ROLE OF TRANSCRIPTION FACTORS IN PAUSING RNA POLYMERASE II IN DROSOPHILA.

A Dissertation in
Biochemistry, Microbiology and Molecular Biology
by
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ABSTRACT

Promoter proximal pausing of RNA Polymerase II (Pol II) occurs on thousands of genes in animal cells. This pausing often correlates with rapid induction of genes, but direct tests of the relationship between pausing and induction rates are lacking. Hsp70 and hsp26 in Drosophila are rapidly induced by heat shock. Contrary to current expectations, depletion of NELF, a key factor in setting up the paused Pol II, disrupted pausing but did not interfere with rapid induction. Instead, depletion of NELF delayed the time when these genes shut off during recovery from heat shock. Further analyses showed that NELF-depletion delayed the dissociation of HSF from hsp70 and hsp26, and a similar delay was observed when CBP was depleted from cells. CBP has been reported to associate with Pol II, and acetylation of HSF by CBP has been implicated in dissociating HSF from heat shock genes. I propose that NELF-mediated pausing allows Pol II to direct CBP-mediated acetylation of HSF, thus causing HSF to dissociate from the gene. Activators are typically viewed as controlling Pol II transcription. My results reveal a reciprocal relationship in which the pausing of Pol II by NELF influences the interaction of the activator.

We have also observed that pausing on hsp70 can be shifted towards the transcription start site (TSS) in a cell free system by reducing the nucleotide concentration. To analyze whether the elongation rate of the Pol II affected where Pol II paused in vivo, permanganate footprinting of the salivary glands of wild type and a slow Pol II mutant polymerase was performed. My results showed that there is an upstream shift of the slower moving Pol II. This revealed that the location of NELF mediated pause
is dictated by the rate of elongation, which in turn may result from the kinetic competition between the rate of elongation and the rate of binding by negative regulators.

NELF also influences the expression of many genes, wherein depleting NELF down-regulated as well as up-regulated the expression of these target genes. Here I have compared using permanganate genomic mapping the effect of NELF depletion on Pol II pausing at NELF target genes in *Drosophila* salivary glands and tissue culture cells. This study demonstrated that *Drosophila* salivary glands can be used as a model tissue for investigating effects of factors regulating paused Pol II.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
</tr>
</tbody>
</table>

**Chapter 1. Introduction**......................................................................................... 1

Summary of transcriptional elongation ........................................................................ 1
Overview of proteins involved in promoter proximal pausing .................................. 2
   Negative Elongation Factor .................................................................................... 2
   DRB Sensitivity Inducing Factor .......................................................................... 6
   Positive Transcription Elongation Factor b ....................................................... 8
DNA binding factors that affect *hsp70* transcription ............................................ 10
   Heat shock factor ................................................................................................. 10
   GAGA factor .......................................................................................................... 12
   Transcription Elongation Factor IIS ................................................................... 14
Promoter proximal pausing .......................................................................................... 16
   Genomewide prevalence of promoter proximal pausing .................................... 16
   Promoter proximal pausing in *Drosophila* heat shock gene *hsp70* .................. 17
Scope and significance of dissertation .................................................................... 20

**Chapter 2. Materials and Methods** ........................................................................ 22

   Chromatin Immunoprecipitation ............................................................................ 22
   Western Blotting ..................................................................................................... 25
   Quantitative Real-time PCR analysis ................................................................... 26
   Reverse transcription PCR analysis .................................................................... 26
   Immunofluorescence analysis of polytene chromosomes .................................... 27
   RNA interference mediated depletion of NELF-D, NELF-E, CBP, Brm and TFIIS ........ 29
   Permanganate genomic footprinting analysis ..................................................... 31
   HSF Binding Assay ............................................................................................... 34

**Chapter 3. Permanganate Genomic Mapping of Genes with Paused Pol II** .......... 36

   Introduction .......................................................................................................... 36
   Results .................................................................................................................... 42
      RNA interference mediated depletion of NELF in salivary glands .................... 42
Down-regulated genes lose paused Pol II from promoter proximal
regions upon NELF depletion .......................................................... 42
Effect of NELF depletion on Pol II elongation in up-regulated genes .... 53
In vivo analysis of the effect of the rate of elongation on the position of
Pol II pausing .................................................................................... 57
Depletion of TFIIS in Drosophila S2R+ tissue culture cells ............... 65
Permanganate footprinting analysis of TFIIS depleted cells .......... 69
Discussion ......................................................................................... 73
Permanganate genomic footprinting of genes regulated by NELF
mediated paused Pol II in Drosophila salivary glands and S2 cells .... 73
Test if depletion of TFIIS may shift site of promoter proximal pausing.... 74
Kinetics of promoter proximal pausing ........................................... 76

Chapter 4. Negative ELongation Factor, NELF, accelerates the rate at which
heat shock genes are shut-off by facilitating the dissociation of heat shock
factor .......................................................................................... 78

Introduction ..................................................................................... 78
Results .............................................................................................. 79
Depletion of NELF disrupts promoter proximal pausing but does not
significantly impact the rate of heat shock induction ..................... 79
Depletion of NELF impairs the shut-off of hsp70 during recovery from
heat shock ......................................................................................... 87
Failure to pause during recovery from heat shock results in hyper-
expression of hsp70 following a brief heat shock ......................... 95
Depletion of NELF impairs dissociation of HSF during recovery from
heat shock ......................................................................................... 97
Disruption of promoter proximal pausing alters interaction of sequence
specific regulators of hsp70 ......................................................... 100
Depletion of NELF slows the rates of shut-off of transcription and alters
interactions of transcription regulators, HSF and GAF, for hsp26 .... 103
Depletion of NELF has little impact on the binding activity of HSF in
cell extracts ..................................................................................... 110
RNAi-mediated depletion of CBP interferes with the dissociation of HSF
from the heat shock genes during recovery from heat shock ........ 114
Depletion of CBP impairs the shut-off of hsp70 and hsp26 during
recovery from heat shock ................................................................. 119
Discussion ...................................................................................... 122
Disruption of promoter proximal pausing on hsp70 does not alter the rate
of heat shock induction .................................................................. 122
NELF functions in fine-tuning gene expression ......................... 123
Paused Pol II promotes dissociation of HSF from the heat shock genes
during recovery from heat shock .................................................. 124
Appendix A. List of LM-PCR primers ................................................................. 128

Appendix B. List of Quantitative Real-time PCR and RNAi primers ................. 131

Appendix C. Evaluate the dynamics of paused Pol II association with DNA ........ 133

Introduction ........................................................................................................... 133

Exploring the dynamics of the paused Pol II ....................................................... 133

Results ................................................................................................................. 136

Effect of α-amanitin on Pol II .............................................................................. 136

Association of paused Pol II with the DNA ......................................................... 140

Selection of genes to analyze the association of Pol II ...................................... 144

Genomic mapping of genes by permanganate footprinting ............................. 145

Discussion ............................................................................................................ 150

Appendix D. Elongation of Pol II is unaffected by the depletion of TFIIS in Drosophila tissue culture cells ............................................................... 151

Introduction ........................................................................................................... 151

Results ................................................................................................................. 151

Depletion of TFIIS in various Drosophila tissue culture cells ........................... 151

TFIIS depletion does not have a significant effect on Pol II arrest ................. 156

Appendix E ............................................................................................................. 161

Quantifications of Permanganate footprinting .................................................... 161

Depletion of NELF does not significantly alter the induction of hsp70 at 
temperatures below 37 °C ................................................................................. 162

References .......................................................................................................... 168
LIST OF FIGURES

Figure 3-1: C4 mutation may interfere with complete folding of the trigger loop. ..... 40
Figure 3-2: NELF depletion modifies levels of Pol II at down-regulated genes.......... 45
Figure 3-3: Down-regulated gene syx4 shows no change in Pol II level.................. 51
Figure 3-4: NELF depletion diminishes pausing at up-regulated genes............... 54
Figure 3-5: Slow mutant of Pol II affects the rate of Pol II elongation. ............... 59
Figure 3-6: Slow mutant of Pol II may pause upstream of the TSS in hsp70 gene.... 63
Figure 3-7: C4 mutant Pol II may hinder the catalytic activity of TFIIS. ............. 66
Figure 3-8: TFIIS was depleted in Drosophila S2R+ tissue culture cells using RNAi................................................................................................. 68
Figure 3-9: Permanganate footprinting in TFIIS depleted cells. ...................... 70
Figure 4-1: Depletion of NELF in salivary glands of Drosophila...................... 81
Figure 4-2: Kinetics of heat shock induction is unaffected following disruption of pausing................................................................. 85
Figure 4-3: NELF is required for normal heat shock gene shut-off.................... 88
Figure 4-4: Chromatin immunoprecipitation analysis of Rpb3 in salivary glands..... 93
Figure 4-5: NELF depletion results in hyper-expression of hsp70 following a brief heat shock. .............................................................................. 96
Figure 4-6: Chromatin immunoprecipitation analysis of HSF in salivary glands. .... 98
Figure 4-7: Chromatin immunoprecipitation analysis of GAF in salivary glands.... 101
Figure 4-8: The effects of NELF depletion on hsp26 shut-off............................. 105
Figure 4-9: Binding activity of HSF in extracts from salivary glands............... 112
Figure 4-10: Chromatin immunoprecipitation analysis of HSF at heat shock gene promoters in salivary glands depleted of CBP .............................. 116
Figure 4-11: Depletion of CBP delays heat shock gene shut off

Figure 4-12: Model for how pausing of Pol II might contribute to acetylation-mediated dissociation of HSF from the heat shock gene

Figure C-1: Schematic representation of Pol II association on hsp70 gene

Figure C-2: C4 mutant Pol II is resistant to α-amanitin

Figure C-3: Heterozygous fly line shows resistance to α-amanitin

Figure C-4: Permanganate genomic footprinting of genes selected to explore dynamic association of Pol II

Figure D-1: Depletion of TFIIS in various Drosophila tissue culture cells

Figure D-2: Depletion of TFIIS in Drosophila tissue culture cells by varying concentrations of dsRNA

Figure D-3: Depletion of TFIIS does not affect the position of Pol II pausing on hsp70

Figure D-4: Effect of TFIIS depletion on hsp70 expression levels

Figure E-1: Quantification of TepII gene

Figure E-2: Analysis of hsp70 induction following heat shock at various temperatures below 37 °C
LIST OF TABLES

Table E-1. One-way Analysis of Variance (Anova) results for levels of hsp70 transcripts after induction at 25, 29, 33 and 37 degrees C..................................................167
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>Pol II</td>
<td>RNA Polymerase II</td>
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<td>DRB</td>
<td>5,6-dichloro-1-β-D-ribofuranosylbenzimidazole</td>
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<td>DRB sensitivity inducing factor</td>
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<td>Transcription start site</td>
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<td>Potassium Permanganate</td>
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<td>Heat shock</td>
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<tr>
<td>hsp</td>
<td>Heat shock protein</td>
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Chapter 1

Introduction.

Summary of transcription elongation.

Transcription by RNA polymerase II (Pol II) proceeds through multiple stages separated into preinitiation, initiation, elongation, and termination. During this process Pol II is acted upon by many positive and negative factors. The pre-initiation and initiation stages of mRNA synthesis have been well characterized by biochemical studies over the past couple of decades. These studies have led to the discovery of general transcription factors (GTF) which are required for binding of Pol II to promoters, formation of the open complex, and synthesis of the first few phosphodiester bonds of nascent transcripts. General elongation factors are involved in the elongation stage of eukaryotic mRNA synthesis, and have been shown to suppress transient pausing or premature arrest of Pol II by directly interacting with the elongation complex. These findings allude to the possibility that Pol II elongation control is an important mechanism in regulating gene expression in eukaryotes. Here I review our current understanding of transcription factors, their mechanisms of action, and delineate their positive and negative roles in Pol II elongation control and pausing.
Overview of proteins involved in promoter proximal pausing.

Negative Elongation Factor.

NELF, the Negative Elongation Factor, was discovered during a study aimed at understanding the inhibitory mechanism of DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) on transcription elongation (Yamaguchi et al. 1999a). Biochemical approaches using a reconstituted system and HeLa nuclear extracts led to the finding that NELF and another protein called DSIF cooperatively associate with Pol II to repress elongation.

Human NELF protein is constituted of four subunits: NELF-A, NELF-B, NELF-C/D and NELF-E (Yamaguchi et al. 1999a). NELF-A (66 kDa) is encoded by WHSC2 (Wolf-Hirschhorn syndrome) and displays weak sequence homology to hepatitis delta antigen (HDAg), a protein encoded by hepatitis delta virus (HDV) (Yamaguchi et al. 2001). NELF-B (62 kDa) is identical to COBRA1, a cofactor of the breast cancer susceptibility gene, BRCA1 (Ye et al. 2001). NELF-C (60 kDa) / NELF-D (59 kDa) are isoforms of a common mRNA species using alternative translation initiation codons. These isoforms exist in distinct NELF complexes (Narita et al. 2003). NELF-E (46 kDa) has Arg-Asp (RD) dipeptide repeats that are essential for transcription repression. NELF-E also contains a putative RNA recognition motif (RRM) in its C-terminus that binds RNA (Yamaguchi et al. 2002; Yamaguchi et al. 1999a).

*Drosophila* has proteins that are homologous to each of the subunits of Human NELF. Alignment of amino acid sequences of the NELF subunits reveals that dNELF-A (135 kDa) is almost twice the size of the hNELF-A due to a non-conserved region between amino acids 300-1100. The sequences of dNELF-B and dNELF-D display more
than 50% identity to the human NELF-B and -D over their entire length. The RRM of hNELF-E displays 30% identity to amino acids 167–232 in dNELF-E. The dNELF-E lacks the RD dipeptide repeats found in hNELF-E. In addition, serines at amino acids 181, 185, 187 and 191 in hNELF-E that are phosphorylated by P-TEFb are absent in dNELF-E (Wu et al. 2005).

To characterize each NELF protein and its interacting partners, different epitope tags were fused to the N-terminus of individual NELF subunits and expressed in a baculovirus system in various combinations. Co-immunoprecipitation results indicated that NELF-E interacted with NELF-B while NELF-D interacted with NELF-A and NELF-B. A pull down assay with glutathione S-transferase fused NELF subunits indicated that Pol II selectively associated with NELF-A (Narita et al. 2003). The RRM of NELF-E is required to inhibit transcriptional elongation of Pol II possibly by associating with the extruding RNA hence pausing Pol II 20-40 nucleotides downstream of the TSS (Rougvie and Lis 1988; Yamaguchi et al. 1999a). NELF is localized in the nucleus in distinct nuclear foci called NELF bodies. NELF is a nuclear transcription factor but fluorescent studies revealed that NELF bodies shuttle between the cytoplasm and nucleus (Yung et al. 2009). This shuttling may be required to maintain a stoichiometry between the individual NELF subunits. Basigin gene (BSG) is known to produce four transcripts via alternative transcription and splicing of its two internal exons, exon I and II. NELF associates with BSG and depletion of NELF decreased the level of expression of BSG from exon I with no significant effect on exon II. Hence NELF can also influence alternative transcription initiation (Sun and Li 2010).
The presence of NELF at hundreds of loci on the polytene chromosomes in *Drosophila* suggested that NELF may play an important role in regulating the expression of many genes. (Wu et al. 2005; Wu et al. 2003). Chromatin immunoprecipitation and microarray analysis (ChIP-chip) determined that NELF is on the promoters of more than 2000 genes in *Drosophila*. Permanganate genomic footprinting of 46 of the 59 selected genes displayed hyper-reactive thymines in the promoter proximal region, demonstrating the co-localization of paused Pol II and NELF. It was also observed that half of the top 10 percentile of expressed genes associates with NELF. Hence, NELF may be positively regulating transcription by Pol II (Lee et al. 2008). Another study revealed that depletion of NELF significantly altered the expression level of 241 genes. Two thirds of these NELF target genes were down-regulated while the others were up-regulated. Chromatin immunoprecipitation (ChIP) of histone H3 indicated increased occupancy of H3 at the promoters of down-regulated genes suggesting that nucleosomes encroach upon the promoters depleted of paused Pol II. These results suggest that NELF might be required for maintaining a nucleosome free promoter. Depletion of NELF also decreased the level of histone H3 trimethylation at Lys 4 (H3-K4-me3) at the promoters of down-regulated genes, thus implying decreased transcriptional activity (Gilchrist et al. 2008). NELF has been implicated in negatively regulating the immediate early genes (IEG) such as junB, c-fos and tis-11. For the junB gene, NELF functions as a dual regulator of transcription. NELF is required for promoter proximal pausing of Pol II and upon activation of junB, it was shown to attenuate transcription as measured by the level of transcripts (Aida et al. 2006).
NELF is an essential protein since depletion of NELF is fatal to the *Drosophila* larvae (Enerly et al. 2002). The importance of NELF has been emphasized in the cause of various diseases and cell function. In HeLa cells, NELF depletion resulted in phenotypes with enlarged nuclei and multi-nucleated cells (Yung et al. 2009). HDAG associates with RNAP II and facilitates transcription elongation of HDV by displacing NELF (Yamaguchi et al. 2001). Knockout of NELF-B (COBRA1) in mice causes lethality early in embryogenesis due to the absence of a discernable inner cell mass (Amleh et al. 2009). Estrogen receptor α (ERα) responsive genes and estrogen dependent proliferation of breast cancer cells were attenuated by COBRA1 (Aiyar et al. 2004). NELF-E has been shown to stimulate the Tat-dependent activation in HIV transcription elongation, but inhibited Tat independent transcription. It has been proposed that NELF-E along with DSIF delays elongation by RNAP II so that the P-TEFb/Tat complex can phosphorylate the RNAP II (Fujinaga et al. 2004). In HIV provirus, permanganate genomic footprinting detected paused Pol II at +45. siRNA-mediated depletion of NELF resulted in a loss of this paused Pol II and an increase in virus production. Thus, NELF represses the expression of HIV provirus (Zhang et al. 2007).

NELF has also been implicated as part of a checkpoint that coordinates elongation and mRNA capping. Capping enzyme (CE) was found to alleviate the NELF mediated repression of Pol II in a reconstituted transcription assay (Mandal et al. 2004). NELF-E was shown to preferentially bind the nuclear cap binding complex (CBC) that associated with RNA forming a NELF-CBC-RNA complex. It was also reported that the absence of NELF-CBC complex leads to aberrant poly-adenylation of histone mRNA (Narita et al. 2007), hence establishing that NELF plays a role in 3’end processing of mRNAs.
**DRB Sensitivity Inducing Factor.**

DRB Sensitivity Inducing Factor, DSIF, was discovered in an attempt to understand the mechanism of Pol II transcription inhibition by the drug DRB (Wada et al. 1998a; Yankulov et al. 1995). Earlier studies reported that DRB does not inhibit a partially purified reconstituted transcription system but that DRB sensitivity was conferred in the reaction by supplementing back a fraction that did not contain any of the general transcription factors (Chodosh et al. 1989).

Human DSIF was purified from HeLa cell extracts after multiple rounds of fractionation. DSIF activity was attributed to two major polypeptides, p160 (160 kD) and p14 (14 kD), that turned out to be homologous to the yeast Spt5 and Spt4 transcription factors (Chiang et al. 1996; Wada et al. 1998a). The mechanism by which Spt4 and Spt5 regulate transcriptional elongation has been investigated. Spt5 contains a number of distinct domains including an acidic amino terminus, four KOW repeats that have homology to the *E. coli* transcriptional regulator NusG (Wada et al. 1998a) and two C-terminal repeat elements designated CTR1 and CTR2 (Stachora et al. 1997). In yeast, Spt4 and Spt5 were implicated as factors that are essential for transcription elongation. In addition, Spt5 was demonstrated to contact the Rpb1 subunit of Pol II. The two peptides formed a complex and co-immunoprecipitated with each other (Hartzog et al. 1998). Deletions of the N-terminal KOW motifs in Spt5 abolished the interactions between Spt4 and Spt5 and Pol II (Ivanov et al. 2000; Yamaguchi et al. 1999b). The consensus sequence of CTR2 is similar to the heptad repeats in the CTD of the largest subunit of Pol II. CTR1 and CTR2 contain multiple Ser and Thr residues and may provide phosphorylation sites for cellular kinases (Stachora et al. 1997). *In vitro* studies determined that phosphorylation
of DSIF was important for the stimulation of transcription in HeLa cells (Wada et al. 1998b).

DSIF plays positive and negative roles in transcription elongation. *In vitro* transcription analysis with whole cell extracts from Spt4 mutant cells showed reduced efficiency of synthesis of full-length transcripts compared to the wild type counterpart (Rondón et al. 2003). Chen et al reported that a recombinant form of DSIF rescued transcription elongation in HeLa nuclear extracts depleted of DSIF (Chen et al. 2009). On the other hand DSIF and NELF cooperatively repressed transcription elongation in a reconstituted system (Renner et al. 2001; Yamaguchi et al. 1999a). In zebrafish, it was inferred that *in vivo* target genes whose transcription is dependent on Spt5 were regulated both positively and negatively during development (Krishnan et al. 2008).

DSIF is recruited to the transcriptionally active loci of *hsp70* in polytene chromosomes of *Drosophila* (Kaplan et al. 2000; Wu et al. 2003). ChIP analysis has revealed that DSIF is associated with the *hsp70, hsp26* and *hsp83* promoters before heat shock induction, suggesting that this factor is involved in promoter proximal pausing (Andrulis et al. 2000). DSIF along with NELF was found to pause Pol II in the promoter proximal region of the *Drosophila hsp70* gene (Wu et al. 2003; Yamaguchi et al. 1999a). Recently, crosslinking studies of reconstituted elongation complexes revealed that DSIF and not NELF contact the 22-mer nascent RNA as it emerges from the Pol II elongation complex. NELF binding was suggested to be upstream from the DSIF binding site when the elongation complex was 70 nucleotides in length. These results present a possible model by which DSIF binds the elongation complex via association with the nascent transcript and subsequently recruits NELF to pause Pol II (Misra and Gilmour 2010).
Besides DSIF’s role in transcription, Wen and Shatkin demonstrated that mammalian Spt5 directly interacts with the capping enzyme. Spt5 was further shown to influence guanylylation of the RNA and positively regulate mRNA capping in the presence of the capping enzyme (Mandal et al. 2004; Wen and Shatkin 1999). In yeast, mutants of Spt5 led to defects in splicing of snRNAs and accumulation of unspliced ribosomal protein RNA (Lindstrom et al. 2003).

**Positive Transcription Elongation Factor b.**

Positive Transcription Elongation Factor b (P-TEFb) is a kinase that was discovered while investigating the mechanism of inhibition by DRB of elongation by Pol II. DRB specifically affected transcription *in vitro* when crude nuclear extracts were used (Chodosh et al. 1989). Later it was established that DRB specifically inhibited P-TEFb dependent transcription in a cell free system (Wada et al. 1998b). In *Drosophila*, P-TEFb was identified by fractionation of nuclear extract during a reconstitution of DRB-sensitive transcription. Further analysis revealed that P-TEFb readily phosphorylated the CTD of the largest subunit of Pol II and was a key factor regulating eukaryotic mRNA transcription at the level of elongation (Marshall et al. 1996; Marshall and Price 1995). In *Drosophila*, upon heat shock, P-TEFb rapidly relocated to the heat shock loci, which are active sites of transcription (Lis et al. 2000). *Drosophila* P-TEFb is a cyclin dependent kinase composed of Cdk9 and cyclin T. In humans, Cdk9 associates with either cyclinT1 or cyclinT2 (Peng et al. 1998).

