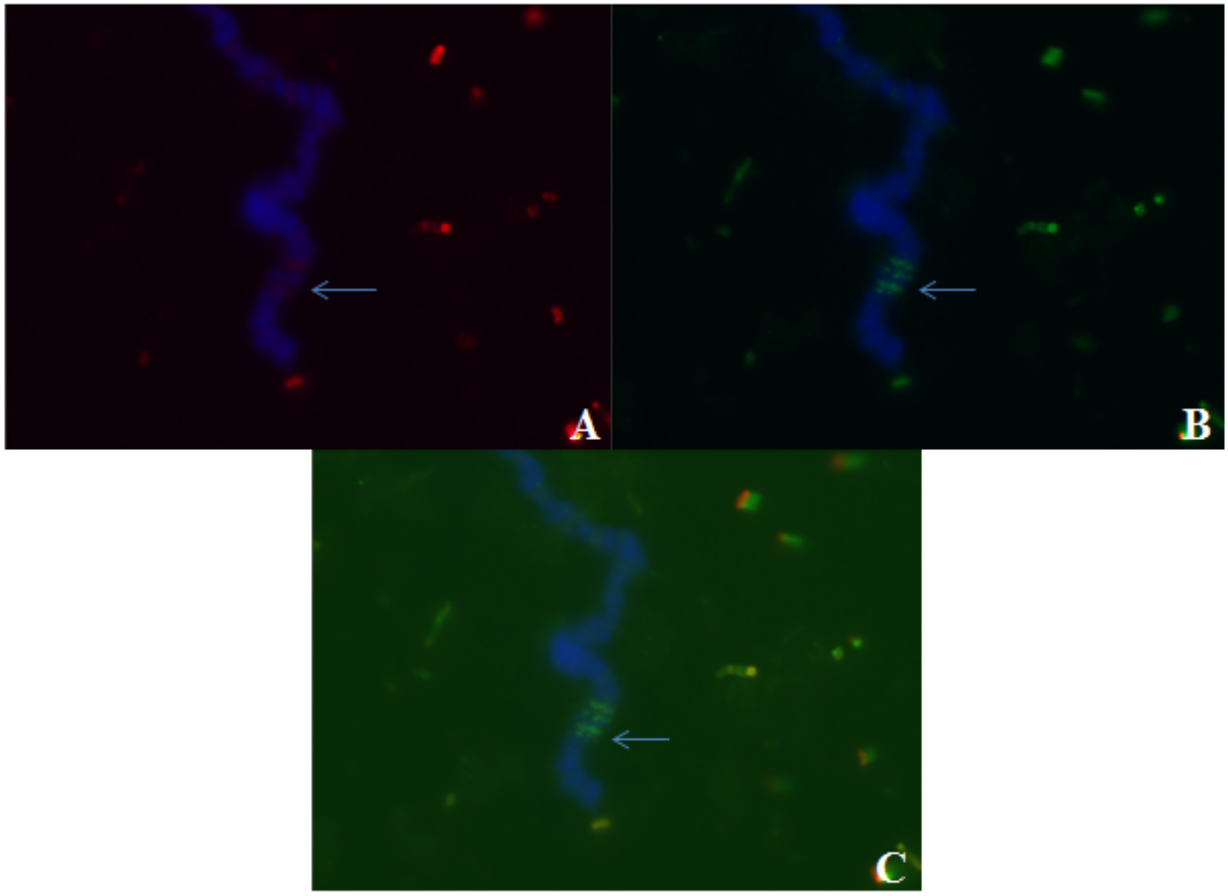

Figure 11 MSL2 and Ser-2P immunostaining of 20 minute HS. Polytene chromosome squashes of 20 minutes heat shocked larvae were stained for MSL2 (A) and Ser-2P (B). (C) is an overlap of MSL-2 and Ser-2P. The blue DAPI stained DNA. The blue arrows denote the approximate 40A region.

