Regulation of the transcriptional activity of poised RNA polymerase II by the elongation factor ELL

Edwin R. Smith*, Benjamin Winter*, Joel C. Eissenberg†, and Ali Shilatifard*‡

*Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, MO 64110; and †Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, MO 63104

Communicated by Michael S. Levine, University of California, Berkeley, CA, May 6, 2008 (received for review February 5, 2008)

Many developmentally regulated genes contain a poised RNA polymerase II (Pol II) at their promoters under conditions where full-length transcripts are undetectable. It has been proposed that the transcriptional activity of such promoters is regulated at the elongation stage of Pol II transcription. In *Drosophila*, the heat-shock loci expressing the *Hsp70* genes have been used as a model for the regulation of the transcriptional activity of poised Pol II. *Drosophila* ELL (dELL) is a Pol II elongation factor capable of stimulating the rate of transcription both *in vivo* and *in vitro*. Although ELL and the elongation factor Elongin A have indistinguishable effects on RNA polymerase *in vitro*, the loss-of-function studies indicate that these proteins are not redundant *in vivo*. In this article, we use RNAi to investigate the physiological properties of dELL and a dELL-associated factor (dEaf) in a living organism. Both ELL and Eaf are essential for fly development. dELL is recruited to heat shock loci upon induction, and its presence with Pol II at such loci is required for proper heat-shock gene expression. Consistent with a role in elongation, dELL knockdown reduces the levels of phosphorylated Pol II at heat-shock loci. This study implicates dELL in the expression of loci regulated by Pol II elongation.

gene expression | heat-shock response | transcription elongation

Efficient transcription by RNA polymerase II (Pol II) is an intricate process that requires multiple contacts with the DNA template and nascent RNA that inevitably leads to frequent stalling during the transcription of a gene (1). The average rate of transcription by Pol II *in vivo* is an order of magnitude higher than that obtained *in vitro* despite additional impediments, such as traversing through nucleosomes. Using biochemical approaches, two Pol II elongation factors, Eleven nineteen lysine-rich leukemia (ELL) and Elongin A, were isolated from cell extracts as factors capable of stimulating Pol II activity by suppressing transient pausing. Despite similar *in vitro* activities, the *Drosophila* orthologs of ELL and Elongin A are each essential for development (2, 3). This observation indicates that their *in vivo* activity is not redundant.

Recent genome-wide studies have found a large number of developmentally regulated genes that contain a paused Pol II at their promoters (4, 5). Therefore, it has been proposed that the transcriptional activity of such poised Pol IIs is regulated at the level of transcription elongation. The classic model for studying genes regulated by promoter-proximal paused polymerase is *Hsp70* gene induction in *Drosophila* (6). Previous studies have shown that several Pol II elongation factors are rapidly recruited to the *Hsp70* genes after heat shock (7–13). Although much work has been done on the role of these factors in gene regulation in cultured cells, less is known about the role of these factors in the regulation of heat-shock gene expression in the whole organism. Although there are several mutants in the gene encoding *Drosophila* ELL (dELL), all of these alleles are embryonic lethal (2). Therefore, we have not been able to use these alleles to further characterize the role of the elongation factor ELL in the regulation of the transcriptional activity of poised Pol II and *Hsp70* loci. To test the role for dELL in gene expression, we have used RNAi to reduce expression levels of both dELL and dELL-associated factor (dEaf) expression levels during development, and we have examined the *in vivo* effect of their reduction on transcription and development. We find that knockdown of dELL and dEaf results in lethality. Furthermore, knockdown of these elongation factors results in reduced *Hsp70* transcript accumulation after heat shock. Immunolocalization of phosphorylated Pol II in heat-shocked dELL knockdown salivary glands demonstrates reduced levels of the elongating form of Pol II at the *Hsp70* loci in the absence of dELL. Our studies demonstrate that dELL is essential for full induction of heat-shock gene expression and are consistent with a role for dELL in Pol II elongation. These findings provide a role for an RNA Pol II elongation factor in the transcriptional regulation of poised Pol II.

Results and Discussion

dELL Knockdown by RNAi Leads to Loss of Viability. dELL was previously shown to be essential; homozygous mutant clones do not survive in the eye (14) and homozygotes for loss-of-function alleles die at the end of embryogenesis or in early first instar (2). To investigate the role of dELL in transcription in flies, we chose to knock down dELL by RNAi, which typically reduces, but does not eliminate, the targeted gene products. A 600-bp portion of the dELL coding region was inserted into a P-element vector that drives the expression of dsRNA through two convergent Gal4 UAS promoters that flank the insert (see *Materials and Methods*). Several transgenic lines were generated and tested for effects on viability by crossing to an *Actin5C-Gal4* driver line that expresses yeast Gal4 under the cytoplasmic actin promoter. All eight dELL RNAi lines show significant loss of viability when expressed under this driver (Table 1). When adult escapers were obtained, very few males were observed, indicating that males are more susceptible to loss of dELL. We also observed greater numbers of females than males at the third instar larval stage, indicating that males are dying earlier than females (data not shown). A significant genome-wide reduction of dELL protein is observed by immunofluorescence analysis of dELL RNAi larval polytene chromosomes (Fig. 1).