Transcriptionally engaged Pol II is phosphorylated by P-TEFb at Ser 2 of the heptameric repeated sequence YSPTSPS of the Pol II CTD (Boehm et al. 2003; Corden
et al. 1985; Ni et al. 2004). The kinase activity of P-TEFb also alleviates the negative effect of DSIF and NELF complexes thus playing a role in stimulating transcription elongation (Hartzog et al. 1998; Wada et al. 1998b; Yamaguchi et al. 1999b). Some activators function by recruiting P-TEFb to the promoter. The human immunodeficiency virus (HIV) encodes a nuclear transactivator called Tat that forms a complex with P-TEFb for efficient transcriptional activation of the HIV LTR (Mancebo et al. 1997; Zhu et al. 1997). Inhibition of P-TEFb by flavopiridol blocked HIV replication (Chao et al. 2000). In a similar assay, c-Myc was linked to the regulator of virion gene expression (Rev) and the stem loop IIB from the Rev response element (RRE) grafted onto the double-stranded stem in TAR recruited P-TEFb to activate transcription in cells (Kanazawa et al. 2003). Peterlin and colleagues also demonstrated that the eukaryotic transcription factor NF-κB required P-TEFb for efficient activation of transcription (Barboric et al. 2001).

Another mechanism regulating the recruitment of P-TEFb to the site of transcriptional activity uses a nuclear protein called Brd4. Analysis in HeLa cells indicated that cyclinT1 and Cdk9 co-immunoprecipitated with Brd4. Fluorescence analysis demonstrated that in living cells, Brd4 interacts with cyclinT1/Cdk9. Reporter assays performed using the HIV-LTR determined that Brd4 stimulated transcription by interacting with P-TEFb. Also Brd4 associated with the mediator complex and a possible mechanism of P-TEFb recruitment may be through the mediator (Jang et al. 2005; Yang et al. 2005).

Active and inactive P-TEFb complexes are in rapid equilibrium. P-TEFb activity is inactivated when it is sequestered by the abundant 7SK snRNA and a protein called
HEXIM1. *In vitro* kinase assays demonstrated the HEXIM1:P-TEFb interaction to be mediated by 7SK snRNA that in turn inhibited transcription by Pol II. But P-TEFb dissociated from 7SK snRNA and HEXIM1 when the cells were subjected to stress inducing agents (Michels et al. 2004; Yik et al. 2003).

Interestingly, flavopiridol an inhibitor of P-TEFb did not significantly alter the processivity of Pol II on the *hsp70* gene. Cells treated with flavopiridol decreased the level of Ser2 phosphorylated Pol II by approximately two fold but the levels of Pol II remained constant throughout *hsp70* (Ni et al. 2008; Ni et al. 2004). This raises the possibility that another kinase may be phosphorylating the CTD of Pol II in the absence of P-TEFb. Park et al reported that cdk8, a component of the mediator complex, may be responsible for phosphorylating both Ser2 and Ser5 of Pol II CTD (Park et al. 2001). Recent evidence indicates that cdk12 is a bona fide CTD kinase, and depletion of cdk12 in HeLa cells decreased the Ser2 phosphorylation of CTD of Pol II (Bartkowiak et al. 2010).

**DNA binding factors that affect *hsp70* transcription.**

**Heat shock factor.**

Heat shock factor (HSF) was originally identified in *Drosophila* nuclear extracts. Binding of HSF to the promoter through DNA–protein interactions, induced heat shock gene expression (Parker and Topol 1984; Wu 1984). Both *Drosophila* and mammalian HSF function as a molecular thermostat that responds to changes in temperature and oxidative stress by converting from a monomeric, inactive form to the active DNA binding trimeric form (Goodson and Sarge 1995; Zhong et al. 1998).
Transcriptional activation by HSF is mediated through regulatory upstream promoter elements called heat shock elements (HSE). The association of HSF to HSE in the heat shock genes is cooperative and occurs at inverted repeats of pentameric sequences nGAAAn (Amin et al. 1988; Perisic et al. 1989; Xiao et al. 1991). A common feature of eukaryotic HSF is the conversion from the monomeric to the high affinity DNA binding trimer upon stress. In mammals, this includes the translocation of inactive HSF1 monomer present in the cytoplasm to the nucleus upon activation (Baler et al. 1993; Sarge et al. 1993).

HSF1 is sequestered in its inactive monomeric form by hsp90 forming an hsp90-HSF1 complex that upon heat stress dissociated from hsp90 to form HSF1 trimers. Hsp90 also aids in disassembly of the trimers following recovery from stress (Ali et al. 1998; Zou et al. 1998). It has also been reported that hsp70 protein interacts with HSF and prolonged heat shock or increased level of hsp70 did not significantly disrupt the dissociation of HSF-DNA binding activity. Although upon recovery from severe heat shock, a modest decrease in the HSF-DNA binding activity was observed in the rat cells expressing either endogenous hsp70 or human hsp70 proteins. However in rat cells expressing a full complement of stress proteins, HSF-DNA binding activity was significantly decreased. These results indicate that a full complement of stress proteins can accelerate recovery to an inactive state (Rabindran et al. 1994). The process of HSF inhibition is still unknown but speculations are that the feedback from heat shock proteins may serve as a control mechanism for HSF activation. This may be through the levels of chaperones, posttranslational modifications or misfolded peptides. Inactivation of HSF was clearly not due to degradation of HSF because HSF activity could be repeatedly
turned on and off with cycles of heat shock and recovery even in the presence of a protein synthesis inhibitor (Zimarino and Wu 1987).

Westerheide et al. (2009) evidenced that HSF is regulated by the acetylation and deactylation by p300/CBP and SIRT1 respectively. DNA binding activity of HSF was enhanced with increased expression of SIRT1, while depletion of SIRT1 enhanced acetylation of HSF and attenuated HSF’s DNA binding activity (Westerheide et al. 2009). I will be presenting evidence later in my thesis that acetylation of HSF might be part of a NELF-dependent mechanism that causes HSF to dissociate from heat shock genes during recovery from heat shock.

Heat shock factors are essential and required for many vital and physiological functions. In Drosophila, mutations in HSF caused an arrest in larval development (Jedlicka et al. 1997). HSF expression was required for normal postnatal growth in mice, and mice lacking HSF exhibited multiple defects, such as prenatal lethality, retarded growth and female infertility (Xiao et al. 1999).

GAGA Factor.

GAGA factor (GAF) associates with numerous genes that have paused Pol II. Mutations in the GAGA binding elements upstream of the hsp70 promoter diminished paused Pol II by approximately four fold (Lee et al. 1992). The binding region of GAF was determined to interdigitate with sequences of HSEs (Gilmour et al. 1989).

In vitro assays demonstrated that GAF altered the organization of nucleosomes previously assembled on DNA in the promoter region of hsp70 (Tsukiyama et al. 1994; Tsukiyama and Wu 1995). GAF may be intricately involved in remodeling the chromatin
structure by preventing the deposition of histone on the promoter of \textit{hsp70} gene in \textit{Drosophila} by interacting with NURF, a nucleosome remodeling factor (Tsukiyama et al. 1994; Tsukiyama and Wu 1995) and FACT, a histone chaperone (Nakayama et al. 2007).

GAF contains a zinc finger DNA-binding domain (DBD) and a BTB/POZ (broad complex tramtrack bric-a-brac/poxvirus and zinc finger) domain that is required for the natural promoter recognition of GAF. The POZ domain was found to mediate the binding of GAF to its promoters by facilitating GAF oligomerization (Katsani et al. 1999). A glutamine rich Q domain located in the C-terminal region of GAF was shown by Wilkins and Lis (1999) to multimerize itself and hence may also contribute to formation of GAF oligomers (Wilkins and Lis 1999).

GAF is encoded by the \textit{Trithorax-like (Trl)} gene, and is necessary for the regulation of developmental genes in \textit{Drosophila} (Farkas et al. 1994). Immunofluorescence analysis of \textit{Drosophila} polytene chromosomes indicated that following heat shock, GAF co-localizes with HSF on the heat shock loci containing heat shock genes such as \textit{hsp70}, \textit{hsp26}, \textit{hsp23} and \textit{hsp22}, thus implying that GAF may be required for maintaining the gene in a transcriptionally active conformation (Tsukiyama et al. 1994). However this is inconsistent with the observation that in the heat shock genes \textit{hsp70} and \textit{hsp26}, HSF has overlapping binding sites with GAF (Lee et al. 1992; Sandaltzopoulos et al. 1995). Hence binding of HSF may require GAF to dissociate from the gene. This is evident in the higher resolution ChIP assay wherein upon heat shock an increase in HSF association was observed along with a concomitant decrease in GAF binding (Chapter 4).
Transcription Elongation Factor IIS.

Transcription elongation factor IIS (TFIIS) facilitates elongation of an arrested molecule of Pol II by stimulating the cleavage activity of Pol II (Fish and Kane 2002). Paused Pol II has a tendency to backtrack, which may lead to Pol II arrest (Adelman et al. 2005). Backtracked Pol II moves upstream on the DNA resulting in the nascent transcript being misaligned at the 3’ end with the catalytic site. TFIIS associates with backtracked Pol II, where it inserts the Zn$^{2+}$ ribbon domain into the secondary channel of Pol II stabilizing the binding of the second active site Mg$^{2+}$ ion required for the transcript cleavage reaction. The nascent RNA now consists of a new 3’end that can be further elongated (Kettenberger et al. 2003).

TFIIS is composed of three domains. Domain I is the least conserved among the mammalian species. The binding of Pol II is attributed to Domain II, which is an $\alpha$-helical region. Domain III is the most conserved of the three domains. Domain III has a Zn$^{2+}$ ribbon motif that shares similarity to several small subunits of nuclear polymerases (Shimasaki and Kane 2000). Domains II and III are connected with a linker region consisting of about 30 amino acids. Mutations in the linker and Zn$^{2+}$ ribbon regions of TFIIS protein caused defective cleavage of the nascent transcript and blocked elongation (Awrey et al. 1998). A study with an artificial RNA–DNA bubble resembling the transcription bubble demonstrated that Pol II is prone to misincorporate nucleotides during transcription. TFIIS readily removed these misincorporated bases at the 3-end (Jeon and Agarwal 1996), hence TFIIS could serve to increase the fidelity of Pol II.

In fractionated Drosophila embryo extracts devoid of TFIIS, Pol II accumulated in the promoter proximal region of hsp70. Upon supplementing this reaction with TFIIS,
rapid elongation of the stalled complex was observed along with cleavage products generated due to TFIIS activity. Depletion of TFIIS also resulted in reduced hsp70 transcripts and reduction in the recruitment of Pol II to the promoter upon heat shock. In polytene chromosomes, TFIIS was observed at over 150 loci and upon heat shock was translocated to the heat shock loci (Adelman et al. 2005). In addition it was proposed that TFIIS catalyzed transcript cleavage was blocked by the association of NELF and DSIF to the region of TFIIS binding on Pol II. This data indicates that NELF prevents the elongation of the arrested Pol II by inhibiting TFIIS. It was also proposed that the interaction of DSIF with Pol II was via the Rpb7 subunit (Palangat et al. 2005). However, a caveat was that these experiments were performed by rinsing the elongation complex with the detergent sarkosyl known to remove the Rpb4/7 sub-complex from Pol II (Ujvári and Luse 2006).

Genomewide analysis has revealed numerous genes that display Pol II stalling. Comparison of the RNA profiles from TFIIS-depleted and mock-treated cells revealed an increase in longer RNA in TFIIS-depleted cells. But it was observed by permanganate footprinting of a subset of genes that the location of Pol II pausing after TFIIS depletion was unchanged compared to the control. Hence it was proposed that paused Pol II was prone to backtracking to a region of the thermodynamically stable RNA-DNA heteroduplex thereby stabilizing the backtracked complex (Nechaev et al. 2010). Lis and colleagues observed that a subset of Pol II could not be efficiently extended to full-length products after a run-on chase (Rasmussen and Lis 1995). This may be due to the subset of Pol II that is in an arrested conformation. Hence the term “stalled” Pol II has been coined (Adelman et al. 2005) to include both “paused” as well as “arrested” Pol II.
Promoter proximal pausing.

Genomewide prevalence of promoter proximal pausing.

Genomewide studies underlining the role of promoter proximal pausing in human and *Drosophila* cells have gained importance. These studies have highlighted the regulatory roles of paused Pol II at the promoters of many active genes. In *Drosophila* cells, permanganate footprinting (Gilmour and Fan 2009) revealed that promoter proximal pausing occurred primarily 20 to 50 nucleotides downstream of the TSS (Lee et al. 2008). Many developmental and stimulus-responsive genes in *Drosophila* involved in cell differentiation, cell-cell signaling and immune response pathways showed enrichment of Pol II at the promoter. Many of these promoters exhibited hyperreactivity of permanganate indicating that the associated promoter was transcriptionally engaged. Additionally, NELF occupancy was detected via ChIP at these genes. Depleting NELF by RNA interference (RNAi) significantly decreased Pol II signals in the promoter region only and not throughout the gene. Finally the paused Pol II was rapidly released in a subset of ultraviolet (UV) inducible genes when induced by UV treatment (Muse et al. 2007).

Zeitlinger et al. (2007) performed a comprehensive ChIP-chip analysis of Pol II in *Drosophila* embryos. They selected a mutant consisting of mesodermal precursor cells that contained repressed neuronal and ectodermal genes. 992 genes displayed Pol II tightly restricted to the TSS. This set of genes was enriched specifically for neuronal and ectodermal developmental genes. A high level of expression in ubiquitously expressed genes such as genes involved in metabolism and cell proliferation displayed uniform Pol II binding signals throughout the entire gene. Genes for adult functions that do not need
to be expressed in the embryo lacked Pol II binding entirely. It was also observed that genes with stalled Pol II were highly enriched among developmental genes that were fated to be transcribed within 12 hours. Hence genes with paused Pol II are poised for rapid induction in response to developmental signals during embryogenesis (Zeitlinger et al. 2007). Another study where Pol II ChIP-chip analysis paired with quantitative in situ hybridization in *Drosophila* embryos was used to study the expression profiles of developmentally controlled genes. Genes which have paused Pol II are synchronously activated whereas genes that do not display paused Pol II are induced stochastically within a population of embryonic tissues. This synchronicity in gene expression can lead to regulation of embryogenesis (Boettiger and Levine 2009).

Lis and colleagues utilized global run-on sequencing (GRO-seq) to identify genomewide transcription and present a direct evaluation of promoter-proximal pausing on all genes. GRO-seq involved doing nuclear run-ons followed by sequencing the newly transcribed RNA. This technique revealed that 41.7% of the active genes in the human fibroblast cells have paused Pol II. This class of genes may be regulated by escape from the pause (Core et al. 2008).

**Promoter proximal pausing at the *Drosophila* heat shock gene *hsp70***.

The *Drosophila hsp70* gene serves as a paradigm for promoter proximal pausing. Under uninduced conditions the *hsp70* gene is not actively transcribed but is poised for rapid induction upon heat shock. Prior to heat shock activation, Pol II initiates transcription but is paused 20 to 40 nucleotides downstream of the transcription start site (TSS) (Rasmussen and Lis 1993; Rasmussen and Lis 1995; Rougvie and Lis 1990).
Crosslinking studies using ultraviolet light followed by immunoprecipitation revealed that Pol II was predominantly located in the 5’ region of the *hsp70* gene between nucleotide -12 and +65 (Gilmour and Lis 1986). Run-on assays aimed at investigating the state of this uninduced Pol II showed that Pol II initiates transcription but remains paused at a region upstream of +34 from the TSS (Rougvie and Lis 1988). Permanganate genomic footprinting was performed to further map the location of the paused Pol II. Thymines in the transcription bubbles are often more reactive to oxidation by permanganate than in double stranded DNA. Permanganate reactivity at +22, +30 and +34 of the non-transcribed strand established that the hyper-reactive pattern was due to pausing of transcriptionally engaged Pol II (Giardina et al. 1992).

Further studies determined that NELF and DSIF caused Pol II to pause in the promoter proximal region of the *hsp70* gene. Immunofluorescence analysis of the polytene chromosomes detected NELF at hundreds of loci and depleting NELF diminished promoter proximal pausing in *hsp70* (Wu et al. 2003).

ChIP results indicated that the *hsp70* gene is induced within 75 sec following heat shock treatment and the first wave of Pol II was observed at the 3’ end of *hsp70* by 150 sec (Boehm et al. 2003). Activation of *hsp70* involves the association of HSF with the HSEs located upstream of the promoter (Perisic et al. 1989) that triggers the release of NELF from Pol II for transcription elongation. ChIP results indicated that DSIF remained associated with the elongation complex (Saunders et al. 2003; Wu et al. 2003). The Pol II CTD prior to heat shock is phosphorylated at Ser5 of the heptad repeat \[Y_1S_2P_3T_4S_5P_6S_7\] (Boehm et al. 2003). Subsequently after heat shock, the CTD is phosphorylated at Ser2 by the kinase P-TEFb. Indirect immunofluorescence analysis of the *Drosophila* salivary
glands revealed that HSF and P-TEFb associated with the hsp70 heat shock loci during heat shock (Lis et al. 2000; Zobeck et al. 2010). ChIP analysis noted that HSF and P-TEFb were recruited to the promoters of the hsp70 gene within 150 seconds of induction of hsp70 (Boehm et al. 2003). The kinase activity of P-TEFb is also known to alleviate the repression due to NELF and DSIF (Renner et al. 2001).

Several genes in Drosophila and mammalian cells displayed paused Pol II. Results from permanganate genomic footprinting of the uninduced Drosophila hsp26 gene showed hyper-reactivity on the coding strand between +25 to +45 and on the transcribed strand between +23 to +56. Hence it was determined that Pol II paused on the hsp26 gene between +23 and +56 from the TSS (Giardina et al. 1992). Promoter proximal pausing was also observed in the two proto-oncogenes c-myc and c-fos. In c-myc, permanganate reactivity was detected downstream of the P2 transcription initiation site at around +30 in both differentiating and proliferating cells. It was observed in nuclear run-on assays that addition of sarkosyl released this paused Pol II at +30 resulting in increased transcriptional activity in P2 at sites +47 - +169 (Krumm et al. 1992).

c-fos is a member of the immediate early response genes. Run-on assay in serum-starved cells showed that Pol II was confined to the first 100 nucleotides downstream from the TSS. After serum-stimulation, Pol II was seen along the entire gene resulting in expression of c-fos. Permanganate footprinting of the non-transcribed strand revealed that Pol II was indeed paused at approximately +40 and upon activation permanganate reactivity was seen further downstream in the gene. These paused Pol II were a result of continuous initiation and were transcriptionally competent upon initiation by serum
(Pinaud and Mirkovitch 1998). Thus promoter proximal pausing of Pol II appears to regulate the transcription of c-myc and c-fos

**Scope and significance of dissertation.**

The goal of my research was to understand how transcription elongation factors and the rate of Pol II elongation affect promoter proximal pausing in *Drosophila* cells. Previous studies indicated that NELF was required for promoter proximal pausing. I was interested in understanding what role NELF plays in regulating the heat shock genes. The well-developed permanganate genomic footprinting assay (Gilmour and Fan 2009) was used to monitor the movement of Pol II on the heat shock genes. Surprisingly it was observed that depleting NELF delayed the shutting off of *hsp70* and *hsp26* when larvae were allowed to recover from heat stress. NELF depletion did not alter the rates of induction of these heat shock genes. Instead, NELF depletion caused hyper-expression of the heat shock genes upon recovery from heat shock. If the heat shock genes showed delayed recovery upon NELF depletion, this would indicate that HSF might still be associated with the promoters of these genes. I developed a ChIP assay for *Drosophila* salivary glands to test for HSF association. Interestingly, in NELF depleted larvae HSF dissociation was delayed upon recovery from heat shock, but the amount of HSF and binding activity showed no significant differences when compared to control cells. This suggests that there may be other factors or posttranslational modification that may be required for the dissociation of HSF from the DNA. CREB-binding protein (CBP) known to acetylate Lys80 of HSF1 in humans was implicated for the dissociation of HSF from the promoter (Westerheide et al. 2009). Therefore, I depleted CBP in salivary glands and
observed that HSF dissociation was delayed in glands depleted of CBP. Hence I posit that NELF is required to pause Pol II and this increases the time provided for Pol II to guide CBP to acetylate HSF thus facilitating the dissociation of HSF.

I also explored whether the rate of elongation affected promoter proximal pausing because other work in the laboratory had shown that varying the NTP concentration altered the location of Pol II pausing in a cell free system. I tested this hypothesis in a C4 mutant Pol II fly line that harbored a mutation in the largest subunit of Pol II. This mutant Pol II was found to have an elongation rate that is half the elongation rate of the wild-type Pol II (Chen et al. 1996). I compared genes that have Pol II paused in narrow as well as widespread regions. Remarkably, the pattern of permanganate reactivity for the slow Pol II mutant shifted upstream relative to the wild-type Pol II genes. This suggests that there may be a kinetic competition between the rate of binding by NELF and the intrinsic rate at which Pol II escapes promoter proximal pausing.

The results presented here will lead future research to study the role of other transcription factors on promoter proximal pausing. Equipped with the biochemical assays and the added tool of Drosophila salivary gland ChIP, this work will direct us to better comprehend the mechanisms and roles of factors that either activate or inactivate a large fraction of genes.
Chapter 2

Materials and Methods.

Chromatin Immunoprecipitation.

ChIP assays were performed as described previously (Petesch and Lis 2008) with some modifications. Drosophila third instar larvae were treated as required and salivary glands were isolated. During dissection, the isolated glands were placed in a drop of Schneider’s Drosophila medium (S2) (Invitrogen). A total of 10 pairs of glands were isolated and transferred to a 1.5 ml Eppendorf tube containing 100 µl dissection buffer (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl$_2$) and 1% formaldehyde (prepared from 37% HCHO from EMD chemicals). The glands were then incubated on ice for 5 min and then at room temperature for 7 min. 5.4 µl of 2.5 M glycine was added to a final concentration of 125 mM to quench the cross-linking reaction and samples were placed on ice for 2 min with intermediate swirling. The glands were then pelleted at 900 x g (3500 rpm in an Eppendorf microcentrifuge) for 2 min at 4 °C. 100 µl sonication buffer (20 mM Tris [pH 8.0], 0.5 % sodium dodecyl sulphate [SDS], 2 mM EDTA [pH 8.0], 0.5 mM EGTA, 0.5 mM PMSF and 2 µl protease inhibitor cocktail containing 1.6 mg/ml Benzamidine HCl, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin A and 1 mg/ml Leupeptin) was added to the pelleted glands and left at room temperature for 10 min. The glands were then moved to ice for 10 min. These glands were then placed on a vortexer for 10 min and later physically homogenized with a micro pestle to achieve complete lysis. The lysates were sonicated at
4 °C in the bioruptor (Diagenode) at full frequency for 15 min at a 30 sec on and 30 sec off cycle to give an average fragment size of 400 base pairs. The lysates were centrifuged in an Eppendorf microcentrifuge at 13,000 rpm for 7 min at 4 °C to remove cell debris. The supernatant containing the cross-linked material for immunoprecipitation was transferred to a fresh siliconized 1.5 ml tube, flash frozen and stored at -80 °C.