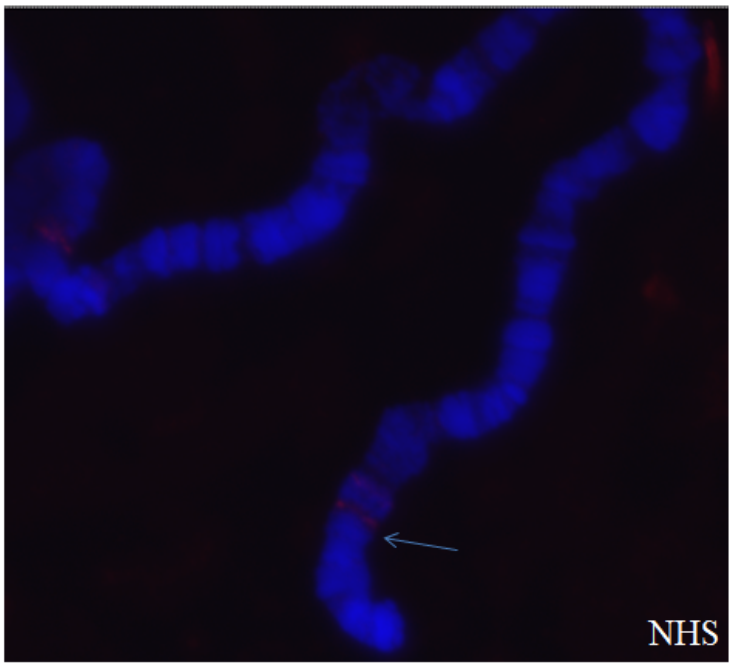

Figure 12 HSF immunostaining of non-heat shocked. Polytene chromosome squashes of non-heat shocked (NHS) larvae were stained for HSF. The blue DAPI stained DNA. The blue arrow denotes the approximate 40A region.

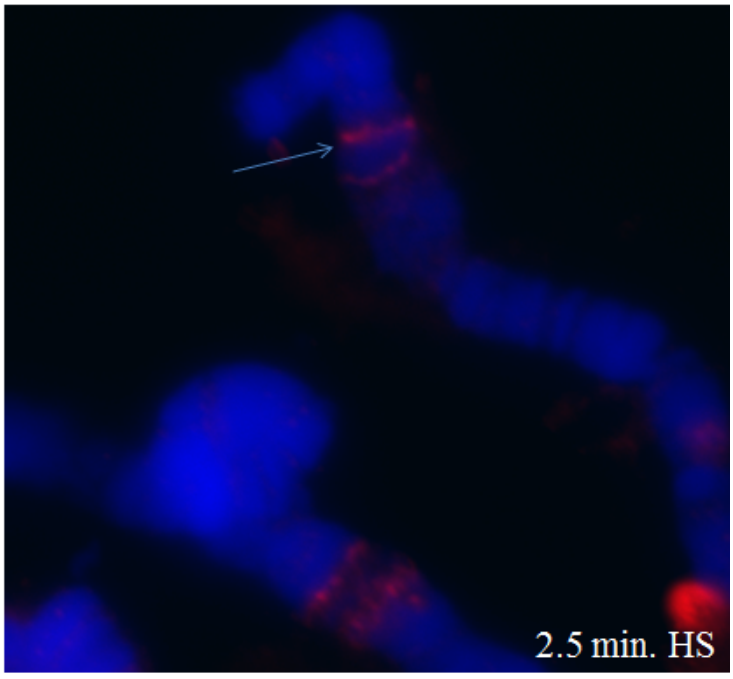

Figure 13 HSF immunostaining of 2.5 minutes heat shocked. Polytene chromosome squashes of 2.5 minutes heat shocked larvae were stained for HSF. The blue DAPI stained DNA. The blue arrow denotes the approximate 40A region.

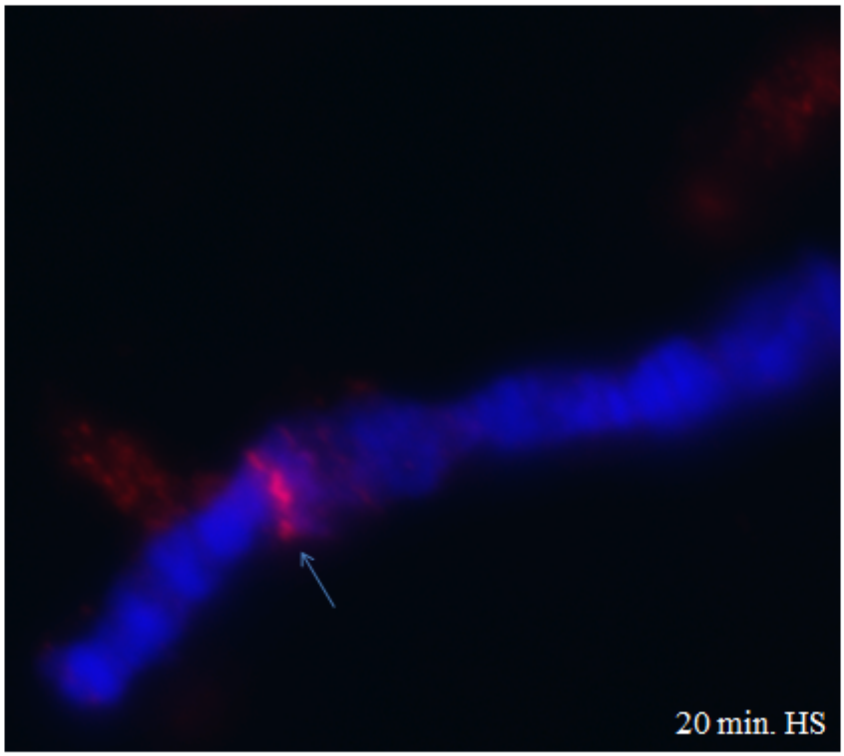

Figure 14 HSF immunostaining of 20 minutes heat shocked. Polytene chromosome squashes of 20 minutes heat shocked larvae were stained for HSF. The blue DAPI stained DNA. The blue arrow denotes the approximate 40A region.

Dosage compensation and gene expression levels of *hsp28* and *hsp83*

A way to see if there is dosage compensation is by checking the RNA ratio between male larvae to female larvae; males need to produce equal amounts of RNA as females do. Following heat shock, the expression of *hsp28* and *hsp83* was increased only by approximately 2 fold (Figure 15). In contrast, the autosomal *hsp 70* gene that we measured as a control exhibited an increase in gene expression of approximately 60 fold (data not shown). After heat shock, the male to female ratios for *hsp28* and *hsp83* are 1.05 and 0.95, respectively (Figure 16). Interestingly, the non-heat shocked male to female ratios for *hsp28* and *hsp83* are 0.99 and 1.07, respectively (Figure 17). This result indicates that the expression of the two non-heat shocked

genes is dosage compensated although the presence of the MSL complex could not be demonstrated by immunofluorescence (Figure 7).

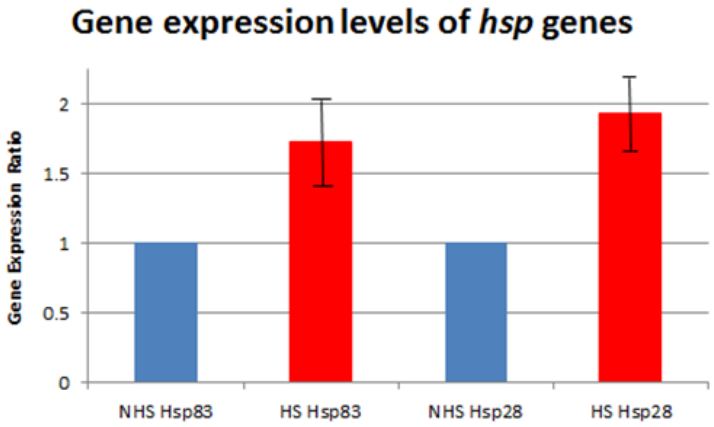

Figure 15 Gene expression levels of *hsp* genes. The RNA from heat-shocked genes were normalized to RNA from non-heat shocked genes. The non-heat shocked genes had gene expression ratios of 1 because they were normalized to their RNA levels. The standard error bars were included for heat shocked *hsp* genes.