For immunoprecipitation, 10 µl of chromatin solution (equivalent to one pair of salivary gland) was transferred to a fresh siliconized 1.5 ml tube and diluted with 390 µl IP buffer (0.5 % Triton X-100, 2 mM 0.5 M EDTA [pH 8.0], 20 mM Tris [pH 8.0], 150 mM NaCl and 10% glycerol) to achieve a final SDS concentration of 0.0125%. The chromatin was precleared by incubating with 15 µl of 50% slurry of Protein A Sepharose (GE Healthcare) in 10 mM Tris-Cl [pH 8.0], 1 mM EDTA [pH 8.0] (TE) and 1 mg/ml acetylated BSA (Promega). The chromatin was then placed on a rotator for 2 hours at 4 °C. The beads were removed by centrifugation at 3000 rpm for 4 min in an Eppendorf microcentrifuge. The chromatin solution was transferred to a fresh siliconized 1.5 ml tube. Antibody was added to the sample and incubated overnight at 4 °C. Immunoprecipitation was performed using 4 µl of anti-Rpb3 (rabbit Ab), 4 µl of anti-GAF (gift from Dr. Wu) (rabbit Ab), 2 µl anti-HSF (rabbit Ab) (gift from Dr. Lis) or 2 µl anti-CBP (rabbit antibody) (gift from Dr. Mazo) antibodies. A sample devoid of antibody was used as a negative control. Next, 30 µl of 50% slurry of Protein A Sepharose was added to the solution and incubated for another 2 hours at 4 °C. The beads were collected by centrifugation at 3000 rpm for 4 min at 4 °C and subjected to washings with 400 µl of wash buffers for 5 min each and centrifuged at 3000 rpm for 3 min in an Eppendorf microcentrifuge. The beads were washed one time with low salt wash buffer (0.1 % SDS,
1% TritonX-100, 2 mM EDTA, 20 mM Tris-Cl [pH 7.8] and 150 mM NaCl), three times with high salt wash buffer (0.1 % SDS, 1% TritonX-100, 2 mM EDTA, 20 mM Tris-Cl [pH 7.8] and 500 mM NaCl) and once with lithium chloride wash buffer (0.1 % SDS, 1% TritonX-100, 1 µM EDTA, 0.01 mM Tris-Cl [pH 7.8], 0.25 M LiCl, 1% NP-40 and 1% Sodium deoxycholate) and placed on the rotator at 4 °C overnight. The beads were collected by centrifugation and washed once more with lithium chloride wash buffer and twice with TE. After the first TE wash the suspension of beads was moved to a fresh 1.5 ml tube to reduce background. Elution buffer (1% SDS and 0.1 M NaHCO₃) was added to elute the protein-DNA complexes from the beads. This step is done twice with 100 µl of elution buffer each for 15 min at room temperature to ensure complete elution. The samples are then centrifuged at 3000 rpm for 3 min at room temperature to avoid precipitation of SDS and then combined together.

To reverse the crosslinks, 8 µl of 5 M NaCl was added to the combined eluates and placed in a water bath at 65 °C for 3.5 hours. At this point 10 µl chromatin was combined with 200 µl of elution buffer and 8 µl of 5 M NaCl for preparation of the input DNA and placed at 65 °C for 3.5 hours. After 3.5 hours, 6 µl 1 M Tris-Cl [pH 7.8], 2 µl pellet paint (Novagen) and 2 µl 10 mg/ml Proteinase K was added to each sample and further incubated for an additional 30 min at 65 °C. DNA was purified by extracting against phenol/chloroform/isoamyl alcohol, 25:24:1 (Sigma) and precipitated with 100% ethanol (Pharmco) at room temperature. The DNA was washed once with 100 µl 75% ethanol and allowed to air dry. The immunoprecipitated DNA was dissolved in 40 µl of ddH₂O whereas the input DNA was dissolved in 400 µl of ddH₂O representing 10 % of input DNA. The input DNA was diluted to 1%, 0.1% and 0.01%. 4 µl of the ChIP and
input DNA were analyzed by qRT-PCR for hsp70 and hsp26 gene specific primers listed in Appendix B.

**Western blotting.**

Immunoblotting was performed in both Drosophila salivary glands and Drosophila tissue culture cells. Drosophila tissue culture cells S2-DRSC, S2R+, Kc167 and ML-DmBG3-c2 cell lines (DGRC) were grown to a density of ~ 1 x 10^6 cells/ml on a 35 mm culture plate in either Schneider’s Drosophila medium (Invitrogen) or Shields and Sang M3 insect medium (Sigma) at 24 °C. The cells were collected by centrifugation at 7000 rpm for 3 min in a 1.5 ml tube. Drosophila salivary glands were isolated from third instar larvae in 15 µl of dissection buffer (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂). The cells were homogenized in 100 µl of lysis buffer (WCE) containing 10 mM Tris-Cl [pH 7.5], 2 mM NaCl, 20 mM EDTA [pH 8.0], 0.5% SDS, 1 mM dithiothreitol and 2 µl proteinase inhibitor cocktail (1.6 mg/ml Benzamidine HCl, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin A, 1 mg/ml Leupeptin). Protein concentration was determined by Bradford assay (Bio-Rad). Proteins were subjected to 10% SDS-PAGE, and transferred at 10 V for 45 min to a 0.2 micron nitrocellulose membrane (BioRad) using a semi dry method (BioRad). Blots were incubated overnight in the cold with specific polyclonal antibodies. NELF-D, NELF-E, DISF, Rpb3 and TFIIS were detected with anti-NELF-D (rabbit Ab) at 1:2000 dilution, anti-NELF-E (rabbit Ab) at 1:2000 dilution, anti-Spt5 (rabbit Ab) at 1:1500 dilution, anti-Rpb3 (rabbit Ab) at 1:3000 dilution and anti-TFIIS (Guinea pig Ab) at 1:2000 dilution respectively. The blots were later probed with Cy5 conjugated or Alexa
568 secondary antibodies at 1:3000 dilution for 90 min. The blots were visualized on a Typhoon (GE Healthcare)

**Quantitative Real-time PCR analysis.**

Quantitative real-time PCR was performed using reaction mix SYBR green PCR master mix (Applied Biosystems Inc.) or SensiMixPlus SYBR (Quantace). The reactions were performed on an Applied Biosystems 7300 real-time PCR system (Applied Biosystems Inc.). Data was analyzed on ABI Prism sequence detection system software (Applied Biosystems Inc.). All the data acquired were in the linear range of amplification. Standard curves for ChIP samples were determined by serial dilution of the input samples to quantify immuno-precipitated samples. A reference standard generated from salivary glands isolated from larvae that were heat shocked for 20 min was used for the reverse transcription-PCR (RT-PCR) samples to quantify the levels of transcript. The data was plotted using Kaleidagraph graphing software (Synergy Software). The specific primers used in this analysis are listed in Appendix B.

**Reverse transcription PCR analysis.**

cDNA synthesis for reverse transcription PCR was performed using either gene specific primers (refer to Appendix B) or using both oligo dT16 (IDT) and random hexamers (Invitrogen). Total RNA was extracted from either *Drosophila* tissue culture cells or salivary glands by homogenizing the cells in Trizol (Invitrogen). Trizol total RNA extraction protocol from Invitrogen was followed and RNA was dissolved in DEPC treated ddH2O. The RNA was quantified by Nanodrop and stored at -80 °C. For RT-PCR,
200 ng of total RNA was combined with the reverse primers for gene specific cDNA synthesis or with oligo dT<sub>16</sub> and random hexamers to a final concentration of 0.65 µM and 0.5 mM dNTP (GE Healthcare). The reaction was placed in a thermal cycler (MJ Research) at 65 °C for 5 min. After 5 min the tubes were placed on ice and a mix containing 1X transcription buffer (50 mM Tris-Cl [pH 8.3], 75 mM KCl, and 3 mM MgCl<sub>2</sub>), 10 µM DTT, 0.4 units of RNasin (Promega), 100 units of RT-MMLV (USB) was added. Reverse transcription was performed at 42 °C for 50 min and later at 70 °C for 15 min to stop the reaction.

The cDNA was then either subjected to qRT-PCR as described above or PCR amplification for 25 cycles. The PCR amplified DNA was then visualized by agarose gel electrophoresis and scanned on the Typhoon. RP49 RNA was used as an internal standard to correct for differences in the recovery of RNA (Petesch and Lis 2008). All primers used in the assay are listed in Appendix B.

**Immunofluorescence analysis of polytene chromosomes.**

Polytene chromosome spreads were prepared as previously described (Champlin et al. 1991). Two pairs of salivary glands were dissected from normal or RNAi larvae in 50 µl of dissection buffer. The glands were then transferred into 50 µl of Solution A (15 mM Tris-Cl, pH 7.4, 60 mM KCl, 15 mM spermine, 1.5 mM spermidine and 1% Triton X-100) for 15 sec, followed by transfer into Solution B (15 mM Tris-Cl, pH 7.4, 60 mM KCl, 15 mM spermine, 1.5 mM spermidine, 1% Triton X-100, and 3.7% formaldehyde) for 15 sec and finally into Solution G (50% glacial acetic acid) for 3 min. During the last three min, the glands were transferred to a siliconized coverslip containing 9 µl of
Solution G. A glass slide was placed onto the coverslip with the glands, and the slide was turned over so the chromosomes could be squashed by tapping the coverslip. Once the desired amount of spreading of chromosomes was observed in the microscope, the slide was flash frozen in liquid nitrogen, the coverslip was removed and the slide was stored overnight in 95% ethanol at 4 °C.

Staining of slides in Fig. 4-1A was carried out at room temperature. The slides were first rehydrated by placing them in TBST (150 mM NaCl, 10 mM Tris-CI pH 7.5 and 0.03% Triton X-100), twice for 5 min each. The slides were incubated in blocking solution (10 % Fetal Bovine Serum in TBST) for 1 hour at room temperature and washed with TBST for 5 min. The primary antibodies (affinity purified NELF D and NELF E antibodies) were diluted 1:10 in TBST and 20 µl of the antibody solution was applied to the slides for 2 hours. The slides were washed with TBST three times for 10 min each. Secondary antibody (anti- rabbit Alexa 568) was diluted 1:250 in TBST and 20 µl of this solution was used to stain each slide for 90 min. The slides were washed with TBST containing 10 ng/ml Hoechst for 10 min to stain the DNA and then washed with TBST for 15 min. The slides were then mounted with a coverslip and 20 µl of mounting solution (100 mM Tris-CI pH 8.5, 80% glycerol, 2% n-propylgallate). Chromosomes were viewed using fluorescence microscopy.

Polytene chromosome spreads in Fig. 4-10A were prepared similarly as described above with a few exceptions. TBS (150 mM NaCl, 10 mM Tris-CI pH 7.5) was used in all subsequent steps following rehydration of the chromosomes. Primary antibodies against CBP (gift from Dr. Alex Mazo) and Pol II were diluted at 1:100 and 1:25 in TBS.
The secondary antibodies (anti-rabbit Alexa 488 and anti-mouse Alexa 568) were diluted 1:200 in TBS.

**RNA interference mediated depletion of NELF-D, NELF-E, CBP, Brm and TFIIS.**

To express NELF RNAi in salivary glands, the parental fly lines NELF-E 17A10 (+/NELF-E17A10) (Enerly et al. 2002) and two transformants of NELF-D, NELF-D8-2 (+/NELF-Di8-2) and NELF-D2-1 (+/NELF-Di2-1) containing the RNAi construct (Gilchrist et al. 2008) were mated with D7,1824 salivary gland specific driver. The D7, 1824 contains and hsp70-LacZ transgene at 87E (Wu et al. 2003) and a transgene (Bloomington Stock 1824) that expresses Gal4 specifically in the salivary glands (Brand and Perrimon 1993). As a control, yw flies were mated with D7, 1824 driver line (+/SG-Gal4). RNAi lines targeting CBP and Brm were obtained from the Transgenic RNAi project (TRIP) and are designated: CBP12806, and brm14019 (the corresponding TRIP designations are JF02806, and HM04019 respectively). RNAi-mediated depletion of CBP and Brm in salivary glands was performed similarly as described above.

RNAi in tissue culture cells were performed in S2-DRSC, S2R+, Kc167 and ML-DmBG3-c2 cell lines (DGRC). Double stranded RNA (dsRNA) required for RNAi in tissue culture cells was generated from PCR products derived by amplifying genomic DNA extracted from *Drosophila* tissue culture cells. The primers contained the T7 promoter sequence (5'-GAA TTA ATA CGA CTC ACT ATA GGG AGA-3'). LacZ RNAi and Spt5 RNAi served as negative and positive controls respectively for TFIIS depletion in the above mentioned cell lines (all primers used for depletion of LacZ, Spt5 and TFIIS are listed in Appendix B). The PCR amplified DNA was extracted with
phenol/chloroform and precipitated with 100% ethanol. *In vitro* transcription reactions containing 350 mM HEPES [pH 7.5], 32 mM Magnesium acetate, 40 mM DTT, 2 mM Spermidine, 28 mM each NTP, and 0.5 µg PCR amplified DNA in a final volume of 20 µl were incubated at 37 °C for 5 min. After 5 min, 1.0 µg of T7 RNA polymerase (purified by me) was added to the reaction and further incubated at 37 °C for overnight. The next day, the reaction was centrifuged for 2 min at 13,000 rpm to pellet the magnesium pyrophosphate. The supernatant was transferred to a new 1.5 ml tube and 2 units of DNase I was added followed by incubation at 37 °C for 30 min. The reaction mixture was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and microcentrifuged at 13,000 rpm for 4 min. The aqueous layer was extracted with chloroform. To the resulting aqueous layer, one-fifth volume ammonium acetate (5 M ammonium acetate + 100 mM EDTA) and 3 volumes of chilled 100% ethanol was added. After incubating on ice for 10 min, the dsRNA was precipitated, washed with 75% ethanol and dissolved in DEPC treated ddH₂O. One 20 µl reaction typically yields ~ 150 µg of dsRNA.

RNAi treatment of *Drosophila* tissue culture cells was performed as described at the *Drosophila* Genomics Research Center (DGRC; https://dgrc.cgb.indiana.edu). 1 x 10⁶ *Drosophila* cells were seeded on a 35 mm tissue culture plate in 10% FBS containing Schneider’s *Drosophila* medium (Invitrogen) or Shields and Sang M3 insect medium (Sigma) (the Sigma media was used as it was recommended by DGRC). The cells were allowed to attach to the tissue culture plate for 45 min after which the media was replaced with 1 ml of fresh media without FBS. The cells were then incubated with dsRNA of the required amount (3 µg to 10 µg per ml of media) for 2 hours at 24 °C. After 2 hours the
cells were supplemented with 2 ml of FBS containing media and incubated at 24 °C for 3 to 5 days.

**Permanganate genomic footprinting analysis.**

Permanganate genomic footprinting analysis was performed in *Drosophila* tissue culture cells as previously described (Gilmour and Fan 2009) and in *Drosophila* salivary glands with a few modifications. Ten pairs of salivary glands were isolated from third instar larvae. The glands were transferred to a drop of S2 medium prior to transferring them to a siliconized 0.65 ml tube containing 10 µl of the S2 media.

For heat shock times less than 10 min, salivary glands were first isolated and then heat shocked in the thermal cycler and immediately incubated on ice for potassium permanganate (KMnO₄) treatment. For heat shock times exceeding 20 min, third-instar larvae were heat-shocked in an Eppendorf tube. This tube was pierced with a fine needle along the sidewall for air circulation to the larvae. Salivary glands were dissected within the next 8-10 min and treated with KMnO₄. Other treatments such as DRB or flavopiridol were carried out on salivary glands for required lengths of time and then these glands were heat-shocked prior to treatment with permanganate.

KMnO₄ (Sigma) crystals were dissolved in dissection buffer on the rocker for 10 min. The resulting 80 mM solution was diluted to a 20 mM solution with dissection buffer and incubated on ice. Isolated salivary glands were treated with 100 µl of 20 mM cold KMnO₄ for 2 min on ice and then stopped with 100 µl 2X KMnO₄ stop solution (20 mM Tris-Cl [pH 7.5], 20 mM NaCl, 40 mM EDTA [pH 8.0], 1% SDS, 0.4M β-mercaptoethanol). The glands were allowed to lyse at room temperature. The lysates were
then incubated at 37 °C for 90 min with 50 µg proteinase K. The samples were then transferred to a fresh siliconized 1.5 ml tube to facilitate extraction and 200 µl of ddH₂O was added. The DNA was extracted sequentially with 300 µl of equilibrated phenol (Sigma), 300 µl of phenol/chloroform/isoamyl alcohol, 25:24:1 and 400 µl of chloroform. The samples were vortexed for 1 min and centrifuged for 3 min for phase separation. 3M sodium acetate [pH 7.0] equivalent to 1/10th the volume of extracted supernatant was added along with 2 µl pellet paint and 1 ml of cold 100 % ethanol. The samples were vortexed and incubated on dry ice for 10 min. The DNA was precipitated by centrifugation at 13,000 rpm in an Eppendorf microcentrifuge for 20 min. The DNA pellet was washed once with 75% ethanol and air dried for 5 min. The resulting DNA was dissolved in 20 µl TE (10 mM Tris-Cl [pH 7.5], 1 mM EDTA [pH 8.0])

The permanganate treated DNA in 20 µl was then subjected to piperidine cleavage by adding 10 µl of piperidine and 70 µl of ddH₂O to the DNA. The samples were incubated at 90 °C for 30 min and then diluted with 300 µl of ddH₂O. The samples were transferred to a 1.5 ml siliconized tube and extracted twice with 800 µl of iso-butanol, once with 400 µl of iso-butanol and once with 100 µl ether. For each extraction, samples were vortexed for 1 min and centrifuged for 3 min. The samples were then ethanol precipitated and dissolved in 20 µl of 0.5X TE [pH 8.0]. The DNA concentration was quantified by Nanodrop (Thermo Scientific).

Soft body tissues were used to generate naked DNA and G/A markers. The soft body tissues were treated with 100 µl TE and 100 µl of 2X KMnO₄ stop solution, and genomic DNA was extracted as described above. To generate a naked DNA control, 1 µg of genomic DNA was subjected to 100 µl KMnO₄ treatment for 0 and 60 sec. The
reaction was stopped using 100 µl of 2X KMnO₄ stop solution and DNA was extracted
by the addition of 20 µl 3M Sodium acetate [pH 7.0] and 1 ml cold 100% ethanol as
described above. The DNA was dissolved in 90 µl of ddH₂O. For generation of G/A
markers, 1 µg of genomic DNA was combined with 20 µl of 0.5X TE and 50 µl of 99%
formic acid. The mix was then incubated at 15 °C for 5 min. Cold depurination stop
solution (0.3 M sodium acetate [pH 7.0] and 50 µg/ml tRNA) was added, and depurinated
DNA was precipitated with 750 µl of cold ethanol. The pelleted DNA was dissolved in
90 µl of ddH₂O. DNA generated for naked DNA control and G/A marker were further
subjected to piperidine cleavage as described above.

Ligation mediated PCR (LM-PCR) as described previously (Gilmour and Fan
2009) was performed on 60 ng of permanganate treated samples including naked DNA
and G/A markers. Pfu polymerase used for DNA amplification in the LM-PCR reactions
was purified by Dr. Song Tan. Primers and their annealing temperatures utilized in LM-
PCR are listed in Appendix A.

Heat shock of Drosophila tissue culture cells was performed in a water bath pre-
heated to 37 °C. Cells were grown in 35 mm tissue culture dish in 10% FBS containing
Schneider’s Drosophila medium (Invitrogen) or Shields and Sang M3 insect medium.
After 4 days, the media from the tissue culture dish containing approximately 3 million
cells was aspirated and substituted with pre-warmed media for instant heat shock. This
culture dish was carefully floated over a support in the water bath such that the cells as
well the media are immersed. After the required period of heat shock the media was
carefully poured off and the cells were treated with 0.8 ml of chilled 10 mM KMnO₄
solution and incubated on ice for 1 min. The reaction was stopped with 0.2 ml of 5X
KMnO₄ stop solution. Further treatment and processing of the samples were performed as previously described (Gilmour and Fan 2009).

**HSF Binding Assay.**

20 pairs of salivary glands from control or NELF-D depleted larvae were isolated in 10 µl dissection buffer following appropriate heat shock treatments. After adding 60 µl of extraction buffer (10 mM HEPES pH 7.9, 0.4 M NaCl, 0.1 mM EGTA, 0.5 mM EDTA, 0.5 mM DTT, 5% Glycerol, 0.5 mM PMSF, 0.4% NP40, 1 % of protease inhibitor cocktail containing 1.6 mg/ml Benhamidine HCl, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin A, 1 mg/ml Leupeptin), the glands were flash frozen in liquid nitrogen. The glands were thawed and homogenized using a small pestle in an Eppendorf tube. The lysates (salt extracts) were centrifuged at 100,000g for 5 min in a Beckman airfuge. The extracts were transferred to a fresh tube, flash frozen and stored at -80 °C.

Radiolabelled HSE6 DNA was prepared by annealing HSE6 oligonucleotides (Fernandes et al. 1995) HSE6 F-5’-ttaagcaagctgtcataagcaattcgg and HSE6 R-3’-tcgcctttcgaagtacttacaagctcttataagcaatt-5’ in 250 mM Tris-HCl pH 7.7 at a concentration of 10 pmol/ µl. The duplex DNA was labeled by Klenow DNA polymerase fill-in using unlabelled α-thio-dATP and α³²P dTTP. The labeled template was purified using a Micro Biospin 6 column (Bio-Rad).

Binding reactions were assembled on ice by mixing 10 µl binding buffer (15 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.1mM EGTA, 0.5 mM DTT, 5% Glycerol and 0.1% NP40) and 2 µl of label mix containing 5 µg of tRNA, 1 µg of Hae III digested E. coli DNA and 5 fmol of³²P labeled HSE6 DNA. 15 µl of extract containing 7.5 µg of protein
was added to this mix and incubated at room temperature for 10 min. The samples were analyzed on a 1 % TAE-agarose gel at 4 °C. The gel was dried onto exposed X-ray film with a hot air blower, and then exposed to a phosphorimager and visualized with a Typhoon (GE Healthcare).
Chapter 3

Permanganate Genomic Mapping of Genes with Paused Pol II.

Introduction.