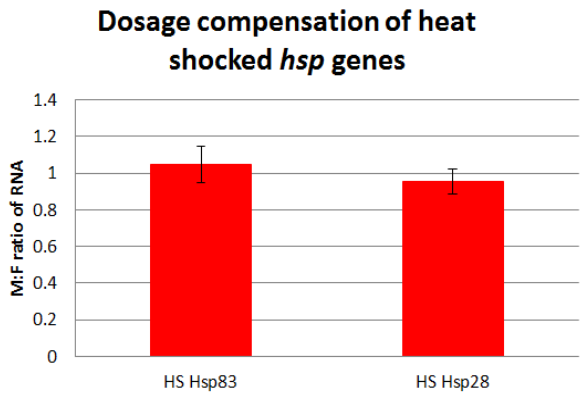

Figure 16 Dosage compensation of heat shocked *hsp* genes. The RNA ratio between heat shocked male and female is listed on the y-axis. This ratio was done for *hsp28* and hsp83. The standard error bars were inserted.

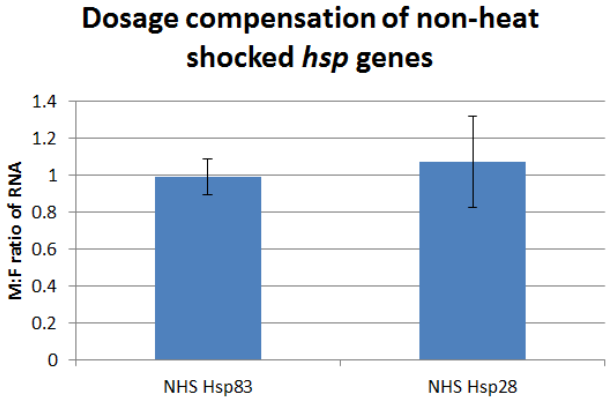

Figure 17 Dosage compensation of non-heat shocked *hsp* genes. The RNA ratio between non-heat shocked male and female is listed on the y-axis. This ratio was done for *hsp28* and hsp83. The standard error bars were inserted.

Cold shock

Because of the HSF localization to the *hsp* gene in the non-heat shocked polytenes and the lower than expected fold increase of gene expressions of the *hsp28* and *hsp83,* the idea that sorting the larvae and dissecting the salivary glands over ice might prevent spurious activation of the heat shock genes under non heat shock conditions was tested. Male larvae were sorted, dissected, and stained with orcein over ice (Figure 18B) and compared to the non-heat shocked polytenes of Figure 18A. The 4°C environment seems to induce the same level of activation of hsp as does handling and dissection at room temperature. Male larvae were also cold-shocked for 30 minutes and then the polytene chromosomes were compared. Figure 18C depicts the outcome of the 30 minute cold shock. When comparing the cold-shocked polytene to the heat-shocked polytene in Figure 18D, the puff resulting from cold shock at 40A is induced though not to the extent that results from heat shock. Therefore, cold shock followed by dissecting larvae over ice would not help attain the non-heat shock, inactive state of the *hsp28*.