Expression of genes by Pol II is regulated at multiple steps during the transcription cycle. In *hsp70*, a molecule of Pol II is paused at the promoter proximal region and escape from this pause into productive elongation occurs upon heat shock stimulus (Rasmussen and Lis 1993; Rougvie and Lis 1988). In *Drosophila*, promoter proximal pausing of Pol II has been reported for 1000 of approximately 18000 genes (Lee et al. 2008; Muse et al. 2007; Zeitlinger et al. 2007). Paused Pol II was seen in *Drosophila* embryos on highly regulated genes that are involved in controlling development (Zeitlinger et al. 2007). Another study showed an enrichment of genes with paused Pol II that respond to developmental or stress stimuli (Muse et al. 2007). These studies indicate that promoter proximal pausing may be part of the mechanism that is necessary to manage the complex network of gene expression.

High-resolution nuclear run on assays reveal that the Pol II pauses in a narrow region but not at a single nucleotide. Quantitative analysis revealed that pausing in *hsp70* and *hsp27* showed a bimodal peak where the peaks were separated by 10 nucleotides (Rasmussen and Lis 1993). Paused Pol II was observed on thousands of genes but no requirement for a specific sequence that dictates the site of Pol II pausing has been determined (Lee et al. 2008). The elongation rate may influence where Pol II pauses,
since data from our lab indicates that in a cell free system the location of the pause changes with the rate of elongation. Nucleosome positioning in Drosophila may also influence promoter proximal pausing. The position of the +1 nucleosome at genes with paused Pol II tend to be 10 bases downstream from genes lacking paused Pol II (Mavrich et al. 2008). A recent study indicated that the melting temperature ($T_m$) of the 9 base pair RNA-DNA heteroduplex may stabilize the transcription bubble causing Pol II to pause in a region 20 – 35 nucleotides downstream from the TSS (Nechaev et al. 2010).

Pol II is paused 20-40 nucleotides downstream of the TSS in the hsp70 gene (Gilmour and Lis 1986). NELF was implicated in inhibiting transcript elongation by pausing Pol II (Yamaguchi et al. 1999a). NELF was also found to be concentrated at promoters of approximately 2000 genes, which are anticipated to have paused Pol II (Lee et al. 2008). RNAi mediated depletion of NELF impairs pausing on the hsp70 gene (Wu et al. 2003). Functionally, NELF-mediated pausing of Pol II regulates a myriad of events such as attenuation of proto-oncogenes, junB (Aida et al. 2006), breast cancer gene, BRCA1 (Aiyar et al. 2004) and HIV provirus (Zhang et al. 2007). In in vitro assays, NELF has been implicated as part of a checkpoint that coordinates elongation and mRNA capping (Mandal et al. 2004). In Drosophila hsp70, NELF dissociates from the uninduced gene during heat shock activation (Wu et al. 2005; Wu et al. 2003). Release from the paused state to the elongation state is extremely quick and can account for an increase in RNA levels (Chapter 4).

To investigate the physiological function of NELF and its role in gene regulation, we collaborated with Dr. Adelman to investigate a genomewide transcriptional profile of NELF regulated genes in Drosophila S2 cells. Of the 18,500 genes, NELF depletion
altered the expression levels of 241 genes. According to our previous observation, depleting NELF diminished pausing in *hsp70* (Wu et al. 2003); hence expression of NELF target genes should increase following NELF depletion. Surprisingly, expression levels of 170 of the 241 genes decreased following NELF depletion, indicating that NELF may be playing a positive role in the expression of these genes. Those genes that decreased in expression were referred to as down-regulated genes. Genes whose expression increased following NELF depletion were referred to as up-regulated genes. Expression levels of 35 candidate NELF-regulated genes determined by RT-PCR corroborated the microarray results (Gilchrist et al. 2008).

Here I will compare using permanganate genomic mapping (Gilmour and Fan 2009) in what way depletion of NELF may influence pausing of Pol II at genes that are either up-regulated or down-regulated in *Drosophila* salivary glands and tissue culture cells.

In this chapter, I also describe results that investigate how the rate of elongation influences pausing in vivo. This helps us distinguish between models based on kinetics verses thermodynamics. I will use salivary glands as a model system to analyze the pausing of Pol II in the C4 mutant *Drosophila* fly line. The C4 mutant harbors a mutation in the largest subunit of Pol II causing its elongation rate to be 2-fold slower than the wild type Pol II (Chen et al. 1996; Greenleaf et al. 1979). This may be explained by a possible interruption of the interaction between Val 1089 of the trigger loop and the mutation of Arg 741 to His. The extension of the imidazole side chain of His may interfere with the complete folding of the trigger loop. The trigger loop is necessary at the active center for delivery of the correct nucleotide (NTP) to the active site followed by catalysis and
further translocation of the DNA-RNA hybrid (Wang et al. 2006) (Fig. 3-1). Disruption of the folding of the trigger loop may slow the rate of phosphodiester bond formation and hence elongation. This single amino acid substitution in the C4 mutant may inhibit the function of the trigger loop thus slowing the rate of Pol II elongation.
**Figure 3-1.** C4 mutation may interfere with complete folding of the trigger loop. Model of Pol II elongation complex representing the proximity of the trigger loop and Arg 726 (yeast) in the Rpb1 subunit Pol II (PDB ID: 3HOY). Nucleic acids, amino acid Arg 726 (yeast) of Rpb1, Rpb1, Rpb2, Val 1089, bridge helix and the trigger loop are highlighted using the color key. Parts of the Pol II protein are omitted for clarity. The proximity of Val 1089 of the trigger loop with Arg 726, the site of the C4 mutation is depicted in the lower panel. Figure was generated using MacPymol.
Results.

RNA interference mediated depletion of NELF in salivary glands.

I investigated if the effects of NELF depletion on Pol II at up-regulated and down-regulated genes in *Drosophila S2* tissue culture cells also occurred in *Drosophila* salivary glands. Down-regulated genes were defined as ones that decrease expression when NELF was depleted while up-regulated genes were one that increased expression. This comparison was performed to gain a high-resolution view by permanganate footprinting of the effect of NELF depletion on the position of Pol II. Permanganate footprinting data for the S2 cells were either adapted from Chanhyo Lee’s thesis 2008 or from data published by Gilchrist et al. (Gilchrist et al. 2008). Hence some genes reported below do not show NELF-knock down results in S2 cells.

To express NELF RNAi in salivary glands, the parental fly line NELF-D 8-2 containing the NELF-D transgene controlled by Gal4 was mated with D7, 1824 that expressed Gal4 in salivary glands of third instar larvae. Mating the D7, 1824 and yw fly lines served as control. Depletion of NELF-D was confirmed by Western blot analysis indicating depletion of NELF-D and NELF-E but not DSIF or Rpb3, a subunit of Pol II (Fig. 4-1 Chapter 4).

**Down-regulated genes lose paused Pol II from promoter proximal regions upon NELF depletion.**

Occupancy of Pol II at the promoters of down-regulated genes that decreased upon NELF depletion also had a negative effect on the level of transcripts (Gilchrist et al. 2008). Permanganate mapping of 46 genes that have paused Pol II also showed
colocalization of NELF (Lee et al. 2008). I utilized permanganate genomic footprinting for a high-resolution view of the effect of NELF depletion on Pol II to compare NELF regulated genes in *Drosophila* salivary glands and S2 cells. Hyper-reactive thymines were observed at the promoters of many genes identified as NELF target genes. Fold changes in the levels of transcripts were calculated based on a correlation between NELF depletion, untreated cells and mock treated cells. Genes were selected from the microarray data that showed a decrease in the transcript levels after NELF depletion.

Permanganate reactivity in the promoter region of the down-regulated genes diminished following NELF depletion. The behavior of Pol II in *Drosophila* S2 cells and salivary gland was monitored by permanganate footprinting in the down-regulated genes CG30456, *TepII*, and *T1* that harbored paused Pol II. In CG30456, the pattern of permanganate reactivity at +25 and +37 was found to be similar in salivary glands and S2 cells (Fig. 3-2A, lane 3 and B, lane 5). This pattern of hyper-reactivity is due to the transcription bubble associated with transcriptionally engaged Pol II (Lis 1998). NELF-D depletion decreased permanganate reactivity at +25 and +37 when compared to the control (Fig. 3-2A compare lanes 3 and 4).

The presence of paused Pol II on *Tep II* in the control caused thymines at +29 and +34 to +40 of the non-transcribed strand to be hyper-reactive to permanganate in salivary glands and S2 cells (Fig. 3-2C, lane 4 and B, lane 3). RNAi against NELF-D diminished the permanganate reactivity at +29 and +34 to +40 in glands (Fig. 3-2C compare lanes 4 and 5) as well as S2 cells (Fig. 3-2D compare lanes 3 and 4) indicating that the level of paused Pol II was reduced. In addition, I observed a decrease in reactivity at +77 in salivary glands when NELF was depleted (Fig. 3-2C compare lane 4 to 5). The
differences in the banding patterns in salivary glands and S2 cells may be due to the ratio of signal to noise being lower in salivary glands or tissue specific. Due to the differences in intensities of the control and NELF-D depleted lanes (Fig. 3-2C, lanes 4 and 5), quantification of these lanes was performed (Appendix E, Fig. E-1). A comparison between the peaks of Pol II intensities in control and NELF-D depleted lanes indicated diminished levels of Pol II at +29, +38 and +77 upon NELF depletion (Appendix E, Fig. E-1).

Another gene T1, also known as Toll, displayed paused Pol II in the regions +26, +37 and +38 in the controls of salivary glands and S2 cells (Fig. 3-2E, lane 4 and F, lane 3). Depleting NELF showed a reduction in levels of Pol II at +26, +37 and +38 in both glands (Fig. 3-2E compare lanes 4 and 5) and S2 cells (Fig. 3-2F compare lanes 3 and 4). The permanganate patterns of these genes show significant reduction in reactivity at the promoter proximal regions, indicating a substantial reduction of paused Pol II at these promoters. Although differences were observed, overall the permanganate footprinting results were similar for both salivary glands and S2 cells. These results also suggest that maintenance of paused Pol II near these promoters requires NELF.
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A+G Ladder DNA 60' Mock-tr. NELF-dep.

- +29
- +34-36
- +38-40
- +46

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**Figure 3-2.** NELF depletion modifies levels of Pol II at down-regulated genes. *(A)* NELF-D was depleted in NELF-D 8-2 fly line while yw, D71824 was used as a control. Permanganate reactivity in salivary glands isolated from third instar larvae was analyzed near the TSS of CG30456. Lanes 1 and 2: naked DNA controls were either untreated or treated with KMnO₄ for 1 min. DNA derived from salivary glands was permanganate treated and subjected to LM-PCR. Lanes 3 and 4: depict control and NELF-D depleted glands. The accurate start sites were based on the new annotated start sites (Nechaev et al. 2010). *(B)* Permanganate reactivity in Drosophila S2 tissue culture cells was analyzed near the TSS of CG30456. Lanes are denoted as G/A marker (lane 1), naked DNA controls were either untreated (lane 2) or treated with KMnO₄ for 30 sec (lane 3), or 60 sec (lane 4). DNA from Drosophila S2 cells was permanganate treated and subjected to LM-PCR (lane 5) (Results in panel *(B)* were obtained by Chanyo Lee and were adapted from his thesis). The accurate start sites were based on the new annotated start sites (Nechaev et al. 2010). *(C)* NELF-D was depleted in NELF-D 8-2 fly line, while yw, D71824 was used as a control. Permanganate reactivity in salivary glands isolated from third instar larvae was analyzed near the TSS of TepII. Lane 1: G/A marker, Lanes 2 and 3: naked DNA controls were either untreated or treated with KMnO₄ for 1 min. DNA derived from salivary glands was permanganate treated and subjected to LM-PCR. Lanes 4 and 5: depict control and NELF-D depleted glands. *(D)* Permanganate reactivity in Drosophila S2 tissue culture cells was analyzed near the TSS of TepII. Lanes are denoted as, G/A marker (lane 1), naked DNA control treated with KMnO₄ for 60 sec (lane 2). Lanes 3 and 4: DNA mock treated or NELF-depleted in Drosophila S2 cells was permanganate treated and subjected to LM-PCR (Results in panel *(D)* were performed by the Adelman lab and were adapted from (Gilchrist et al. 2008)). *(E)* NELF-D was depleted in NELF-D 8-2 fly line while yw, D71824 was used as a control. Permanganate reactivity in salivary glands isolated from third instar larvae was analyzed near the TSS of T1. Lane 1: G/A marker, Lanes 2 and 3: naked DNA controls were either untreated or treated with KMnO₄ for 1 min. DNA derived from salivary glands was permanganate treated and subjected to LM-PCR. Lanes 4 and 5: depict control and NELF-D depleted glands. *(F)* Permanganate reactivity in Drosophila S2 tissue culture cells was analyzed
near the TSS of *T1*. Lanes are denoted as G/A marker (lane 1), naked DNA control treated with KMnO₄ for 60 sec (lane 2). Lanes 3 and 4: DNA mock treated or NELF-depleted in *Drosophila* S2 cells was permanganate treated and subjected to LM-PCR (Results in panel (F) were performed by the Adelman lab and were adapted from (Gilchrist et al. 2008)).
Surprisingly, syx4, a gene that was down-regulated upon NELF depletion did not show a significant change in permanganate reactivity. This is partly because the strong permanganate reactivity that was seen in the tissue culture cells at +36, +37 and +40 to +43 (Fig. 3-3A) was absent in the salivary glands (Fig. 3-3B) (Fig. compare 3-3A lane 3 and 3-3B lane 5). This variation may be a tissue specific effect.
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**GA markers**

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Figure 3-3. Down-regulated gene *syx4* shows no change in Pol II level. *(A)* NELF-D was depleted in NELF-D 8-2 fly line while yw, D71824 was used as a control. Permanganate reactivity in salivary glands isolated from third instar larvae was analyzed near the TSS. Lanes 1 and 2: naked DNA controls were either untreated or treated with KMnO$_4$ for 1 min. DNA derived from salivary glands was permanganate treated and subjected to LM-PCR. Lanes 3 and 4: depict control and NELF-D depleted glands. *(B)* Permanganate reactivity in *Drosophila* S2 tissue culture cells was analyzed near the TSS. Lanes were denoted as G/A marker (lane 1), naked DNA controls were either untreated (lane 2) or treated with KMnO$_4$ for 30 sec (lane 3), or 60 sec (lane 4). DNA from *Drosophila* S2 cells was permanganate treated and subjected to LM-PCR (lane 5) (Results in panel *(B)* were obtained by Chanhyo Lee and were adapted from his thesis).
Effect of NELF depletion on Pol II elongation in up-regulated genes.

I selected genes that showed a 2-5-fold increase in transcripts for footprinting by potassium permanganate. If these genes were repressed by NELF, upon its depletion I expect an increase in permanganate reactivity downstream of the promoter and diminished pausing. *Hsp70* was up-regulated upon NELF depletion and the permanganate mapping showed reduced pausing at +22 and +30. It would be interesting to compare the pattern of permanganate reactivity seen in tissue culture cells to that of salivary glands to evaluate if the effect of NELF depletion is not limited to S2 cells.

At the NELF repressed gene *oaf*, Pol II pausing was observed in the regions +30 to +35 in both salivary glands and S2 cells (Fig. 3-4A, lane 2 and B, lane 5). Depletion of NELF-D in salivary glands did not change permanganate reactivity in the region from +27 to +47 (Fig. 3-4A compare lanes 2 and 3), but increased the reactivity downstream from +52 to +83 (Fig. 3-4A lane 3), demonstrating that Pol II recruitment and pausing persists at this gene. Also the increase in permanganate reactivity upstream of position +27 in NELF-D depleted glands could be due to Pol II initiating transcription.

We observed that NELF depletion in *Drosophila* S2 cells led to increased permanganate reactivity at sites further downstream from the promoter of up-regulated gene *mfas* at positions +45, +55 (Fig. 3-4D compare lanes 3 and 4), but a similar result was not observed for salivary glands. (Fig. 3-4C compare lanes 2 and 3). This may be a case where Pol II escapes from the pause and rapidly enters the gene and hence no significant increase in permanganate reactivity was detected. This may be a tissue specific effect that is limited to the S2 cells and not salivary glands. Another explanation is that the signal to noise ratio is much weaker in glands.
A

Control
NELF-D depleted

B

GA markers

0'' Naked DNA
30'' Cells, 60''

1 2 3 4 5

oaf

1 ! 2 ! 3 ! 4 ! 5

+30, +31
+27
+34, +35
+44, +45
+50 ~ +53
+63, +64
+77
+80
+82, +83
+3, +4
+6 ~ +10
+13, +14
+17, +18
+22, +23
+26, +27
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+64, +65
+68, +69
+72, +73
+76, +77
+80, +81
+84, +85

Naked DNA (1')

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+77
+71
+66, +67
+60, +61
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+40 ~ +42
+37, +38
+31, +32
+24, +25
+17, +18
+11, +12
+6, +7
+3, +4
+2, +3
**Figure 3-4.** NELF depletion diminishes pausing at up-regulated genes. (A) NELF-D was depleted in NELF-D 8-2 fly line while yw, D71824 was used as a control. Permanganate reactivity in salivary glands isolated from third instar larvae was analyzed near the TSS of *oaf*. Lane 1: naked DNA control treated with KMnO₄ for 1 min. DNA derived from salivary glands was permanganate treated and subjected to LM-PCR. Lanes 2 and 3: depict control and NELF-D depleted glands. (B) Permanganate reactivity in *Drosophila* S2 tissue culture cells was analyzed near the TSS of *oaf*. Lanes are denoted as G/A marker (lane 1), naked DNA controls were either untreated (lane 2), or treated with KMnO₄ for 30 sec (lane 3), or 60 sec (lane 4). Lane 5: DNA from *Drosophila* S2 cells was permanganate treated and subjected to LM-PCR. (Results in panel (B) were obtained by Chanhyo Lee and were adapted from his thesis). (C) NELF-D was depleted in NELF-D 8-2 fly line while yw, D71824 was used as a control. Permanganate reactivity in salivary glands isolated from third instar larvae was analyzed near the TSS of *mfas*. Lane 1: naked DNA control treated with KMnO₄ for 1 min. DNA derived from salivary glands was permanganate treated and subjected to LM-PCR. Lanes 2 and 3: depict control and NELF-D depleted glands. (D) Permanganate reactivity in *Drosophila* S2 tissue culture cells was analyzed near the TSS of *mfas*. Lanes are denoted as G/A marker (lane 1), naked DNA control treated with KMnO₄ for 60 sec (lane 2). Lanes 3 and 5: DNA mock treated or NELF-depleted in *Drosophila* S2 cells was permanganate treated and subjected to LM-PCR (Results in panel (D) were performed by the Adelman lab and were adapted from (Gilchrist et al. 2008)).
In vivo analysis of the effect of the rate of elongation on the position of Pol II pausing.

A recent study reported that in an in vitro transcription system, addition of DSIF or NELF alone had no effect on the elongation rate of Pol II but addition of both DSIF and NELF to the reaction inhibited transcription (Missra and Gilmour 2010). In my preceding analysis, the appearance of permanganate reactivity in oaf downstream from normal pause site after depleting NELF could be due to a decrease in the rate at which NELF associates with the elongation complex.

Hence, there might be a kinetic competition between the rate of elongation and the rate of association of NELF with the elongation complex. To test this hypothesis, I used permanganate footprinting to evaluate promoter proximal pausing in salivary glands of flies expressing a slow form of Pol II known as the C4 mutant. In the event that the kinetics of elongation determined the sites of Pol II pausing, I expect the patterns of permanganate reactive thymines to show an upstream shift towards the TSS.

I selected genes that had paused Pol II distributed over either a broad region such as in oaf or narrow region but paused further downstream from the TSS as in CG9008. These genes were selected based on published analysis (Lee et al. 2008). Permanganate genomic footprinting of oaf was performed in the salivary glands of both control Pol II and the slow mutant of Pol II. The results indicate a shift in the pattern of permanganate reactive bands in the slow Pol II. The hyper-reactive bands at +40, +41, +42, +44, +45 and +47 are of stronger intensity in the control Pol II as compared to the slow Pol II where the stronger bands were detected at +27, +30, and +31 (Fig. 3-5A compare lanes 3 and 4). Subsequently, promoters of CG9008, \textit{Fhos}, \textit{cora}, and \textit{ppa} were analyzed by
permanganate genomic footprinting. Permanganate mapping of CG9008 showed increased permanganate reactivity at +20, +21 and +31 in the slow Pol II mutant when compared to the control Pol II denoting an upward shift of paused Pol II (Fig. 3-5B compare lanes 3 and 4). A similar upstream shift in paused Pol II was observed in the slow Pol II mutant in *Fhos* at sites +20, +25, +26, and +30 (Fig. 3-5C compare lanes 3 and 4), *cora* at sites +15, +17, +18, +21, +32 and +37 (Fig. 3-5D compare lanes 3 and 4) and *ppa* at sites +13, +16, +21, +22, and +25 (Fig. 3-5E compare lanes 3 and 4). These results suggest that slowing the rate of elongation causes the location of the pause to shift closer to the TSS.
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**Figure 3-5.** Slow mutant of Pol II affects the rate of Pol II elongation. Salivary glands of control (yw) and homozygous (stock number 3663 v1 RpII2154) α-amanitin+ fly lines were isolated and treated with KMnO₄ as indicated earlier. Naked DNA controls in Lanes 1 and 2 in all panels were either untreated or treated with KMnO₄ for 1 min respectively. Pattern of permanganate footprinting for genes with primers near the TSS are illustrated for control (lane 1) and homozygous (lane 2) in (A) oaf, (B) CG9008, (C) Fhos, (D) cora, and (E) ppa genes.
I further analyzed the paused Pol II on our model gene, *hsp70* by permanganate footprinting. I observed that the permanganate reactivity at +22 in the slow mutant Pol II was shifted upstream compared to the control Pol II (Fig. 3-6 compare lanes 3 and 4). The intensity of the +22 site in the slow mutant was approximately 1.5 fold more than the control pol II measured by the Imagequant trace (Fig. 3-6). Due to the narrow region of pausing observed in *hsp70*, the upward shift of Pol II was subtle.
Figure 3-6. Slow mutant of Pol II may pause upstream of the TSS in *hsp70* gene. Salivary glands of control (+/SG-Gal4) and homozygous (stock number 3663 v¹ RplI215¹) α-amanitin⁷ fly lines were isolated and treated with KMnO₄ as indicated earlier. Naked DNA control in Lanes 1 and 2 were either untreated or treated with KMnO₄ for 1 min respectively. Pattern of permanganate footprinting of *hsp70* gene near the TSS are indicated for control (lane 1) and Pol II mutant (lane 2) fly lines. The graph shown is a representation of the intensity of thymine reactivity at +22, +30 and +34 was quantified using Imagequant software from GE healthcare.
Depletion of TFIIS in Drosophila S2R+ tissue culture cells.