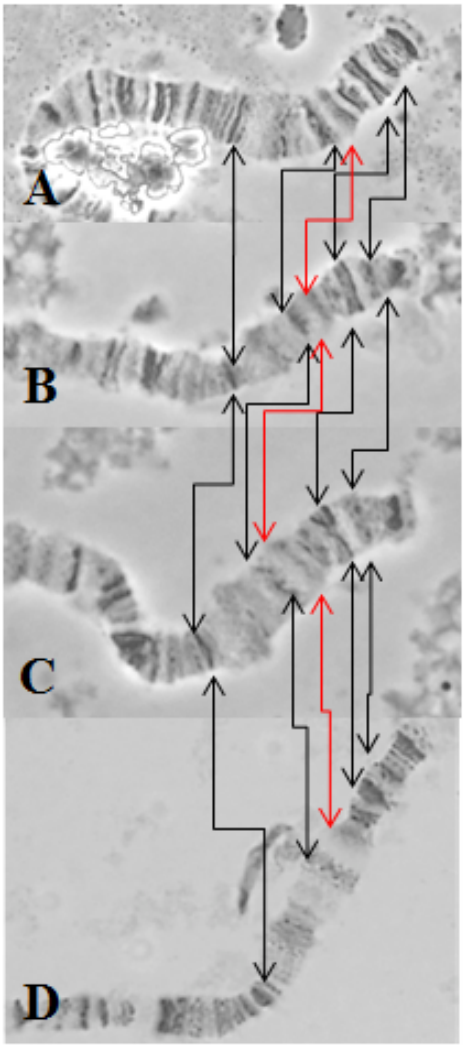

Figure 18 Chromosome orcein staining of the XR chromosome of non-heat shocked, dissected under ice, cold shocked, and heat-shocked larvae. Polytene chromosome squashes stained with orcein from *D. pseudoobscura* third instar male instar larvae that were non-heat shocked (A), dissected under ice (B), cold shocked for 30 minutes (C), and heat shocked for 30 minutes (D) The black arrows denote the band regions. The red arrows denote the 40A region where the puffing occurs.

DISCUSSION

Heat-shock activation

The cytological work presented here provides evidence that upon heat shock the *hsp* genes become more transcriptionally active. Polytene chromosome immunostainings for the presence of the MSL complex, which acetylates H4K16 to loosen the chromatin, and of Serine 2 phosphorylation of RNAPII, which is an indicator of active transcriptional elongation, support this. As the time of heat shock increases, the presence of the MSL complex and Serine 2 phosphorylation of RNAPII increases at the *hsp* genes, which means that the MSL complex is changing the chromatin structure and RNAPII is elongating, sure signs of transcription. The orcein-stained polytene chromosomes also support the finding that there is transcriptional activation occurring by the relative increase in puffing, which is another sign of an increase in transcription.

Dosage compensation of the *hsp28* and *hsp83* genes

An RNA ratio between male and female at a specific gene on the X chromosome of about 1 means that the male is producing equal amounts of transcripts as the female; in other words, the gene is dosage compensated. Dosage compensation of *hsp28* and *hsp83* in heat-shocked larvae is an encouraging result but the dosage compensation of these genes in non-heat shocked larvae suggests that some level of transcription is occurring and that the MSL complex is recruited and working. Yet, the immuno-FISH of MSL2 and *hsp28* gene shows that when no heat shock is applied, the *hsp28* gene does not appear to have recruited the MSL complex. This inconsistency may be due to the relatively low resolution obtained with immunofluorescence.

Occurrence of some activation in non-heat shocked larvae

The immunostaining of HSF in non-heat shocked polytene chromosomes is interesting, because there is HSF binding to the 40A region, which can be assumed to be binding to the HSEs of *hsp28*. O'Brien and Lis (1993) state that the HSF in uninduced cells is kept inactive and is unable to bind to HSEs *in vitro*. However, Boehm et al. (2003) did find low levels of HSF associated with *hsp70* in non-heat shocked larvae by immunostaining. Guertin and Lis (2010) uphold this finding in stating that HSF is inactive yet is meagerly bound to HSEs in unstressed cells. So although some HSF binding is normal, its association with the non-heat shocked *hsp28* appears abnormally high indicating the possibility that there is some stress preceding the polytene chromosome preparation. In the activation of *D. melanogaster hsp* genes, HSF recruitment is detectable as early as 5 seconds of heat shock and is maximal by 75 seconds (Boehm et al., 2003). Our results indicate that the MSL2 recruitment and Ser-2P occurrence happen at a later time during heat shock induction. The quantitative real-time RT PCR data seems to reinforce the idea that some stress is being induced in the non-heat shocked larvae.

The data reveal that the heat shock increases *hsp28 and hsp83* transcription by 2 fold and of *hsp70* by approximately 60 fold. The pattern of fold increase aligns with previous reports that in *D. melanogaster*, the increase in *hsp70* expression is greater than those of *hsp28* and *hsp83* (Shopland and Lis, 1996). Gonsalves et al. (2011) administered 30 minutes of heat shock to third instar larvae and found increases in gene expression for *hsp28, hsp83,* and *hsp70Bb* to be 7.5 fold, 15.1 fold, and 103.3 fold, respectively. In comparison to these data, our increase in expression of the same genes in *D. pseudoobscura* levels is significantly lower. It appears that some level of activation and transcription is already occurring in our non-heat shocked larvae. The *D. pseudoobscura* larvae, raised in 18°C, are exposed to the lab room temperature 24-25°C

for some time, because male and female larvae need to be separated. This temperature shift in the larvae's environment probably induces some expression, and may be an explanation for the presence of some puffing in a few orcein-stained and immunostained polytene chromosomes from non-heat shocked larvae. The larvae to be used in qRT-PCR are exposed to 24-25°C for a longer duration than those used for polytene stainings: separating 10 male and 10 female larvae takes a longer time than finding a single male larva for polytene chromosome staining. So the degree of *hsp* activation in the non-heat shocked larvae utilized in the qRT-PCR might be higher than the degree of *hsp* activation in the non-heat shocked larvae used in polytene stainings.

In the lab, the change in the living condition temperature from 18C to 24-25°C or vice-versa seems to be stressful to *D. pseudoobscura*. After raising at the 18°C temperature for several generations, the flies did not reproduce well when maintained in culture at 24-25°C. *D. pseudoobscura* raised 24-25°C for several generations did not reproduce well when transferred to 18°C. The inability to adapt and reproduce in the new temperature environment might stem from being stressed for a long duration, which activates the *hsps*. I believe that there can be some activation in the 18°C larvae when they are brought to the lab room temperature of 24.4°C. Lerman and Feder (2001) observed that HSF induction takes place at lower temperatures in organisms inhabiting colder environments than in those from warmer environments. Three *D. melanogaster* stocks raised at 18°C, 25°C, and 28°C were heat shocked with temperatures ranging between 25°C and 38°C for 1 hour. At lower temperatures, flies raised in 18°C activated more HSF than flies raised in 25°C and 28°C did (Lerman and Feder, 2001). This HSF activation is synonymous with *hsp* activation.

An option for future studies would be to separate male and female larvae and carry out all of the experimental manipulations at 18°C. An alternative would be to attempt to adapt the flies to room temperature by raising them at 24-25°C.

Which *hsp* gene to use for the study of dosage compensation?

D. melanogaster Schneider 2 cells that are normally maintained at 23°C, were incubated at different temperatures (23°C, 26°C, 29°C, 31°C, 33°C, 35°C, 37°C, or 38°C for 1 hour) and the heat-shock proteins displayed on electrophoretic gels. These experiments revealed that *hsp* genes have their individual induction characteristics, different temperatures of maximal induction, different ranges of temperature in which induction occurs, and different kinetics of induction (Lindquist, 1980). Interestingly, the presence of different heat shock proteins varied upon temperature. Hsp83 protein was detected at 26°C, which is only 3°C above the 23°C normal cell culture temperature. Hsp83 was also present at 23°C, but to a lesser degree. It has a broad induction range from 26°C to 37°C. Hsp28 has a narrow induction range of 35°C-37°C, 35°C being optimal. To go along with the fact that *hsp70* is the most highly induced gene, Hsp70 was produced significantly higher than any other Hsp proteins and was maximally induced at 37°C; lower levels were detected from 26°C to 33°C.