Recently, Adelman and colleagues reported that pausing by Pol II may be favored by the thermodynamic stability of the RNA-DNA heteroduplex. Fluctuations in the stability make Pol II backtrack to a more thermodynamically favored site (Nechaev et al. 2010). I observed an upstream shift in the pause site of the C4 mutant Pol II (slow mutant of Pol II) compared to the wild type. This may be because the mutation on the Rpb1 subunit of C4 Pol II (Arg 741 to His) is close enough to the TFIIS binding site to interfere with its activity (Fig. 3-7). If the C4 mutant Pol II backtracks after initiation and is arrested upstream, TFIIS may not be able to catalyze the release of this arrested Pol II. This may result in Pol II that is arrested upstream creating the illusion of a slow moving Pol II. If this is the case, I predict that depleting TFIIS will have a similar effect to the slow mutation and cause a similar shift in the permanganate reactivity.

To test this prediction, TFIIS was depleted in the S2R+ Drosophila tissue culture cells using RNAi technique. Using LacZ, a gene that encodes β-galactosidase in E.coli as a control, TFIIS was depleted by approximately 82 percent (Fig. 3-8).
**Figure 3-7.** C4 mutant Pol II may hinder the catalytic activity of TFIIS. A model of Pol II elongation complex representing the Pol II-TFIIS complex (PDB ID: 1Y1V). Nucleic acids, amino acid Arg 726 (yeast) of Rpb1, Rpb1, Rpb2, TFIIS, bridge helix and the trigger loop are highlighted using the color key. Parts of the Pol II protein are omitted for clarity. TFIIS binding cleft with proximity to Arg 726, the site of the C4 mutation, is depicted in the lower panel. Figure was generated using MacPymol.
TFII was depleted in *Drosophila* S2R+ tissue culture cells using RNAi. LacZ depletion was performed as a control. Cells were treated with 5 µg of dsRNA for 4 days. After lysis, an equivalent of 10 µg of protein was applied to a 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed with antibodies raised against TFIIS at 1:5000 dilution and Rpb3 at 1:2500 dilution. Rpb3 was used as a loading control. The blot was visualized on typhoon and the bands were quantified against Rpb3 protein.

**Figure 3-8.** TFIIS was depleted in *Drosophila* S2R+ tissue culture cells using RNAi. LacZ depletion was performed as a control. Cells were treated with 5 µg of dsRNA for 4 days. After lysis, an equivalent of 10 µg of protein was applied to a 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed with antibodies raised against TFIIS at 1:5000 dilution and Rpb3 at 1:2500 dilution. Rpb3 was used as a loading control. The blot was visualized on typhoon and the bands were quantified against Rpb3 protein.
Permanganate footprinting analysis of TFIIS depleted cells.

To evaluate the possibility of loss of TFIIS activity in the C4 mutant Pol II caused the upstream shift in the C4 Pol II, I mapped the promoters of genes by permanganate footprinting. If the permanganate pattern is shifted upstream towards the TSS it would indicate an arrested Pol II due to the loss of TFIIS activity. The expected permanganate patterns would resemble the patterns in the C4 mutant Pol II.

On analysis of the results in the *Fhos* gene, the pattern of permanganate reactivity for LacZ depleted cells at +41, +44, +51, +65 and +70 are indicative of the regular pause sites as seen earlier in salivary glands. But increased intensity in the pattern of permanganate reactivity in TFIIS depleted cells at +20, +25, +26, and +30 that would indicate an upstream shift of Pol II was not observed (Fig. 3-9A compare lanes 3, 4 and 7, 8). Results shown are representative of biological replicates. Similarly promoters of the genes *cora*, *ppa* and *CG9008* were also analyzed and showed no upstream shift in the permanganate patterns when TFIIS was depleted (Fig. 3-9B, C and D compare lanes 3, 4 and 7, 8). These results are consistent with the conclusion that the upstream shift in the location of the C4 Pol II is not a consequence of the ability of Pol II to function with TFIIS.
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**C**

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- +72, +73
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- +94
- +97
- +102, +103

**D**

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Figure 3-9. Permanganate footprinting in TFIIS depleted cells. RNAi in *Drosophila* S2R+ cells was performed to deplete TFIIS. Cells were treated with dsRNA for 4 days. LacZ depletion was used as a control. Naked DNA controls in Lanes 1 and 2 in all panels were either treated or untreated with KMnO₄ for 1 min respectively. Pattern of permanganate footprinting for genes with primers near the TSS are illustrated for control (lane 3) and TFIIS RNAi (lane 4) in (A) *Fhos*, (B) *cora*, (C) *ppa*, and (D) *CG9008* genes. The data generated is a representation of two biological repeats.
Discussion.

Permanganate genomic footprinting of genes regulated by NELF mediated paused Pol II in *Drosophila* salivary glands and S2 cells.

Results from the microarray analysis identified 241 out of 18,000 genes in *Drosophila* S2 cells that change expression upon depletion of NELF. Previous studies have identified promoter proximal pausing in *hsp70* as a target of NELF (Wu et al. 2003). We found that NELF stimulates transcription of nearly 70% of NELF target genes (Gilchrist et al. 2008). Previous reports of genome wide analyses of NELF-depleted human cells show significant numbers of NELF down-regulated transcripts (Aiyar et al. 2007; Narita et al. 2007). Our analysis reveals that NELF can attenuate the expression of inducible genes that possess paused Pol II that are poised for rapid induction upon activation (Gilchrist et al. 2008).

Adelman and colleagues identified genes that are up-regulated and down-regulated upon NELF depletion (Gilchrist et al. 2008). I analyzed these genes in salivary glands by permanganate footprinting to gain insight into how NELF influences these two groups of genes. Based upon previous studies, NELF binds to Pol II and, along with DSIF and other factors, induces promoter proximal pausing (Renner et al. 2001; Wu et al. 2003; Yamaguchi et al. 2002). Depletion of NELF diminishes pausing, hence allowing Pol II to resume transcript elongation. In the case of heat shock genes, depletion of NELF diminishes Pol II pausing at +22 in *hsp70* and at +33 in *hsp26* (Wu et al. 2003) (Chapter 4, Fig. 4-2A). Permanganate footprinting of the NELF up-regulated gene *oaf*, whose expression increased upon NELF depletion, shows significant presence of Pol II downstream of the pause site (Fig. 3-4A). In *mfas*, which is also up-regulated upon NELF
depletion, presence of Pol II was seen further into the body of the gene in S2 cells (Fig. 3-4D, lane 4), while in the case of salivary glands (Fig. 3-4C, lane 3) a different permanganate pattern was observed compared to the S2 cells (Compare lane 3 in Fig. 3-4C to lane 4 in Fig. 3-4D). This may be because Pol II escapes from the pause and rapidly enters the gene and hence no significant increase in permanganate reactivity was detected. In comparison, in case of the down-regulated genes I observed a decrease in permanganate reactivity upon NELF depletion (Fig. 3-2) in salivary glands and S2 cells. This implies that there is no new initiation of Pol II after the molecule of paused Pol II was released following NELF depletion.

Chromatin remodeling factors may be involved in maintaining the promoters of these NELF target genes accessible for elongation. Dr. Adelman’s lab observed that histone H3 occupancy at the up-regulated genes such as hsp70 and oaf did not change upon NELF depletion, whereas a significant increase was observed in down-regulated genes TepII, T1 and CG30456. This indicates that paused Pol II may be preventing the encroachment of the nucleosome on these genes. Previous analysis suggests that a shift in position of the canonical +1 nucleosome downstream may be a result of paused Pol II (Mavrich et al. 2008). Hence paused Pol II may affect the occupancy as well as nucleosome positioning in the promoter proximal region.

Test if depletion of TFIIS shifts the site of promoter proximal pausing.

At hsp70, Pol II pauses in the promoter proximal region after transcribing about 20 – 40 nucleotides (Rasmussen and Lis 1993). If Pol II pauses for an extended period of time then it could have a tendency to backtrack on the gene and displace the 3’ end of the
RNA from the active site of Pol II. This arrested state of Pol II is relieved by the action of TFIIS by cleavage of the 3’ extruding RNA (Wang et al. 2009). Adelman et al, has observed that Pol II appears to accumulate in an arrested state in the promoter proximal region of cells that have been depleted of TFIIS (Adelman et al. 2005). Furthermore, she suggests that the mechanism resulting in the pausing of Pol II in the promoter proximal region is one in which a Pol II transcribes beyond its ultimate site of pausing, backtracks, and finally engages TFIIS to realign the 3’ end of the nascent transcript with the active site of the Pol II (Nechaev et al. 2010). Since the C4 mutation (Arg 741 to His) is located near where TFIIS enters the Pol II (Fig. 3-7), the mutant might be defective in cutting the RNA and become trapped in a position upstream from normal. Several points argue against this possibility. First, Greenleaf and colleagues showed that this mutation does not interfere with TFIIS-dependent escape from arrest sites in vitro (Chen et al. 1996). Second, Adelman does not detect any difference in the location of the transcription bubble on specific genes in control and TFIIS-depleted cells (Nechaev et al. 2010). Indeed, here a yoyo model in which Pol II overshoots the pause and then backtracks up to a final location does not predict the upstream shift that I observe.

I attempted to test if the upstream shift of the C4 pol II is due to its inability to be acted upon by TFIIS. If this were the case, then depleting TFIIS should have the same effect as the C4 mutation. I failed to see any shift in the location of the transcription bubbles upon depletion of TFIIS from tissue culture cells. My observation for the gene CG9008 is in accordance with reports from the Adelman lab where depletion of TFIIS does not shift the location of the transcription bubble (Nechaev et al. 2010). A limitation of my TFIIS depletion experiment is that I do not know if the extent of depletion is
sufficient to inhibit TFIIS activity in vivo. Further analysis of RNA lengths from TFIIS depleted cells and control may reveal the extent of TFIIS depletion. Nechaev et al. 2010, reported a global increase in RNA lengths in cells depleted of TFIIS compared to the control. This would also resolve whether the upstream shift in Pol II pausing is an effect of the rate of elongation or due to Pol II arrest owing to loss of TFIIS activity. In the event that the shift is due to slower rate of elongation, I anticipate that the length of RNA will be shorter than if Pol II has backtracked and arrested upstream where the length of RNA will be longer.

**Kinetics of promoter proximal pausing.**

Biochemical evidence indicates that NELF and DSIF are involved in promoter proximal pausing (Wu et al. 2003; Yamaguchi et al. 1999a). No requirement for a specific sequence that dictates the site of Pol II pausing has been determined (Lee et al. 2008). Jian Li, a colleague in our lab, has results indicating that location of the pause shifts closer to the transcription start site when the nucleotide concentration is lowered in an *in vitro* transcription reaction. My discovery that the C4 mutant shifts the location of the pause suggests that the rate of elongation affects where the Pol II pauses. The elongation rate of the C4 mutant was determined to be half that of normal Pol II *in vitro* (Chen et al. 1996). I propose that this reduction in the rate of elongation allows NELF and DSIF to capture the mutant Pol II before it advances to the location normally reached by wild-type Pol II. As the nascent transcript extrudes from the elongating Pol II, DSIF and NELF associate with it and cause Pol II to pause once it has transcribed at least 20 nucleotides (Missra and Gilmour 2010). The upstream shift in the pause site that I have
observed suggests that there is kinetic competition between rate of elongation and binding of DSIF and NELF.

A recent model for promoter proximal pausing argues that the location of the pause is dictated by the thermodynamic stability of RNA-DNA duplex in the transcription elongation complex (Nechaev et al. 2010). This sequence specific mechanism of pausing seems at odds with my kinetic model because it places major emphasis on contributions of the transcribed sequence. While the thermodynamic model may be well suited for a molecule of Pol II that may be arrested due to backtracking, I propose that DSIF and NELF can be rapidly deployed to efficiently bind the extruding RNA and pause Pol II when it has transcribed 18-20 nucleotides (Missra and Gilmour 2010).

Portions of this chapter are published in Genes & Development (Gilchrist et al. 2008).
Chapter 4

Negative ELongation Factor, NELF, accelerates the rate at which heat shock genes are shut-off by facilitating the dissociation of heat shock factor.

Introduction.

Recent analyses of the distribution of Pol II in animal cells reveal that Pol II is concentrated at the 5’ end of thousands of genes (Core et al. 2008; Guenther et al. 2007; Muse et al. 2007). In most cases, the Pol II is transcriptionally engaged but paused within the first 50 nucleotides of the TSS (Core et al. 2008; Gilchrist et al. 2008; Lee et al. 2008). The pause is mediated by the cooperative activity of DRB sensitivity-inducing factor (DSIF) and Negative ELongation Factor (NELF), which inhibit transcription elongation (Yamaguchi et al. 1999a). The duration of the pause varies for different genes and is a rate-limiting step in the expression of many genes (Fuda et al. 2009). The kinase, P-TEFb appears to control the duration of the pause by phosphorylating Pol II, NELF, and DSIF (Cheng and Price 2007; Renner et al. 2001).

Possible functions for promoter proximal pausing are beginning to be identified. Genes that respond rapidly to external signals tend to have paused Pol II (Adelman et al. 2009; Kininis et al. 2009). The paused state could accelerate the rate of induction by allowing the chromatin remodeling and histone modifications that precede recruitment of Pol II to be uncoupled from transcription elongation, thus establishing a state that is poised for rapid induction. For some genes, the paused Pol II appears to prevent a
nucleosome from assembling over the core promoter region, which would otherwise repress transcription initiation (Gilchrist et al. 2010; Gilchrist et al. 2008). Finally, paused Pol II has been shown to function as an insulator of enhancer function, thus serving as a way to demarcate functional domains in chromatin (Chopra et al. 2009).

Promoter proximal pausing was first identified on the \textit{hsp70} gene (Rougvie and Lis 1988). A long-standing hypothesis is that promoter proximal pausing allows for the rapid induction of the heat shock genes (Lis 1998). I set out to test this hypothesis by determining if disrupting promoter proximal pausing affects the kinetics of induction. RNAi-mediated depletion of NELF impairs pausing on \textit{hsp70} (Gilchrist et al. 2008; Wu et al. 2003). Surprisingly, I observed that depletion of NELF has no significant impact on the rates at which \textit{hsp70} or \textit{hsp26} are induced. Instead, depletion of NELF impairs the rate at which these genes shut-off during recovery from a heat shock. Further analysis reveals that NELF depletion impairs the rate at which HSF, the transcriptional activator of heat shock genes, dissociates from the heat shock gene during recovery from heat shock.

\textbf{Results.}

\textbf{Depletion of NELF disrupts promoter proximal pausing but does not significantly impact the rate of heat shock induction.}

To test if promoter proximal pausing contributes to the rapid induction of \textit{hsp70}, I disrupted this pausing by using RNAi to deplete the NELF-D subunit from \textit{Drosophila} salivary glands. \textit{NELF-Di}^{8-2} or \textit{NELF-Ei}^{17A10} was mated with \textit{SG-Gal4} to express NELF-D or NELF-E RNAi in salivary glands of larval offspring. As a control, \textit{yw} was mated
with *SG-Gal4*. Indirect immunofluorescence microscopy of polytene chromosomes derived from the salivary glands revealed that RNAi against NELF-D depleted both NELF-D and NELF-E from the chromosomes (Fig. 4-1A i, compare panels e to f and g to h). In contrast, only the level of NELF-E associated with the chromosome is reduced by the RNAi against NELF-E (Fig. 4-1A ii, compare panels e to f and g to h). These results are in agreement with our previous finding that depleting NELF-D in *Drosophila* cells with RNAi results in loss of both NELF-D and NELF-E whereas depletion of NELF-E with RNAi only results in loss of NELF-E (Gilchrist et al. 2008). I propose that the loss of NELF-D destabilizes the NELF complex and results in degradation of NELF-E. Immunoblot analysis confirmed that the RNAi against NELF-D decreased the level of D and E and depletion of NELF-E decreased levels of NELF-E but had no effect on Spt5, the largest subunit of DSIF and Rpb3, the third largest subunit of Pol II (Fig. 4-1B). Although the decrease in NELF-D is more pronounced than NELF-E on the immunoblot in the NELF-D RNAi case, the inability to assemble complete complexes results in the loss of both proteins from polytene chromosomes.
A

i

a  NELF-D RNAi

b  NELF-D RNAi

c  NELF-D RNAi

d  NELF-D RNAi

e  NELF-E

f  NELF-E

g  NELF-E

h  NELF-D

Control and NELF-D depleted

ii

a  NELF-E RNAi

b  NELF-E RNAi

c  NELF-E RNAi

d  NELF-E RNAi

e  NELF-E

f  NELF-E

g  NELF-D

h  NELF-D

Control and NELF-E depleted
**Figure 4-1.** Depletion of NELF in salivary glands of *Drosophila.* (A), Immunodetection of NELF-D and NELF-E on polytene chromosomes from +/SG-Gal4 (control), *NELF-D*\textsuperscript{8-2}/SG-Gal4 (NELF-D RNAi) and *NELF-E*\textsuperscript{i7A10}/SG-Gal4 (NELF-E RNAi) larvae. The chromosome spreads were stained with either affinity-purified, rabbit anti-NELF-D or anti-NELF-E antibodies. The primary antibodies were visualized with Alexa 568-conjugated anti-rabbit antibody. Panels a, b, c & d show chromosomes stained with the DNA stain, Hoechst. Panels e & f, were stained with anti-NELF-E antibody in control, NELF-D (i) and NELF-E (ii) depleted larvae. Panels g & h were stained with anti-NELF-D antibody in control, NELF-D (i) and NELF-E (ii) depleted larvae. (B), Immunoblot analysis of lysates from control, NELF-D and NELF-E RNAi-expressing salivary glands. Amounts of total protein are indicated at the top of each lane. The blot was probed with antibodies raised against NELF-D, NELF-E, Spt5 and Rpb3. Spt5 is the largest subunit of DSIF. A magnified image of the middle panel shows the depletion of NELF-D, which is the faster moving band of the doublet.
The behavior of Pol II on hsp70 in non-heat shocked and heat shocked salivary glands was monitored by permanganate genomic footprinting (Gilmour and Fan 2009). Thymines in single stranded DNA such as transcription bubbles are often more reactive to oxidation by permanganate than in double stranded DNA. Paused Pol II on hsp70 causes thymines at +22, +30 and +34 of the non-transcribed strand to be hyper-reactive to permanganate oxidation (Fig. 4-2A, lane 3). Previous work demonstrates that this pattern of hyper-reactivity is due to the transcription bubble associated with transcriptionally engaged Pol II (Lis 1998). RNAi against the two NELF subunits, NELF-D and NELF-E, reduced the permanganate reactivity at these three positions indicating that the level of paused Pol II is diminished upon depletion of NELF (Fig. 4-2A, compare lanes 3, 6 and 9).

Heat shock induction of hsp70 dramatically alters the pattern of permanganate reactivity on hsp70 in accordance with transcriptional activation. Permanganate hyper-reactive sites associated with the train of Pol II molecules transcribing the gene are observed to extend well beyond the pause site (Fig. 4-2A, lanes 4 and 5). To determine if depletion of NELF and the concomitant loss of paused Pol II affected the rate of induction, I compared the permanganate reactivity at 2.5 and 5 min of heat shock for the control and NELF depleted salivary glands. No significant difference was observed in the level of reactivity between control and NELF depleted samples suggesting that the rate of induction was not affected by the loss of paused Pol II (Fig. 4-2A, compare lanes 4 and 5 to 7 and 8 and 10 and 11).

As an additional test of the contribution of NELF to the rate of induction, I compared the levels of hsp70 RNA synthesized during a 5 min heat shock in control and
NELF-depleted salivary glands. *Hsp70* transcript levels in glands from the various control glands (+/SG-Gal4, +/-NELF-Di\(^{8-2}\), and +/-NELF-Di\(^{2-1}\)) following the 5 min heat shock varied by approximately 30% percent (Fig. 4-2B). Importantly, levels of *hsp70* transcripts in NELF-depleted glands (NELF-Di\(^{8-2}\)/SG-Gal4 and NELF-Di\(^{2-1}\)/SG-Gal4) were within the range of variation exhibited by the controls. Thus, I conclude that the presence of paused Pol II is not required for rapid induction of *hsp70*. 
Figure 4-2. Kinetics of heat shock induction is unaffected following disruption of pausing. (A) Permanganate genomic footprinting analysis of hsp70 in salivary glands isolated from +/SG-Gal4 (Control), NELF-D\textsuperscript{8-2}/SG-Gal4 (NELF-D depleted) and NELF-D\textsuperscript{17A10}/SG-Gal4 (NELF-E depleted) larvae. Lanes 1 and 2: naked DNA untreated or treated with KMnO\textsubscript{4} for 1 min. Lanes 3, 6 and 9: DNA derived from non heat shocked salivary glands and permanganate treated. Lanes 4, 7 and 10: DNA derived from salivary glands heat shocked for 2.5 min and permanganate treated. Lanes 5, 8 and 11: DNA derived from salivary glands heat shocked for 5 min and permanganate treated. Numbers along the right margin are the locations of thymines on the non-transcribed strand of DNA relative to the TSS. (B) Quantitative RT-PCR analysis of hsp70 RNA levels in salivary glands. Salivary glands were isolated from NELF-D depleted glands (NELF-D\textsuperscript{8-2}/SG-Gal4 and NELF-D\textsuperscript{2-1}/SG-Gal4) or control glands (+/SG-Gal4, +/NELF-D\textsuperscript{8-2}, and +/NELF-D\textsuperscript{2-1}). Glands were then either untreated (NHS) or heat shock treated at 37 °C for 5 min. (5’ HS). The relative values were normalized to the +/SG-Gal4 NHS sample. Results are from two biological replicates, and error bars indicate the range of measurements from the replicates.
Depletion of NELF impairs the shut-off of \textit{hsp70} during recovery from heat shock.