There are differences between *hsp83* and *hsp28*. *Hsp83* is constitutively expressed in non-heat shocked *D. melanogaster* and *D. pseudoobscura,* and upon heat shock is further induced several fold (O'Connor and Lis, 1981; Blackman and Meselson, 1986; Lindquist and Craig, 1988). Its continuous expression and increased expression upon heat shock reveal that it functions in both housekeeping and stress regulation. Pauli et al, (1990) performed in situ hybridization with *hsp28* and found that in the first, second, and third instar larvae, *hsp28* was

only present in the brain and gonads. Later in the late third instar larvae and prepupa stage, *hsp28* is also present in imaginal discs, due to the induction by ecdysterone, a molting hormone (Lindquist, 1986; Pauli et al, 1990; Zimmerman et al., 1983). Avoiding the late third instar larvae stage and having the flies acclimate to 24.4°C could enable the use of *hsp28* as a gene that can be quickly activated and used to study when and where various proteins, such as those of the MSL complex, interact along the gene.

MSL Complex's role in transcription upregulation

It is unknown how dosage compensation upregulates transcription. Since the escape of Pol II is the rate-limiting step in transcription speed, a possible answer is that the MSL complex reduces RNAPII pausing in compensated genes to increase transcription and produce equal amounts of RNA. *Hsp70,* the prototypical example, and *hsp28* have been shown to possess paused RNAPII (O'Brien and Lis, 1993). In recent years, it has been revealed that many genes in *Drosophila* have paused RNAPII at the promoter region (Lis, 2007). Therefore, RNAPII stalling is not only specific to *hsp* genes, but is widespread, taking place at hundreds of genes that react to stimuli and developmental signals (Muse et al., 2007). Using this model of *hsp28* in *D. pseudoobscura* would allow investigation of whether the MSL complex does cause a change in RNAPII pausing levels.

Conclusion

After appropriate modifications to ensure the inactive state in *hsp28* of non-heat shocked larvae, the dosage compensated *hsp28* in *D. pseudoobscura* can be used to investigate the sequence of events in transcription and give a better understanding of where the MSL complex is recruited and how it enhances transcription.

FUTURE DIRECTION

(1) Determine experimental conditions to minimize the non-heat shock induction and maximize the difference in gene expression following heat shock. For QRT-PCR done on the 18°C raised larvae non-heat shock activation could be minimized by taking one larva at a time, sorting the sex, and flash freezing the larva in liquid nitrogen. As an alternative, I would like to raise *D. pseudoobscura* at 24-25°C for several generations, so the flies acclimate to the new temperature setting. Immunostaining for HSF on non-heat shocked polytene chromosomes would reveal if this factor's localization to *hsp28* weakens or disappears over time. Once the *D. pseudoobscura* that have become accustomed to 24-25°C, RNA would be extracted from heat shocked and non-heat shocked larvae and quantitative real time RT-PCR would be performed to determine the increase in expression following heat shock.

(2) Repeat the detection of the MSL complex, of RNAPII phosphorylated at S5 (paused) and S2 (elongating), of H4K16Ac and P-TEFb using chromatin immunoprecipitation (ChIP).

(3) As for determining whether the MSL complex reduces pausing, the ratio of elongating to total RNAPII along the hsp28 gene following induction would be measured in males and females. ChIP would be done with antibodies for Ser-5P and Ser-2P and then quantitative real time PCR with *hsp28* primers to measure the quantity at various points across the gene. The DNA level ratio between Ser-5P (paused polymerase) to the total polymerase (Ser-5p+Ser2P) would be a way to measure the level of pausing. If there is less pausing, the ratio would get smaller.

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