I investigated the possibility that NELF functions in shutting off the \textit{hsp70} promoter while cells recover from heat shock. Control, NELF-D- and NELF-E-depleted larvae were heat shocked for 20 min to induce \textit{hsp70} and then returned to normal temperature for recovery. Using permanganate footprinting, I found that reestablishment of the paused state following the shift of control larvae from heat shocked to non-heat shocked conditions occurred within 45 min of the temperature shift (Fig. 4-3A, lanes 3 to 7 and lanes 15 to 18). In contrast significant transcriptional activity still occurred on \textit{hsp70} in NELF-D depleted salivary glands after 45 min of recovery, as evidenced by the permanganate reactivity at +7 and +8 and in the region downstream from +34 (Fig. 4-3A, lanes 9 to 12 and lanes 20 to 23). I also tested if NELF-E depleted salivary glands displayed a defect in recovery from heat shock after the larvae were recovered for 45 min. There was no defect in recovery detected. The NELF-E depleted glands behaved similarly as the control (Fig. 4-3B compare lanes 5 and 8). Eventually after 120 min of recovery, transcription of \textit{hsp70} was repressed to the non-heat shocked state (Fig. 4-3C). Because NELF-E showed similar effect as the control, all further experimentation to evaluate the role of NELF were performed in NELF-D depleted salivary glands.
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Recovery (min) 0 45 0 45

Heat Shock (20 min)

+7, +8
+22
+30
+45
+53
+67
+76
+83, +85
+90
+95
+101, +102
+107
+111
+115, +116
+122
+137
+141
+146
+152
+155
+160
+162
+172
+175, 176
+178, 179
+183

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Recovery (min) 0 0 0

Heat shock (20 min)

+7, +8
+22
+30
+34
+45
+53
+67
+76
+83, +85
+90
+95
+101, +102
+107
+111
+115, +116
+122
+137
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+178, 179
+183
Figure 4-3. NELF is required for normal heat shock gene shut-off. (A) Permanganate genomic footprinting analysis of hsp70 in glands from control and NELF-D depleted larvae subject to heat shock and recovery. Displayed are representative results from the analysis of control glands (+/SG-Gal4: lanes 3-7 and lanes 14-18) and two different NELF-D depleted glands (NELF-Dr8-2/SG-Gal4 and NELF-Dr2-1/SG-Gal4: lanes 8-12 and 19-23 respectively). Larvae were heat shocked at 37 °C for either zero (NHS) (lanes 3, 8, 14 and 19) or 20 min. (lanes 4, 9, 15 and 20). The heat-shocked larvae were then allowed to recover at 22 °C for 15 min (lanes 5, 10, 16 and 21), 30 min (lanes 6, 11, 17 and 22) or 45 min (lanes 7, 12, 18 and 23). Salivary glands were dissected from the larvae and subjected to permanganate treatments. Lanes 1, 2 and 13: naked DNA either untreated or treated with KMnO4 for 1 min. (B) Permanganate genomic footprinting analysis of hsp70 in glands from control and NELF-E depleted larvae subject to heat shock and recovery. Displayed are representative results from the analysis of control glands (+/SG-Gal4: lanes 3-5) and NELF-E depleted glands (NELF-Ei17A10/SG-Gal4): lanes 6-8). Larvae were heat shocked at 37 °C for either zero (NHS) (lanes 3 and 6) or 20 min. (lanes 4 and 7). The heat-shocked larvae were then allowed to recover at 22 °C for 45 min (lanes 5 and 8). Salivary glands were dissected from the larvae and subjected to permanganate treatments. Lanes 1 and 2: naked DNA either untreated or treated with KMnO4 for 1 min. (C) Permanganate genomic footprinting analysis following 120 min of recovery from heat shock. Lanes 3-5 are from control glands (+/SG-Gal4). Lanes 6-8 are from NELF-D depleted glands (NELF-Dr2-1/SG-Gal4). Lanes 9-11 are from NELF-D depleted glands (NELF-Dr8-2/SG-Gal4). Larvae were heat shocked at 37 °C for either zero (NHS) (lanes 3, 6 and 9) or 20 (lanes 4, 7 and 10) min. The heat-shocked larvae were then recovered for 120 min (lanes 5, 8 and 11). Lanes 1 and 2: naked DNA either untreated or treated with KMnO4 for 1 min.
To corroborate the permanganate footprinting results, I used ChIP to monitor the association of Pol II with *hsp70* in salivary glands before heat shock, during heat shock, and during recovery from heat shock. Similar levels of Pol II were detected on the promoter region of *hsp70* in non-heat shocked control and NELF-D-depleted salivary glands (Fig. 4-4A) in accordance with what was previously observed in *Drosophila* tissue culture cells (Gilchrist et al. 2008). The similar levels of Pol II detected on the *hsp70* promoter by ChIP in control and NELF-D depleted glands seems inconsistent with the greater level of permanganate reactivity detected on *hsp70* in control and NELF-D depleted glands, but similar results were obtained with *Drosophila* tissue culture cells (Gilchrist et al. 2008). One explanation for this is that NELF causes Pol II to pause in a narrow region of the promoter (Lee et al. 2008). Loss of NELF broadens the distribution of Pol II without increasing the overall amount of Pol II such that the local density of Pol II in one place is insufficient to generate a permanganate footprint.

Heat shock induction increased the level of Pol II detected in the promoter proximal region of *hsp70* in both the control and NELF-depleted glands (Fig. 4-4A and 4-4B, 20’ HS). No significant differences were observed for the Pol II levels at the *hsp70* promoter region of NELF-depleted or control salivary glands during heat shock or throughout the recovery from heat shock (Fig. 4-4A).

I also monitored Pol II at +600 relative to the transcription start to determine if the depletion of NELF influenced Pol II that was actively transcribing the gene. Prior to heat shock induction, significantly less Pol II was detected at +600 than the promoter for both control and NELF-depleted glands (Fig. 4-4A and B, NHS). Heat shock caused a marked increase in the level of Pol II detected at +600, consistent with the gene being transcribed
During recovery from heat shock, the level of Pol II detected at +600 was similar in control and NELF-depleted glands at 15 and 30 min, but 2-fold less in control glands than in NELF depleted glands after 45 min (Fig. 4-4B). This coincides with the permanganate reactivity detected on \textit{hsp70} after 45 min of recovery by NELF-depleted but not control salivary glands (Fig. 4-3A). Thus both assays indicate that the absence of NELF delays the time needed for \textit{hsp70} to shut off.
Figure 4-4. Chromatin immunoprecipitation analysis of Rpb3 in salivary glands. Larvae of control (+/SG-Gal4) (checkered bars) and NELF-D RNAi (NELF-Dt<sup>8-2</sup>/SG-Gal4) (solid bars) fly lines were either not heat activated (NHS) or heat activated for 20 min at 37 °C and then recovered for 15, 30, 45 and 120 min at 22 °C. Salivary glands were then isolated and processed for ChIP analysis. Chromatin was precipitated with Rpb3 antibody. qRT-PCR was performed to amplify a region -79 to +25 in (A) and +552 to +662 in (B) from TSS region of hsp70. Results are indicative of two individual repeats. The error bars denote a range of variation in biological samples.
Failure to pause during recovery from heat shock results in hyper-expression of $hsp70$ following a brief heat shock.

If depletion of NELF impairs the rate at which $hsp70$ shuts off, then I should observe hyper-expression of $hsp70$ in response to a brief heat shock. To test this prediction, I heat shocked larvae for 5 min and then returned the larvae to 22 °C for 45 min. Two different NELF-D RNAi transgenic lines exhibited 3.5 to 4-fold more $hsp70$ mRNA than any of the control lines (Fig. 4-5). This contrasts with the comparable level of $hsp70$ mRNA that is produced during a 5 min heat shock (Fig. 4-2B). Thus, the decrease in NELF impairs the ability of cells to attenuate expression of $hsp70$ during recovery from the heat shock.
**Figure 4-5.** NELF depletion results in hyper-expression of *hsp70* following a brief heat shock. NELF-D depleted salivary glands (*NELF-Di$^{8-2}$/SG-Gal4 and *NELF-Di$^{2-1}$/SG-Gal4) or control glands (+/SG-Gal4, +/NELF-Di$^{8-2}$, and +/NELF-Di$^{2-1}$) were analyzed. Salivary glands were isolated from non-heat shocked larvae (NHS) or from larvae that were heat shocked for 5 min at 37 °C and recovered for 45 min at 22 °C (5' HS 45' Rec). Total RNA isolated from the salivary glands was then subjected to cDNA synthesis and qRT-PCR analysis for the region from +1916 to +2036 in *hsp70*. Results are from two separate experiments and the error bars represent the range of the measurements. The relative values were calculated by normalizing the data to the (+/SG-Gal4) NHS sample.
Depletion of NELF impairs dissociation of HSF during recovery from heat shock.

HSF is a sequence-specific, DNA-binding protein that rapidly associates with hsp70 and activates transcription upon heat shock (Boehm et al. 2003; Lis and Wu 1993). Resetting hsp70 to its pre-induced state presumably involves dissociation of HSF, although this has never been directly tested in Drosophila. I was curious to learn if the capacity of the cell to establish the paused state during recovery from heat shock could be influencing the association of HSF with hsp70.

ChIP was used to monitor the association of HSF with hsp70. ChIP analysis of HSF in control glands revealed a significant increase in the association of HSF with hsp70 upon heat shock induction and complete dissociation of HSF by 45 min of recovery (Fig. 4-6A). Like the control glands, HSF associated with the hsp70 gene in the NELF-D depleted glands upon heat shock, but in contrast to the control glands, the level of HSF associated with hsp70 in NELF-depleted glands remained high after 45 min of recovery (Fig. 4-6A, 20’ HS 45’ Rec). Similar results were observed with the other NELF-D RNAi transgenic line (Fig.4-6B). These results indicate that NELF somehow contributes to the dissociation of HSF from hsp70, thus providing a possible explanation for the delay in the rate at which hsp70 recovers from heat shock.
HSF ChIP on *hsp70*

**A**

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**B**

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Figure 4-6. Chromatin immunoprecipitation analysis of HSF in salivary glands. Effect of NELF depletion on the association of the transcription activator, HSF, with hsp70. Salivary glands of control larvae (+/SG-Gal4) and two NELF-D depleted larvae (NELF-Di8-2/SG-Gal4 and NELF-Di2-1/SG-Gal4) were analyzed. (A) The larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 °C, or heat shocked 20 min and then recovered for 15, 30, 45 and 120 min. (B) The larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 °C, or heat shocked 20 min and then recovered for 45 and 120 min. Salivary glands were then isolated and ChIP was performed with anti-HSF antibody. qPCR was performed for the region from -79 to +25 of hsp70. Results are derived from two independent experiments, and the error bars denote the range of values.
Disruption of promoter proximal pausing alters interaction of sequence specific regulators of hsp70.

Another sequence-specific, DNA-binding protein that contributes to expression of hsp70 is the GAF. GAF associates with hsp70 prior to heat shock and is involved in establishing the paused Pol II (Lee et al. 1992). I was curious to know if pausing of Pol II might also influence the association of this transcription factor.

Heat shock caused a 3-fold decrease in the level of GAF associated with hsp70 (Fig. 4-7A). Several of the HSF binding sites overlap with the binding sites for GAF so it is possible that HSF is displacing GAF from the DNA. In control glands during the recovery from heat shock, GAF reassociates with hsp70 in a fashion that coincides with the dissociation of HSF. The reassociation of GAF with hsp70 in the NELF-depleted glands is markedly less than in control glands (Fig. 4-7A 20’ HS 45’Rec) and coincides with the failure of HSF to dissociate from hsp70. Depletion of NELF-D in another transgenic line yielded comparable results (Fig. 4-7B). Thus, interactions of both GAF and HSF are altered by the capacity of Pol II to pause on the hsp70 promoter.
GAF ChIP on hsp70

A

[Bar chart showing percent input for different conditions and time points for +SG-Gal4 and NELF-Di^2/SG-Gal4]

B

[Bar chart showing percent input for different conditions and time points for +SG-Gal4 and NELF-Di^2/SG-Gal4]
Figure 4-7. Chromatin immunoprecipitation analysis of GAF in salivary glands. Effect of NELF depletion on the association of the GAF with hsp70. Salivary glands of control larvae (+/SG-Gal4) and two NELF-D depleted larvae (NELF-Di^8-2/SG-Gal4 and NELF- Di^8-1/SG-Gal4) were analyzed. (A) The larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 °C, or heat shocked 20 min and then recovered for 15, 30, 45 and 120 min. (B) The larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 °C, or heat shocked 20 min and then recovered for 45 and 120 min. Salivary glands were then isolated and ChIP was performed with anti-GAF antibody. qPCR was performed for the region from -79 to +25 of hsp70. Results are derived from two independent experiments, and the error bars denote the range of values.
Depletion of NELF slows the rates of shut-off of transcription and alters interactions of the transcription regulators, HSF and GAF, for *hsp26*.

I analyzed the impact of NELF depletion on the shut-off of *hsp26* to assess whether its role in shutting off *hsp70* was unique. As I saw for *hsp70*, depletion of NELF resulted in the continued presence of Pol II downstream from the pause site after 45 min of recovery from heat shock (Fig. 4-8A). Also, expression of *hsp26* was significantly more in NELF depleted larvae than in controls following recovery from a brief heat shock (Fig 4-8B).

Next I evaluated the occupancy of the two DNA binding factors GAGA and HSF that are reported to associate with multiple sites in *hsp26*. (Glaser et al. 1990; Lu et al. 1993; Sandaltzopoulos et al. 1995). The dissociation of HSF from the *hsp26* promoter region was significantly impaired during recovery in NELF-depleted glands (Fig. 4-8 C and D). Unexpectedly there was more HSF associated with *hsp26* in the *NELF-Dr^2-1/SG-Gal4* (Fig 4-8D) than *hsp70* (Fig 4-6B) after the 20 min heat shock. There are multiple HSEs in the *hsp26* gene. This may be a case where all or most of the HSEs were saturated with HSF in *NELF-Dr^2-1/SG-Gal4* larvae compared to the control. Thus NELF is involved in shutting off transcription and dissociating HSF for *hsp26* during the recovery from heat shock.

Levels of GAF association in *hsp26* gene, in the control and NELF-D RNAi larvae were comparable prior to heat shock. When these larvae were heat shocked, GAF dissociated from *hsp26* but re-associated to the GAGA binding regions following recovery in both, control and NELF-D depleted (*NELF-Dr^8-2/SG-Gal4*) larvae (Fig. 4-8 E). A similar observation was made using the second NELF-D depleted fly line *NELF-
Di²¹/SG-Gal4 before heat shock and following recovery from heat shock (Fig. 4-8 F). But the level of GAF associated in the NELF-Di²¹/SG-Gal4 larvae after 20 min heat shock seemed two-fold higher than in the control (Fig. 4-8 F, 20’ HS). This discrepancy may be due to the degree of accessibility of the GAF to its binding sites. This result is not in agreement with the observation in hsp70. This may be because hsp26 contains two regions of HSF and GAF binding sites at regions -135 to -85 and another further upstream at -320 and -370 (Glaser et al. 1990; Lu et al. 1993; Sandaltzopoulos et al. 1995). Another set of HSF binding region is at -256 to -280 bases distal to the promoter. GAF overlaps the HSF binding sites distal to the promoter (Sandaltzopoulos et al. 1995).

My ChIP resolution encompassed approximately 400 nucleotides and amplification of the DNA was performed in the region proximal to the promoter. Association of GAF in NELF-D depleted larvae may be delayed in the regions distal to the promoter where binding regions of GAF and HSF overlap, which needs further evaluation. This may explain the prolonged association of HSF in NELF depleted larvae.
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{Heat Shock (20 min)
Recovery (min)

+4
+9, +10
>+17, +25
+33
+43--+45
+61, +62
+82, +83
+90--->93
+96, +97
+103
+112, +113
+116, +117
+122, +123
+138
>140 to +142
+146, +148
+162
+167
+178
+184
+186
B  Transcript measurements

Relative levels of hsp26 transcripts

NHS  5' HS  45' Rec

Heat shock / recovery (min)

C  HSF ChIP on hsp26

Percent input

NHS  20' HS  20' HS  15' Rec  20' HS  30' Rec  20' HS  45' Rec  20' HS  120' Rec

Heat shock / recovery (min)

D  HSF ChIP on hsp26

Percent input

NHS  20' HS  20' HS  20' HS  45' Rec  20' HS  120' HS Rec

Heat shock / recovery (min)
E  
**GAF ChIP on hsp26**

![Graph E](image)

F  
**Heat shock / recovery (min)**

![Graph F](image)
Figure 4-8. The effects of NELF depletion on hsp26 shut-off. (A) Permanganate genomic footprinting analysis of hsp26 in salivary glands of control (+/SG-Gal4) and two different NELF-D depleted larvae (NELF-Di^8-2/SG-Gal4); Lanes 7-10 and (NELF-Di^2-1/SG-Gal4); Lanes 11-14. Larvae were heat shocked at 37 ºC for either zero (NHS) (lanes 3, 7 and 11) or 20 min (lanes 4, 8 and 12). The heat-shocked larvae were then allowed to recover at 22 ºC for 45 min (lanes 5, 9 and 13) and 120 min (lanes 6, 10 and 14). (B) Levels of hsp26 transcripts upon induction and recovery. NELF-D depleted salivary glands (NELF-Di^8-2/SG-Gal4 and NELF-Di^2-1/SG-Gal4) or control glands (+/SG-Gal4, +/NELF-Di^8-2, and +/NELF-Di^2-1) were analyzed. Salivary glands were isolated from non-heat shocked larvae (NHS), and larvae that were heat shocked at 37 ºC for 5 min (5’ HS) or heat shocked for 5 min at 37 ºC and recovered for 45 min at 22 ºC (5’ HS 45’ Rec). Total RNA was isolated, subjected to cDNA synthesis and then quantified by qRT-PCR by amplifying the region from +620 to +733 in hsp26. Results are for two individual repeats. The relative values are calculated by normalizing the data to the control NHS samples. (C) and (D) Effect of NELF depletion on HSF at hsp26. Salivary glands of control larvae (+/SG-Gal4) and two NELF-D depleted larvae (NELF-Di^8-2/SG-Gal4 and NELF-Di^2-1/SG-Gal4) were analyzed. (C) The larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 ºC, or heat shocked 20 min and then recovered for 15, 30, 45 and 120 min. (D) The larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 ºC, or heat shocked 20 min and then recovered for 45 and 120 min. Salivary glands were then isolated and ChIP was performed with anti-HSF antibody. qPCR was performed for the region from -77 to +24 of hsp70. Results are derived from two independent experiments, and the error bars denote the range of values. (E) and (F) Effect of NELF depletion on the association of the GAF with hsp26. Salivary glands of control larvae (+/SG-Gal4) and two NELF-D depleted larvae (NELF-Di^8-2/SG-Gal4 and NELF-Di^2-1/SG-Gal4) were analyzed. (E) The larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 ºC, or heat shocked 20 min and then recovered for 15, 30, 45 and 120 min. (F) The larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 ºC, or heat shocked 20 min and then recovered for 45 and 120 min. Salivary glands were then isolated and ChIP was performed with anti-GAF antibody. qPCR was
performed for the region from -77 to +24 of hsp70. Results are derived from two independent experiments, and the error bars denote the range of values.
Depletion of NELF has little impact on the binding activity of HSF in cell extracts.

HSF stimulates transcription of heat shock genes by binding to HSEs located upstream from the core promoter region of the heat shock genes (Lis and Wu 1993). Prior to heat shock, HSF exists as a monomer that does not bind DNA. During heat shock, HSF forms trimers that tightly associate with HSEs (Orosz et al. 1996; Perisic et al. 1989). Resetting heat shock genes to their preinduced state involves the inactivation of HSF DNA binding activity and its dissociation from the heat shock genes (Voellmy and Boellmann 2007).

I tested if the differences in the binding of HSF to hsp70 in control and NELF-D depleted glands might be due to differences in HSF binding activity by monitoring levels of HSF DNA binding activity in extracts from salivary glands. Electrophoretic mobility shift assays (EMSA) were done to evaluate the level of HSF binding activity in extracts from control and NELF-depleted glands. I found it necessary to use a high affinity HSE polymer containing 6 binding sites for HSF (Fernandes et al. 1995) to detect HSF binding activity because the amount of extract obtained from salivary glands was limiting. A heat shock dependent gel-shift complex was detected, which disappeared when HSF antibody was added, indicating that this complex contained HSF (Fig. 4-9A, compare lane 2 to 7 and lane 5 to 8). HSF binding activity decreased by approximately 2-fold after the control larvae were allowed to recover for 45 min from heat shock (Fig. 4-9A, compare lanes 2 and 3). The continued presence of HSF binding activity in the extracts after 45’ of recovery is in agreement with what has been observed for adherent Drosophila tissue culture cells (Fritsch and Wu 1999). This modest decrease in binding activity is in stark contrast to the 19-fold decrease in HSF detected on hsp70 in the control salivary glands.
after the 45 min recovery from heat shock (Fig. 4-6), and suggests that HSF’s dissociation from hsp70 involves more than dissociation driven by the loss of HSF binding activity in the nucleoplasm and mass action.

Depletion of NELF had a modest impact on the inactivation of HSF binding activity relative to control glands. In NELF-depleted glands, HSF binding activity was the same at 20 min of heat shock and after 45 min of recovery (Fig. 4-9B). Within experimental variation, HSF behaved essentially the same in control and NELF-depleted cells. Western blot analysis of HSF indicated that comparable levels of HSF were present in both cells (Fig. 4-9C). Moreover, the slight shift in mobility of HSF on SDS-PAGE that accompanied heat shock and persisted throughout the 45 min of recovery was the same. This slight shift in mobility could result from phosphorylation (Fritsch and Wu 1999). Overall, the striking difference in the dissociation of HSF from heat shock genes in control and NELF-depleted salivary glands does not appear to be due to significant differences in the binding activity of HSF. Rather, I propose that the paused Pol II generated through the action of NELF is actively involved in dissociating HSF from the heat shock genes.
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B

HSF binding activity

Ratio relative to control 20' HS

Heat Shock / Recovery

C

Heat shock (20 min) / Recovery (min)

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HSF

Rpb3
Figure 4-9. Binding activity of HSF in extracts from salivary glands. Depletion of NELF does not significantly affect HSF binding activity. (A) Extracts were made from salivary glands isolated from control (+/SG-Gal4) and NELF-D depleted (NELF-Di8-2/SG-Gal4) larvae. Larvae were heat shocked at 37 °C for either zero (lanes 1 and 4) or 20 min (lanes 2 and 5). The heat-shocked larvae were then recovered at 22 °C for 45 min (lanes 3 and 6). Extracts from glands were incubated with a radiolabelled, double-stranded oligonucleotide containing 6 heat shock elements (HSE6). HSF antibody was added to samples in lanes 7 and 8 to identify the HSF containing complex. The upper panel shows the entire gel with free DNA and a spurious band of unknown identity marked by an asterisk. The lower panel is a cutout of the HSF-containing region digitally darkened with Adobe Photoshop. (B) The HSF shifted complex was quantified relative to the band intensity of the control (+/SG-Gal4) after 20 min heat shock treatment. The error bars represent standard deviation of three independent experiments. (C) Depletion of NELF has no effect on the expression of HSF. Twenty micrograms of protein from salivary gland extracts were analyzed by western blotting using antibodies against HSF and the Rpb3 subunit of Pol II. Lanes 1 to 6 correspond to lanes 1 to 6 in panel A.
RNAi-mediated depletion of CBP interferes with the dissociation of HSF from the heat shock genes during recovery from heat shock.

A recent study concluded that the association of HSF1 with heat shock genes in human cells is regulated by acetylation of lysine 80 in HSF (Westerheide et al. 2009), a residue that is conserved in Drosophila HSF. Acetylation of this residue causes HSF to dissociate from hsp70. Overexpression of the acetyl transferases CBP or p300 in human cells resulted in increased acetylation of HSF, thus identifying these proteins as candidates for causing HSF to dissociate from the heat shock genes during recovery from heat shock. Previous work showed that CBP associates with the Drosophila hsp70 promoter region during heat shock (Smith et al. 2004).

Drosophila has only one protein homologous to the closely related CBP and p300. To test if CBP affected the dissociation of HSF from the heat shock gene promoters, I examined the effects of depleting CBP from salivary glands. Immunofluorescence staining of polytene chromosomes showed that RNAi against CBP caused loss of CBP from the chromosomes (Fig. 4-10A, compare panels b and e) without affecting Pol II (Fig. 4-10A, compare panels a and d). ChIP analysis revealed that significantly more HSF remained associated with hsp70 and hsp26 following the 45 min recovery in the CBP depleted glands than in the control glands (Fig. 4-10). I also tested if depletion of a subunit of the Swi/Snf chromatin remodeling complex, brm, affected the dissociation of HSF, since a previous study indicated that Swi/Snf was involved in dissociating the glucocorticoid receptor from the MMTV gene (Fletcher et al. 2002). No effect was detected (Fig. 4-10 B and C). For my experimental purposes, brmi serves as a
negative control as the genotypes of the $CBP\text{I}$ and $brmi$ are the same except for the RNAi transgenes that have been inserted into the identical locus.
B

HSF ChIP on \textit{hsp70}

C

HSF ChIP on \textit{hsp26}
**Figure 4-10.** Chromatin immunoprecipitation analysis of HSF at heat shock gene promoters in salivary glands depleted of CBP.  

(A) Immunodetection of CBP on polytene chromosomes from +/SG-Gal4 (control) and CBP-depleted (CBPl<sup>2806</sup>/ SG-Gal4) larvae. The chromosome spreads were stained with CBP antibody and the Pol II antibody, 8WG16. The primary antibodies were visualized with Alexa 568-conjugated anti-mouse and Alexa 488-conjugated anti-rabbit antibodies respectively. Panels a & d were stained with 8WG16 antibody in control and CBP-depleted larvae. Panels b & e, were stained with anti-CBP antibody in control and CBP-depleted larvae. Panels c & f show chromosomes stained with the DNA stain, Hoechst.  

(B and C) Depletion of CBP from glands interferes with dissociation of HSF from heat shock genes. Control (+/SG-Gal4), CBP-depleted (CBPl<sup>2806</sup>/ SG-Gal4), and brm-depleted (brm<sup>4019</sup>/ SG-Gal4) larvae were either not heat shocked (NHS), heat shocked for 20 min at 37 °C, or heat shocked 20 min and then recovered for 45 min. Salivary glands were then isolated and processed for ChIP analysis. Chromatin immunoprecipitation was performed with anti-HSF antibody. qPCR was performed for the region from -72 to +29 of *hsp70* and -77 to +24 of *hsp26*. Results are derived from two independent experiments, and the error bars denote the range of values.
Depletion of CBP impairs the shut-off of *hsp70* and *hsp26* during recovery from heat shock.

Similar to what I observed for the depletion of NELF, my ChIP analyses revealed that dissociation of HSF from *hsp70* and *hsp26* was delayed by depletion of CBP protein (Fig. 4-10). Therefore, I tested if this also caused transcription to persist longer than normal during recovery from heat shock. Using permanganate footprinting, I found that Pol II returned to its paused state on *hsp70* in control and brm-depleted salivary glands after 45 min of recovery (Fig. 4-11A compare lanes 3 to 5 and 9 to 11). In contrast transcriptionally engaged Pol II was still readily detected outside the region of the pause on *hsp70* in CBP-depleted glands (Fig. 4-11 A, compare lanes 5, 8, and 11). Depletion of CBP also delayed the recovery of *hsp26* as evident by comparing the permanganate reactivity in lane 8 to lanes 5 and 11 in Figure 4-11B. These results corroborate that depletion of CBP delays shutting off of *hsp70* and *hsp26* due to the slower dissociation of HSF.
A  Hsp70

B  Hsp26

<table>
<thead>
<tr>
<th>Naked DNA</th>
<th>Control</th>
<th>CBP depleted</th>
<th>brm depleted</th>
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</tr>
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<tr>
<td>0'</td>
<td>0</td>
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</table>

Heat Shock 20 min

Recovery (min)

+7, +8
+22
+30
+34
+45
+53
+67
+76
+83, +85
+90
+95
+101, +102
+107
+111
+115, +116
+122
+137
+141
+146
+152
+155
+160
+162
+172
+175, 176
+178, 179
+183

+9, +10
+17, +25
+33
+43 to +45
+61, +62
+82, +83
+90 to +93
+96, +97
+103
+112, +113
+116, +117
+122, +123
+138
+140 to +142
+146, +148
+162
+167
+172
+178
+184
+186
+190
+192
+194
+197
+199
+202 to +204
Figure 4-11. Depletion of CBP delays heat shock gene shut off. Permanganate genomic footprinting analysis of (A) hsp70 and (B) hsp26 in glands from control, CBP, and brm depleted larvae subject to heat shock and recovery. Displayed are representative results from the analysis of control glands (+/SG-Gal4: lanes 3-5), CBP-depleted (CBPt2806/ SG-Gal4: lanes 6-8), and brm-depleted (brmi4019/ SG-Gal4: lanes 9-11). Larvae were heat shocked at 37 °C for either zero (NHS) (lanes 3, 6 and 9) or 20 min. (lanes 4, 7 and 10). The heat-shocked larvae were then allowed to recover at 22 °C for 45 min (lanes 5, 8 and 11). Salivary glands were dissected from the larvae and subjected to permanganate treatments. Lanes 1 and 2: naked DNA either untreated or treated with KMnO₄ for 1 min.
Discussion.

**Disruption of promoter proximal pausing on hsp70 does not alter the rate of heat shock induction.**

Correlations between the presence of paused Pol II and rapid induction of gene expression have led to the hypothesis that promoter proximal pausing provides the basis for rapid induction (Adelman et al. 2009; Boettiger and Levine 2009; Kininis et al. 2009). However, none of these studies have actually tested if disruption of promoter proximal pausing delays expression of genes. My analysis reveals that disruption of promoter proximal pausing at hsp70 does not significantly alter the rate of heat shock induction. This was evident by monitoring the association of Pol II with hsp70 using permanganate genomic footprinting and also the synthesis of hsp70 RNA (Fig. 4-2).

It has been proposed that the establishment of paused Pol II at a promoter allows for rapid induction because steps that precede initiation such as chromatin remodeling and Pol II recruitment are bypassed (Boettiger and Levine 2009). In the case of the heat shock genes, these steps are still likely to occur prior to heat shock induction even when promoter proximal pausing is impaired. TFIID, which provides the foundation for assembling a preinitiation complex, makes multiple sequence-specific contacts with hsp70 including the TATA box, the initiator, and downstream sequences (Emanuel and Gilmour 1993; Purnell et al. 1994; Purnell and Gilmour 1993; Wu et al. 2001). In addition, GAF maintains the promoter region in an accessible state even when TFIID binding is impaired (Lu et al. 1994; Weber et al. 1997). Since HSF associates with a transgenic hsp70 promoter deleted of its TATA box, the accessible state of the promoter in lieu of TFIID appears to be sufficient for HSF binding (Smith et al. 2004). Thus access
of DNA by the general transcriptional machinery and HSF may not be limiting for *hsp70* even when promoter proximal pausing is disrupted.

The extent to which promoters retain an accessible state in the absence of paused Pol II is likely to vary among promoters. The heat shock genes could represent one end of a spectrum where accessibility is retained in the absence of paused Pol II because of the binding of GAF and TFIID. At the other end of the spectrum may be genes that require the paused Pol II to maintain a potentially inducible state. Gilchrist et al. (Gilchrist et al. 2010; Gilchrist et al. 2008) have identified cases where the paused Pol II appears to contribute to induction by preventing a nucleosome from assembling over the promoter.

**NELF functions in fine-tuning gene expression.**

I discovered that disruption of pausing impacts the ability of *hsp70* to shut-off during recovery from heat shock. Permanganate genomic footprinting provides a high resolution view of the behavior of Pol II. Permanganate reactivity on *hsp70* at positions +7 and +8 are indicative of newly initiated Pol II whereas reactivity downstream from +34 corresponds to Pol II that has read through the pause (Fig. 4-2A). Pol II ChIP at the promoter region is consistent with the permanganate footprinting showing pol II at the promoter (Fig. 4-4). Similarly for *hsp26*, permanganate reactivity at +9 and +10 are indicative of newly initiated Pol II whereas reactivity downstream from +45 corresponds to Pol II that has read through the pause (Fig. 4-8). The patterns of permanganate reactivity I observe indicate that both initiation and read-through persist for a longer time in NELF-depleted glands than in control glands during recovery from heat shock for both *hsp70* and *hsp26*. This defect could account for the over expression of these two heat
shock genes in response to a brief heat shock. Limiting the production of *hsp70* protein to a level appropriate to the degree of stress is important for proper cell development, since ectopic expression of *hsp70* in salivary glands under non-heat shock conditions inhibited growth of the cells in this tissue (Feder et al. 1992).

My results now expand the types of scenarios in which NELF and promoter proximal pausing serve to attenuate gene expression. In the case of the estrogen-mediated activation of the pS2 gene (Aiyar et al. 2004), direct interaction between the NELF-B subunit and the estrogen receptor causes estrogen-mediated association of NELF with the target gene. Depletion of NELF results in hyper-activation of the pS2 gene, indicating that the estrogen receptor is mediating both activation and repression to achieve a particular level of expression in the presence of stimuli. In the case of *junB* (Aida et al. 2006), depletion of NELF increased the expression of *junB* both before and after serum-mediated activation. My results now reveal a role for NELF in shutting off gene expression upon withdrawal of the stimulus – this case being heat shock.

**Paused Pol II promotes dissociation of HSF from the heat shock genes during recovery from heat shock.**

Pol II paused in the promoter proximal region of a gene physically blocks initiation by a second Pol II. Thus one explanation for the delay in inactivating the heat shock promoters during recovery from heat shock when NELF was depleted could have been the inability to establish a paused Pol II to block reinitiation. However, an analysis of the activator of heat shock genes, HSF, revealed an alternative explanation: depletion of NELF somehow impaired the dissociation of HSF from the heat shock genes (Fig. 4-6
and 4-8C and D). This does not appear to be due to a defect in HSF caused by the absence of NELF because there was no difference in the ability of HSF to induce heat shock genes during heat shock (Fig. 4-2) nor was there any substantial differences in the amount of HSF or HSF DNA-binding activity in control and NELF depleted glands (Fig. 4-9).

In hsp70, paused Pol II might stimulate dissociation of HSF by influencing the association of GAF with its promoter. My ChIP analysis of control glands revealed that GAF dissociates from hsp70 during heat shock and returns during recovery from heat shock. The reassociation of GAF was impaired upon depletion of NELF. Several of the binding sites for GAF in the hsp70 promoter overlap binding sites for HSF, so the reassociation of GAF might displace HSF. The ability of Pol II to influence binding of GAF could be through direct or indirect interactions between the two proteins. But GAF factor behaves differently in hsp26. An explanation for the differences between hsp26 and hsp70 could be that in the promoter proximal region of hsp26 there are independent binding regions for GAF that do not overlap with HSEs (Lu et al. 1993). Hence ChIP analysis detected no change in the levels of GAF association.

I was prompted to explore the role of CBP on the dissociation of HSF from heat shock genes during recovery from heat shock because acetylation of lysine 80 in human HSF1 had been implicated in causing HSF1 to dissociate from hsp70 in human cells (Westerheide et al. 2009). p300 and CBP were implicated in acetylating HSF because ectopic expression of p300 or CBP in human cells increased the level of acetylated HSF in the cell. However, no measurements were made to determine if ectopic expression of CBP had any effect on heat shock gene expression or the association of HSF with the
heat shock genes. My results show that the association of HSF with heat shock genes persisted longer in salivary glands depleted of CBP than in control glands (Fig 4-10), and this correlates with delays in shutting off *hsp70* and *hsp26* (Fig. 4-11).

The available data indicates that the delay in the dissociation of HSF from the heat shock genes can be linked to acetylation of HSF. How might NELF impact HSF acetylation? I propose that NELF-mediated pausing of Pol II could increase the time provided for Pol II to position CBP to acetylate HSF thus triggering the dissociation of HSF (Fig. 4-12). Several observations provide additional support for this model. CBP and p300 associate with Pol IIa and not Pol IIo (von Mikecz et al. 2000) and the Pol II paused in the promoter proximal region is in the IIa state (O'Brien et al. 1994). Also, CBP associates with the heat shock genes in *Drosophila* during heat shock induction (Smith et al. 2004).
Many stimulus-responsive and developmental genes are found to have paused Pol II (Muse et al. 2007), and attenuation of transcription of these genes is likely to be critical for establishing appropriate physiological or developmental programs of gene expression. My finding that paused Pol II influences dissociation of an activator from the promoter provides a mechanism for rapidly shutting off gene expression. The pausing of Pol II could function more broadly by allowing the Pol II to remain stationary at a promoter while it serves to recruit modulators of chromatin structure and gene expression.

Portions of this chapter are part of manuscript that has been submitted to Molecular and Cellular Biology (Ghosh et al. 2011).
Appendix A

List of LM-PCR primers.

The list of primers denoted below was used in the LM-PCR analysis indicated in the order LM1, LM2 and LM3. The calculated Tms were provided by IDT.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Calculated Tm</th>
<th>Annealing Temp.</th>
</tr>
</thead>
<tbody>
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<td>LM1 CG2210(awd)+171</td>
<td>5’-GACCATGATGAAAGTCTCT</td>
<td>51.7</td>
<td>51.0</td>
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<tr>
<td>awd</td>
<td>LM2 CG2210(awd)+171</td>
<td>5’-TGAAAGTCTCTCTTGTTAGC</td>
<td>54.3</td>
<td>55.0</td>
</tr>
<tr>
<td>awd</td>
<td>LM3 CG2210(awd)+171</td>
<td>5’-TCTCTTTGTTAGCCGCCCATT</td>
<td>56.6</td>
<td>58.0</td>
</tr>
<tr>
<td>β1-tubulin</td>
<td>Tub-250-LM1</td>
<td>5’-GCGAAGTCTGCAAAAATG</td>
<td>60.4</td>
<td>54.0</td>
</tr>
<tr>
<td>β1-tubulin</td>
<td>Tub-250-LM2</td>
<td>5’-GTCGTTGCAAAAATGACGCA</td>
<td>65.3</td>
<td>58.0</td>
</tr>
<tr>
<td>β1-tubulin</td>
<td>Tub-250-LM3</td>
<td>5’-GCAAAAAATGACGCCATTTTGCA</td>
<td>69.7</td>
<td>62.0</td>
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<td>LM3 CG15916 +246</td>
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<td>58.0</td>
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<td>58.0</td>
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<td>55.0</td>
<td>51.0</td>
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Appendix B

List of Quantitative Real-time PCR and RNAi primers.

The list of primers denoted below was used in qRT-PCR analysis. Primers with RealT in the nomenclature were used for qRT-PCR while the other primers were used for Reverse transcription PCR and analyzed on AGE. Primers with (*) were used for generating dsRNA for RNAi in *Drosophila* tissue culture cells.

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Appendix C

Evaluate the dynamics of paused Pol II association with DNA.

Introduction.

This chapter describes my attempt to investigate the stability of paused Pol II. I tested this by expressing a mixture of α-amanitin sensitive (α-amanitin<sup>s</sup>) and resistant (α-amanitin<sup>r</sup>) Pol II molecules and determining if α-amanitin<sup>s</sup> Pol II molecules would exchange with the α-amanitin<sup>r</sup> Pol II molecules at promoters with paused Pol II. This failed but in the course of doing these experiments, I discovered that the location where the resistant and sensitive Pol II paused differed for certain promoters. Further analyses lead to the idea that the kinetics of elongation affects where Pol II pauses.

Exploring the dynamics of the paused Pol II.

Transcription by Pol II is essential for gene expression and hence is the basis of all cellular activities. Regarding the association of Pol II with DNA a fundamental question needs to be addressed: Is the interaction of Pol II with DNA stable or dynamic? If this association were dynamic, I would expect the bound molecule of Pol II to be associated for a short period after which a new Pol II would replace this. The half-life of the paused Pol II on hsp70 in uninduced cells is 10 min after which it escapes from the pause site (Lis 1998).
An α-amanitin\textsuperscript{r} \textit{Drosophila} fly line known as C4 harbors a mutation in the largest subunit of Pol II (Greenleaf et al. 1979). This mutant exhibited weaker binding of α-amanitin with a $K_d$ of $10^{-5}$ to $10^{-4}$ M as compared 3.8 X $10^{-7}$ M for the wild type Pol II (Coulter and Greenleaf 1982). C4 mutation is a change of amino acid Arg 741 to His in the Rpb1 subunit of Pol II that renders this Pol II resistant to α-amanitin. \textit{In vitro} the C4 mutant Pol II displayed fifty percent slower elongation rate than the wild type Pol II (Chen et al. 1993).

A heterozygote of α-amanitin\textsuperscript{r} (C4 mutant) and α-amanitin\textsuperscript{s} fly line should express a pool of mixed Pol II. If the association of Pol II is dynamic, I envision that in the presence of α-amanitin, if an α-amanitin\textsuperscript{r} Pol II encountered a pause it would dissociate and be replaced with a new Pol II. If this new Pol II were an α-amanitin\textsuperscript{s} one, it would be trapped by the alpha-amanitin at the TSS resulting in a shift in the position of the transcription bubble from the +30 region to the +1 region. This shift should be visible by permanganate genomic footprinting, which detects the paused Pol II by preferentially oxidizing thymines in single stranded regions. In the case where the interaction would be stable, either both types of Pol II that initiates transcription will pause and remain stably associated with the DNA in the +30 region (Fig. C-1).
Figure C-1. Schematic representation of Pol II association on hsp70 gene. (A) In the dynamic situation in the presence of amanitin when $\alpha$-amanitin resistant Pol II associates with hsp70 first, it will achieve productive elongation. But if the next initiating Pol II is $\alpha$-amanitin sensitive; it will pause upstream (+1) from the regular pause site mapped at +30. In the event that an $\alpha$-amanitin sensitive Pol II initiates elongation first then this will pause at +30. Therefore, $\alpha$-amanitin will cause the permanganate pattern to shift upstream from the pause site to the TSS. (B) If Pol II stably associates with hsp70 then both the $\alpha$-amanitin resistant and $\alpha$-amanitin sensitive Pol II will be located at the +30 pause site and remain there even after addition of $\alpha$-amanitin.
Upon executing this experiment, I was unable to detect a definitive shift in the permanganate reactivity from the +30 to the +1 region for numerous genes including ones that were transcriptionally active. In the ensuing time since doing these experiments, we have discovered that the transcription by Pol II in the promoter proximal regions is surprisingly resistant to α-amanitin. Even α-amanitin concentration of 300 ug/ml, which is at least 100 times the amount needed to inhibit Pol II in vitro, do not provide full inhibition of transcription.

Although the unexpected level of resistance of Pol II to α-amanitin in vivo compromised my original plan, an unexpected observation was that the α-amanitin Pol II seemed to pause upstream from where the wild-type Pol II paused on numerous promoters. This observation contributes a significant piece to understanding the process of promoter proximal pausing.

Results.

Effect of α-amanitin on Pol II.

A fly line containing a mutation in the largest subunit of Pol II in Drosophila was obtained from the Bloomington Drosophila Stock Center (stock number 3663 v RplI2154). This mutation (C4 mutant) changes amino acid Arg 741 to His in Rpb1, which in yeast corresponds to a change of amino acid Arg 726 to His. In vitro assays demonstrated that the C4 mutant Pol II was 50% slower in elongation rates and less efficient in reading through the intrinsic elongation blocks (Chen et al. 1993).

I planned to analyze the dynamics of Pol II in salivary glands because of the successful application of permanganate footprinting to this tissue. However, a priori, I
was unaware if $\alpha$-amanitin will enter the glands and inhibit Pol II and whether I would be able to detect the resistance of the C4 Pol II in vivo. The salivary glands from control and homozygous $\alpha$-amanitin$^r$ third instar larvae were treated with 10$\mu$g/ml $\alpha$-amanitin for 15 min followed by KMnO$_4$ footprinting to analyze the behavior of Pol II on the *hsp70* gene. In non-heat shocked glands, Pol II showed no change in its behavior to $\alpha$-amanitin treatment (Fig. C-2A compare lanes 3 and 5). In the control glands, $\alpha$-amanitin inhibited the increase in permanganate reactivity upon heat shock (Fig. C-2A compare lanes 4 and 6). Low levels of Pol II were seen on the *hsp70* gene indicating a low level of transcription. This was in accordance that transcription in the presence of $\alpha$-amanitin occurs with 1 percent the efficiency of the normal transcriptional rate (Rudd and Luse 1996). In contrast, $\alpha$-amanitin had no effect on Pol II in the $\alpha$-amanitin$^r$ salivary glands (Fig. C-2B compare lanes 3 and 6). In the presence of the $\alpha$-amanitin, elongation in the control tissues is dramatically reduced but there is no effect in the mutant tissues indicating that the C4 mutant fly line was resistance to $\alpha$-amanitin.
A

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<th>Heat Shock +37°C 15 min</th>
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B

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Annotations:
- +7, +8
- +22
- +30
- +34
- +45
- +53
- +67
- +76
- +83, +85
- +90
- +95
- +101, +102
- +107
- +111
- +115, +116
- +122
- +137
- +141
- +146
- +152
- +155
- +160
- +162
- +172
- +175, +176
- +178, +179
- +183
Figure C-2. C4 mutant Pol II is resistant to α-amanitin. Salivary glands of control (+/SG-Gal4) and homozygous (stock number 3663 v^1 RpII215^4) α-amanitin^r fly lines were isolated and either untreated or treated with α-amanitin at 10 μg/ml for 15 min. These glands were then either not heat shocked or heat shocked for 15 min at 37 °C. Permanganate genomic footprinting of hsp70 of the region downstream of TSS was analyzed. Naked DNA control in Lanes 1 and 2 in both panels were either untreated or treated with KMnO_4 for 1 min respectively. (A) Control, Lanes 3 and 5: non-heat shocked salivary glands were either untreated or treated with α-amanitin and Lanes 4 and 6: heat shocked salivary glands either untreated or treated with α-amanitin respectively as mapped by KMnO_4. (B) α-amanitin^r, Lanes 5 and 4: non-heat shocked salivary glands were either untreated or treated with α-amanitin and Lanes 6 and 3: heat shocked salivary glands either untreated or treated with α-amanitin respectively as mapped by KMnO_4.
Association of paused Pol II with the DNA.

To test if the paused Pol II is stably associated with the promoter, heterozygous larvae (α-amanitin\(^{+/s}\)) containing a mixture of α-amanitin\(^{r}\) and α-amanitin\(^{s}\) Pol II were produced by mating virgin D7, 1824 flies with male α-amanitin\(^{r}\) flies. The female progeny were selected because Rpb1 is on the X-chromosome (Greenleaf et al. 1979). As mentioned earlier, Pol II pauses 20 to 40 nucleotides downstream from the TSS in the hsp70 gene. I used the technique of permanganate footprinting using LMPCR to map the location of Pol II in the heterozygotes. If the association of Pol II is dynamic, hyper-reactive thymines should appear upstream of the pause site at +30 in non-heat shocked glands because an α-amanitin sensitive Pol II would block further initiation of a new molecule of Pol II resulting in a laddering effect (Fig. C-1).

Comparison of the permanganate footprints in non heat shocked heterozygotes before and after α-amanitin treatment (Fig. C-3A, lanes 5 and 9 and Fig. C-3B, lanes 5 and 6) showed no reproducible increase in the permanganate reactivity near the TSS suggesting that Pol II was statically bound to the promoter in the time frame of the α-amanitin treatment. The experiments in Fig. C-3A and C-3B were done under slightly different α-amanitin conditions to see if this might alter the outcome of the experiment but no changes were observed.

A comparison of the permanganate footprints in heat shocked heterozygotes before and after α-amanitin treatment was done to test if my approach could detect the dynamics of Pol II that were expected when hsp70 was actively transcribed. Unfortunately, the results were inconclusive. Comparison of lanes 6 and 10 in Fig. C-3A shows a loss of permanganate reactivity downstream from the +30 region and a
persistence of permanganate reactivity upstream. This is consistent with α-amanitin sensitive Pol II piling up at the transcription start. However, this conclusion is compromised by the finding that there was a loss of Pol II from the +30 region in larvae expressing only the α-amanitin sensitive Pol II (Fig. C-3A, compare lanes 7 and 8). Had α-amanitin worked as anticipated, the sensitive Pol II should have been frozen in place, thus preventing any change in permanganate reactivity upon heat shock. Raising the α-amanitin concentration from 10 ug/ml to 20 ug/ml failed to clarify the results because α-amanitin had no effect on the permanganate pattern in heat shocked, heterozygous larvae (Fig. C-3B, lanes 9 and 10).
Heat Shock
37°C 15 min

A

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+7, +8
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+7, +8
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+101, +102
+107
+111
+115, +116
+122
+137
+141
+146
+152
+155
+160
+162
+172
+175, 176
+178, 179
+183
**Figure C-3.** Heterozygous fly line shows resistance to α-amanitin. Salivary glands of control (+/SG-Gal4) and heterozygotes created by mating α-amanitin^r^ (stock number 3663 v^1^ Rpl1215^4^) and D7, 1824 fly lines were isolated and appropriate treatment with α-amanitin were performed. These glands were then either not heat shocked or heat shocked for 15 min at 37 °C where indicated. Permanganate genomic footprinting of hsp70 gene near +250 region downstream of TSS was analyzed. Naked DNA control in Lanes 1 and 2 in both panels were either untreated or treated with KMnO₄ for 1 min respectively. (A) Salivary glands were incubated in 10 µg/ml of α-amanitin for 15 min. , Lanes 3 and 7: Control non-heat shocked salivary glands were either untreated or treated with α-amanitin and Lanes 4 and 8: heat shocked salivary glands either untreated or treated with α-amanitin respectively. Heterozygous (α-amanitin^r/s^), Lanes 5 and 9: non-heat shocked salivary glands were either untreated or treated with α-amanitin and Lanes 6 and 10: heat shocked salivary glands either untreated or treated with α-amanitin, respectively. (B) Salivary glands were incubated in 20 µg/ml of α-amanitin for 10 min. Control, Lanes 3 and 4: non-heat shocked salivary glands were either untreated or treated with α-amanitin and Lanes 7 and 8: heat shocked salivary glands either untreated or treated with α-amanitin respectively. Heterozygous (α-amanitin^r/s^), Lanes 5 and 6: non-heat shocked salivary glands were either untreated or treated with α-amanitin and Lanes 9 and 10: heat shocked salivary glands either untreated or treated with α-amanitin respectively.
Selection of genes to analyze the association of Pol II.

Since the α-amanitin treatment failed to cause a change in the permanganate pattern on hsp70 in the non heat shocked, heterozygous glands, it appears that the paused Pol II is stably bound to the hsp70 promoter. I extended my analysis to other genes that contained a paused Pol II using salivary glands that were treated with 20 µg/ml of α-amanitin for 10 min. This treatment was sufficient to inhibit transcription (Fig. C-3B lanes 7 and 8), as well as minimize the exposure of the glands to ambient surroundings that may elicit stress responses. The genes were selected based on the genome wide analysis (Gilchrist et al. 2008) and permanganate footprinting analysis (Lee et al. 2008) using the UCSC browser (http://genome.ucsc.edu). The selected genes showed localized peaks of paused Pol II near the promoter proximal region. These four genes: CG14709, mfas, β1-tubulin and oaf, display paused Pol II at the promoter proximal region (Gilchrist et al. 2008) as also evidenced by permanganate footprints (Lee et al. 2008). Paused Pol II was observed in the promoter proximal regions of CG14709 between +11 and +21, mfas between +37 and +45, β1-tubulin between +20 and +40 and oaf between +27 and +37. To test if dynamic association of Pol II is an event of constitutively active genes, another set of genes with increased Pol II levels in the downstream region of the gene was also selected. Four genes RpS17, CG15916, awd and CG14782 were selected after analysis with the UCSC browser, which displayed high levels of Pol II in the promoter (Gilchrist et al.) and in the downstream regions. RpS17 and CG15916 also constituted a pause site.
Genomic mapping of genes by permanganate footprinting.

The selected genes were then analyzed to determine if they displayed the characteristics of Pol II dynamically cycling at the promoter proximal region by permanganate footprinting. CG14709 (Fig. C-4A) has paused Pol II concentrated in a narrow region. Paused Pol II is evident in untreated salivary glands from control and α-amanitin r/s larvae (Fig. C-4A compare lanes 3 and 5). In the heterozygotes, α-amanitin treatment would cause an increase in permanganate reactivity at the -4 site if the association of Pol II were dynamic (Fig. C-4A lane 6). This gene does not display the characteristics of a dynamic Pol II. The -4 band that contains a permanganate reactive thymine, did not show increased intensity after the α-amanitin r/s gland was treated with α-amanitin (Fig. C-4A compare lanes 4 and 6). mfas (Fig. C-4B), β1-tubulin (Fig. C-4C), oaf (Fig. C-4D) and RpS17 (Fig. C-4E) also did not show any change in the permanganate pattern upon α-amanitin treatment suggesting that Pol II was stably associated with these promoters. The results for CG15916 were equivocal due to the high background of bands in the naked DNA control (Fig. C-4F lane 2) as compared to the experimental lanes. Results for awd (Fig. C-4G) and CG14782 (Fig. C-4H) showed no detectable differences for Pol II levels between the α-amanitin treated control and α-amanitin r/s glands (compare lanes 5 and 7). These genes seem to have low levels of transcribing Pol II in regions that were mapped by permanganate footprinting.

These results did not provide evidence of a dynamic association of Pol II. Given that pausing is transient (O'Brien and Lis 1993), the conclusion suggesting that Pol II association is stable across genes with paused Pol II seemed implausible.
Figure C-4. Permanganate genomic footprinting of genes selected to explore dynamic association of Pol II. Salivary glands of control (+/SG-Gal4) and heterozygotes created by mating α-amanitin\textsuperscript{r} (stock number 3663 v\textsuperscript{1} Rpl215\textsuperscript{4}) and D7, 1824 fly lines were isolated. These glands were then either untreated or treated with 20 µg/ml of α-amanitin for 10 min. After KMnO4 treatment LM-PCR with primers near the promoter proximal regions of the selected genes were analyzed. Naked DNA control in Lanes 1 and 2 of panels A, B, C, D and Lanes 2 and 3 of panels E, F, G, H were either untreated or treated with KMnO\textsubscript{4} for 1 min respectively. Lane 1 of panels E, F, G, H are G/A markers. Patterns of permanganate genomic footprinting for genes with primers near the TSS are illustrated in lanes 2 and 3 for control and 4 and 5 for α-amanitin\textsuperscript{r/s} glands of genes in panels (A) CG14709, (B) MFAS, (C) beta 1tubulin, (D) oaf, and lanes 4 and 5 for control and 6 and 7 for α-amanitin\textsuperscript{r/s} glands of genes in panels (E) RpS17, (F) CG15916, (G) awd, (H) CG14782.
Discussion.

The mechanism of promoter proximal pausing by Pol II has still eluded us. The half-life of the paused Pol II on hsp70 in uninduced cells is 10 min after which it escapes from the pause site (Lis 1998). A new Pol II initiates transcription and pauses at the promoter proximal region. Hence the association of Pol II may be assumed to be dynamic. Salivary glands from the heterozygotes treated with α-amanitin for periods of either 10 or 15 min may be sufficient to capture Pol II in its dynamic state where an α-amanitin′ Pol II would be released from the pause while an α-amanitin″ Pol II would create a blockade to the next initiating Pol II. Albeit my results suggest that the association of paused Pol II with template may be a stable one. There are technical issues that need addressing for any definitive conclusions. These issues can be tested by increasing the concentration of α-amanitin or the varying the time of heat shock.
Appendix D

Elongation of Pol II is unaffected by the depletion of TFIIS in *Drosophila* tissue culture cells

Introduction.

During transcription, Pol II may misincorporate nucleotides. To correct for this mistake Pol II has a built in mechanism that enables it to cleave the 3’ end of the nascent transcript. To achieve this the Pol II molecule has to backtrack and rearrange the active center for the oncoming chemistry. Eukaryotic transcription elongation factor IIS (TFIIS) is required for communicating to Pol II and catalyze the cleavage reaction. In the absence of TFIIS Pol II becomes arrested in the backtracked conformation (Kettenberger et al. 2003).

In the *hsp70* gene, a molecule of Pol II may backtrack causing its arrest. TFIIS efficiently rescues Poll II to an elongating Pol II upon heat shock induction (Adelman et al. 2005). If this is true then I hypothesize that when the *hsp70* gene is induced in the absence of TFIIS, Pol II might be arrested in a promoter proximal region.

Results.

Depletion of TFIIS in various *Drosophila* tissue culture cells.

To test my hypothesis, I depleted TFIIS protein in *Drosophila* tissue culture cells. Depletion was performed in a spectrum of 4 different *Drosophila* cell lines: S2R+, S2DRSC, KC167 and ML-DmBG3-c2 (Fig. D-1 A-D). S2R+ cells were treated with
dsRNA against TFIIS for either 3 days (Fig. D-1A lanes 4-6) or 5 days (Fig. D-1A lanes 7-9). Untreated and LacZ treated cells served as controls, while DSIF depletion served as negative control. Depletion of TFIIS in the other three cell lines were performed for an intermediate of 4 days and TFIIS was significantly depleted in all these cell lines (Fig. D-1 B, C, and D).

To test if maximum depletion of TFIIS was obtained, the cells were incubated in either 3, 9 or 27 µg of dsRNA. Results indicated that the level of protein depletion was similar in both 3 µg or 27 µg of RNAi treatments (Fig. D-2). Immunoblot analysis confirmed that the RNAi against TFIIS decreased the level of TFIIS protein but had no effect on Spt5, the largest subunit of DSIF and Rpb3, the third largest subunit of Pol II (Fig. D-1A) and maximum depletion was obtained under experimental limitations.
**Figure D-1.** Depletion of TFIIS in various *Drosophila* tissue culture cells. Immunoblot analysis shows depletion of Spt5 and TFIIS by RNAi in *Drosophila* cells. LacZ depletion was performed as a control. The amount of protein applied to the SDS-PAGE was compared to protein from untreated cells at concentrations mentioned in lanes 1, 2 and 3. *(A)* S2R+ cells were treated with 10 µg of dsRNA for either 3 days; Lanes 4-6, or 5 days; Lanes 7-9. After lysis, an equivalent of 10 µg of protein was analyzed by SDS-PAGE. *(B)* S2DRSC cells were treated with 10 µg of dsRNA for 4 days. After lysis, an equivalent of 10 µg of protein was analyzed by SDS-PAGE. *(C)* KC167 cells were treated with 10 µg of dsRNA for 4 days. After lysis, an equivalent of 10 µg of protein was analyzed by SDS-PAGE. *(D)* ML-DmBG3-c2 cells were treated with 10 µg of dsRNA for 4 days. After lysis, an equivalent of 9 µg of protein was analyzed by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed with antibodies raised against TFIIS at 1:5000 dilution, Spt5 at 1:1500 dilution and Rpb3 at 1:2500 dilution. Rpb3 was used as loading control. The blot was visualized on typhoon.
Figure D-2. Depletion of TFIIS in *Drosophila* tissue culture cells by varying concentrations of dsRNA. Immunoblot analysis shows depletion of Spt5 and TFIIS by RNAi in *Drosophila* cells. LacZ depletion was performed as a control. The amount of protein applied to the SDS-PAGE was compared to protein from untreated cells at concentrations mentioned in Lanes 1, 2 and 3. *Drosophila* tissue culture cells (*A*) S2DRSC and (*B*) KC167, were treated with 3 µg (lanes 4, 7 and 10), 9 µg (lanes 5, 8 and 11) or 27 µg; Lanes 6, 9 and 12 of dsRNA for 4 days. After lysis, an equivalent of 9 µg of protein was analyzed by a 10 % SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and probed with antibodies raised against TFIIS at 1:5000 dilution, Spt5 at 1:1500 dilution and Rpb3 at 1:2500 dilution. Rpb3 was used as loading control. The blot was visualized on typhoon.
TFIIS depletion does not have a significant effect on Pol II arrest.

I further tested if depletion of TFIIS arrested Pol II in a region proximal or downstream of the TSS by depleting TFIIS in S2R+ *Drosophila* tissue culture cells. LacZ and DSIF were also depleted as controls. Paused Pol II on *hsp70* caused thymines at +22, +30 and +34 of the non-transcribed strand to be hyper-reactive to permanganate oxidation that is due to the transcription bubble being associated with transcriptionally engaged Pol II (Fig. D-3A, lanes 2, 5, 8 and 11). Upon heat shock induction, permanganate reactivity on *hsp70* was altered dramatically, which represents transcriptional activation. In the S2R+ untreated as well as the LacZ RNAi treated cells, permanganate reactivity was observed beyond the Pol II pause sites as evidenced by the +7, +8 and regions downstream from the TSS (Fig. D-3A lanes 3, 4, 6 and 7). To determine if depletion of TFIIS led to Pol II arrest, I compared the permanganate reactivity at 2.5 and 5 min of heat shock for the control and TFIIS depleted cells. No significant difference was observed in the level of reactivity between control and TFIIS depleted cells (Fig. D-3A, compare lanes 6-7 to 12-13). Similar comparison between control and TFIIS depleted cells of permanganate reactivity at regions +2000 nucleotides from the TSS also showed no significant differences (Fig. D-3B, compare lanes 4 and 8). This suggested that the depletion of TFIIS did not affect elongating Pol II in *hsp70*. Additionally, to test if TFIIS depletion affected the levels of *hsp70* RNA synthesized, *Drosophila* tissue culture cells S2R+, S2DRSC and ML-DmBG3-c2 were heat shocked for 2.5 and 5 min. *Hsp70* transcript levels in cells from LacZ control and TFIIS depleted cells showed no significant difference (Fig. D-4). Thus, my observations suggest that depletion of TFIIS does not affect elongating Pol II in *hsp70*. 

### A

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<th>Spt5 RNAi</th>
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### B

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Figure D-3. Depletion of TFIIS does not affect the position of Pol II pausing on hsp70. RNAi in Drosophila S2R+ cells was performed to deplete TFIIS and Spt5. Cells were treated with dsRNA for 4 days. LacZ depletion was used as a control. KMnO₄ treatment was performed after appropriate heat shock treatments. (A) Permanganate mapping was performed near the promoter region with primers listed in Appendix A. Lanes 2, 5, 8 and 11 are NHS, lanes 3, 6, 9 and 12 are cells that were heat shocked for 2.5 min and lanes 4, 7, 10 and 13 are cells that were heat shocked for 5 min. Naked DNA control in lane 1 was treated with KMnO₄ for 1 min. (B) Permanganate mapping was performed near the 3’ region with primers listed in Appendix A. Lanes 3, 5 and 7 are NHS, lanes 4, 6 and 8 are cells that were heat shocked for 2.5 min. Naked DNA controls in lanes 1 and 2 were either untreated or treated with KMnO₄ for 1 min respectively.
Transcript measurements

A  S2R+

B  S2DRSC

C  ML-DmBG3-c2

Relative levels of hsp70 transcripts

Heat shock (min)
Figure D-4. Effect of TFIIS depletion on hsp70 expression levels. TFIIS (green bars) and Spt5 (red bars) were depleted in (A) S2R+, (B) S2DRSC, and (C) ML-DmBG3-c2 cells. Lac Z depletion (blue bars) was used as a control. Cells were then either not heat shock treated (NHS) or heat shock treated for 2.5 or 5 min. Cells were heat shocked by adding media at 37 °C and floating the plates in a 37 °C water-bath. Total RNA isolated by Trizol was subjected to cDNA synthesis and qRT-PCR using primers at the 3’ region of the hsp70 gene tabulated in Appendix B. Each sample was normalized to its NHS condition.
Appendix E

Quantifications of Permanganate Footprinting.

**Figure E-1.** Quantification of *TepII* gene. Imagequant quantification of Fig. 3-2C, lanes 4 and 5. The ratio of sum of total counts of control and NELF-D depleted lanes was used to normalize the NELF-D lane. The graph was plotted using Kaleidagraph software.
Depletion of NELF does not significantly alter the induction of hsp70 at temperatures below 37 °C.

The heat shock genes are maximally induced at 37 °C within 60 sec of initiating a heat shock (Boehm et al. 2003; O'Brien and Lis 1993). To investigate whether the depletion of NELF altered the temperature at which hsp70 was induced, I monitored the behavior of Pol II in the 5’ region of the gene with permanganate footprinting. Salivary glands from control larvae and larvae that were depleted of NELF-D or NELF-E were isolated after heat shock induction for 20 min at 25, 29, 33 and 37 degrees C. This analysis revealed that the hsp70 gene was fully induced at 37 °C irrespective of the levels of NELF-D or NELF-E as evidenced by the permanganate reactivity at +7 and +8 (Fig. E-2A compares lanes 6, 10 and 14). In the NELF-D depleted larvae, a subtle increase in reactivity at the +7 and +8 region in the 33 °C induction was observed when compared to the control (Fig. E-2A compare lanes 5 and 9).

To verify if induction of hsp70 at 33 °C was significantly different in NELF-D – depleted and control glands, a closer investigation was performed by analyzing hsp70 at narrower increments of temperature change between 25 and 33 °C. This revealed that the hsp70 was induced at 31 and 33 degrees C when NELF-D was depleted as evidenced by the appearance of the +7, +8 bands (Fig. E-2B compare lanes 5-6 to 9-10 and 13-14). Also there was significant reactivity observed upon NELF-D depletion at regions downstream of +45 when the larvae were treated at 25 and 29 degrees C (Fig. E-2B, lanes 7 and 8). These results suggest this batch of NELF-D depleted larvae had inadvertently become stressed since there is significant reactivity in the downstream region of the 25 °C treated larvae.
If the NELF-D depleted larvae were indeed induced at 33 °C, I would expect the expression of *hsp70* to be higher than the control. To test for this, I performed RT-PCR and measured the levels of *hsp70* transcripts (Fig. E-2C). My observations revealed that there was no significant increase in the expression of *hsp70* in NELF depleted larvae when compared to the control. I performed one-way Analysis of Variance (ANOVA) of the transcripts from the control and NELF-D and –E depleted larvae induced at various temperatures using Kaleidagraph (Table E-1). The differences in levels of transcript observed after induction at 33 °C had a high p-value of 0.08 (Table E-1). The results indicate that the P-value of all the samples tested were high rendering the differences observed in levels of transcript by reverse transcription insignificant (Table E-1).

The subtle differences I observed in the permanganate footprinting along with intermittent stress that was sometimes observed in the non-heat shocked larvae prompted me to look for more robust impacts of depleting NELF on *hsp70*.
A

<table>
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<th>Naked DNA</th>
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B

Heat Shock 20 min (°C)

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Relative Levels of hsp70 transcripts

Heat shock (°C)

+/-SG-Gal4
NELF-Dih2/SG-Gal4
NELF-Ei17A10/SG-Gal4
Figure E-2. Analysis of hsp70 induction following heat shock at various temperatures below 37 °C. Permanganate genomic footprinting analysis of hsp70 in glands from +/-SG-Gal4 (Control), NELF-Dt^{8-2}/SG-Gal4 (NELF-D depleted) and NELF-Dt^{17A10}/SG-Gal4 (NELF-E depleted) larvae were subjected to heat shock for 20 min at; (A) 25, 29, 33, and 37 degrees C or (B) 25, 29, 31 and 33 degrees C. Displayed are representative results from the analysis of control glands (lanes 3-6), NELF-D depleted glands (lanes 7-10) and NELF-E depleted glands (lanes 11-14). Lanes 1 and 2; naked DNA either untreated or treated with KMnO₄ for 1 min. (C) Salivary glands were isolated from third instar larvae of the aforementioned matings that were heat shocked for 20 min at 25, 29, 33, and 37 degrees C. cDNA was synthesized from total RNA isolated by Trizol. Reverse transcription was performed in gene specific regions of hsp70 followed by qRT-PCR. RP49 RNA was used as an internal standard to correct for differences in the recovery of RNA.
**Table E-1.** One-way Analysis of Variance (Anova) results for levels of *hsp70* transcripts after induction at 25, 29, 33 and 37 degrees C.

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<td>Total</td>
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<tr>
<td>A</td>
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</table>

| **29 °C**  |
| One-way ANOVA |
| Factor A: 3 Groups |
| Control, NELF-D depleted, NELF-E depleted |
| Analysis of Variance Results |
| Source | DF | SS | MS | F | P |
| Total | 8 | 0.054682562 | 0.0068353203 | | |
| A | 2 | 0.013018881 | 0.0065094404 | 0.93742657 | 0.44231 |
| Error | 6 | 0.041663682 | 0.006943947 | | |

| **33 °C**  |
| One-way ANOVA |
| Factor A: 3 Groups |
| Control, NELF-D depleted, NELF-E depleted |
| Analysis of Variance Results |
| Source | DF | SS | MS | F | P |
| Total | 8 | 0.9295049 | 0.11618811 | | |
| A | 2 | 0.53099758 | 0.26549879 | 3.9973989 | 0.07881 |
| Error | 6 | 0.39850732 | 0.066417887 | | |

| **37 °C**  |
| One-way ANOVA |
| Factor A: 3 Groups |
| Control, NELF-D depleted, NELF-E depleted |
| Analysis of Variance Results |
| Source | DF | SS | MS | F | P |
| Total | 14 | 6.4623452 | 0.46159609 | | |
| A | 2 | 1.7261037 | 0.86305187 | 2.1866753 | 0.15498 |
| Error | 12 | 4.7362415 | 0.39468679 | | |
References.


Missra A, Gilmour DS. 2010. Interactions between DSIF (DRB sensitivity inducing factor), NELF (negative elongation factor), and the Drosophila RNA polymerase II transcription elongation complex. *Proc Natl Acad Sci USA*.


VITA

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1. Negative elongation factor, NELF, accelerates the rate at which the hsp70 heat shock gene is shut-off during recovery from heat shock by facilitating dissociation of the activator. Ghosh, S.K.B., Missra, A., & Gilmour, D.S. Manuscript submitted to MCB.