Through two-hybrid analysis, two interacting partners of ELL have been characterized in humans, Eaf1 and Eaf2 (15, 16). Eaf1 and Eaf2 are highly related and can stimulate the elongation activity of ELL *in vitro* (17). Recently, the association of Eaf with ELL was shown to be evolutionarily conserved, with the finding that *Schizosaccharomyces pombe* homologs SpEaf and SpELL directly interact with each other (18). Additionally, SpEaf enhances the stimulation by SpELL of Pol II transcription *in vitro* (18). Because *Drosophila* also has a single Eaf homolog, we used RNAi to knock down dEaf levels and assessed the viability of

Author contributions: E.R.S., J.C.E., and A.S. designed research; E.R.S., B.W., J.C.E., and A.S. performed research; A.S. contributed new reagents/analytic tools; E.R.S., B.W., J.C.E., and A.S. analyzed data; and E.R.S., J.C.E., and A.S. wrote the paper.

The authors declare no conflict of interest.

‡To whom correspondence should be addressed. E-mail: ASH@Stowers-Institute.org.

Table 1. Effect of dELL RNAi on viability

RNAi line	Act5c-Gal4			CyO, y^+		
	Female	Male	Total	Female	Male	Total
dELL 1	0	0	0	58	55	113
dELL 3	2	0	2	46	37	83
dELL 4	0	0	0	43	58	101
dELL 5	0	0	0	78	82	160
dELL 6	0	0	0	54	46	100
dELL 7	0	0	0	62	54	116
dELL 8	45	1	46	62	80	142
dELL 9	14	0	14	67	57	124

Table 2. Effect of dEaf RNAi on viability

RNAi line	Act5c-Gal4			CyO, y^+		
	Female	Male	Total	Female	Male	Total
dEaf 1	143	60	203	228	240	468
dEaf 2	204	73	277	241	221	462
dEaf 3	195	64	259	189	152	341
dEaf 4	60	5	65	223	228	451
dEaf 6	235	97	332	288	324	612
dEaf 8	268	139	397	240	310	547

dEaf-knockdown flies in six different transgenic RNAi lines (Table 2). In all lines, we observed significant reductions in the number of adult progeny of RNAi-expressing flies compared with control siblings. In addition, a consistent reduction in the male–female sex ratio was observed for dEaf RNAi, suggesting that the male-enhanced lethal phenotype (not observed by us for other elongation factors) is due to loss of a dELL–dEaf complex.

To test for the effectiveness of the RNAi knockdowns, we measured dELL and dEaf mRNA levels in knockdown larvae and their control siblings. Significant reductions in dELL transcripts are observed in the dELL RNAi larvae (Fig. 2*A*). dELL transcripts, as measured by RT-PCR, are not reduced by RNAi to the same level as dELL protein, as assessed by immunofluorescence on polytene chromosomes. Previously, we observed that knockdown of dRTF1 by RNAi was more effective at the protein than the RNA levels presumably because the long dsRNAs produced are processed as miRNAs and interfere with translation (13). Because dELL is nested in an intron of the gene encoding the chromatin remodeling enzyme dMi-2, we also measured transcript levels for this gene and found no reduction of dMi-2 RNA in dELL RNAi larvae (Fig. 2*A*). Additionally, we find that dEaf RNA levels are reduced in dEaf RNAi larvae (Fig. 2*B*). Interestingly, a significant increase in dELL levels is observed in dEaf RNAi larvae, possibly compensating for the lower dEaf levels (Fig. 2*B*).

dELL Is Required for Heat-Shock Gene Expression. dELL was previously shown to be recruited to heat-shock genes upon heat shock (7). To determine whether dELL is required for heat-shock gene expression, we compared the levels of *Hsp70* transcripts after heat shock in dELL knockdown larvae and their control siblings. By immunofluorescence analysis, little or no dELL is seen at the *Hsp70* gene after heat shock in dELL knockdown larvae, whereas the control siblings without the Gal4 driver showed the expected recruitment of dELL to the *Hsp70* gene (Fig. 3*A*). Northern blot analysis showed reduced levels of *Hsp70* mRNA levels in the dELL RNAi larvae (Fig. 3*B*). A similar analysis was done with dEaf RNAi larvae, and reduced *Hsp70* mRNA also occurs after heat shock, although the deficit was less than observed for the dELL RNAi larvae (Fig. 3*B*). Similar results were observed when *Hsp70* levels were measured by RT-PCR, showing greater reductions in *Hsp70* RNA levels in dELL RNAi than dEaf RNAi larvae (Fig. 3*C*).

Chromosomal levels of dELL are markedly reduced in the absence of Cdk9, the catalytic subunit of the Pol II C-terminal

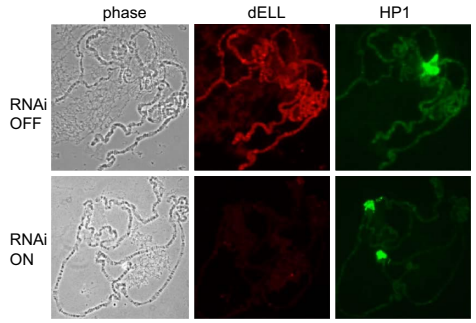

Fig. 1. Reduction of dELL levels on polytene chromosomes after RNAi. Flies carrying a Gal4-responsive dsRNA construct were crossed to flies expressing one copy of Gal4 driven by the Actin5C promoter. (*Upper*) Salivary gland polytene chromosomes from control larvae (RNAi OFF) carrying the second chromosome balancer *CyO* were seen to express dELL at numerous interband sites of polytene chromosomes. (*Lower*) In contrast, RNAi larvae (RNAi ON), expressing Gal4, have greatly reduced levels of dELL throughout the salivary gland nucleus. HP1 levels on control and dELL knockdown larvae were comparable.

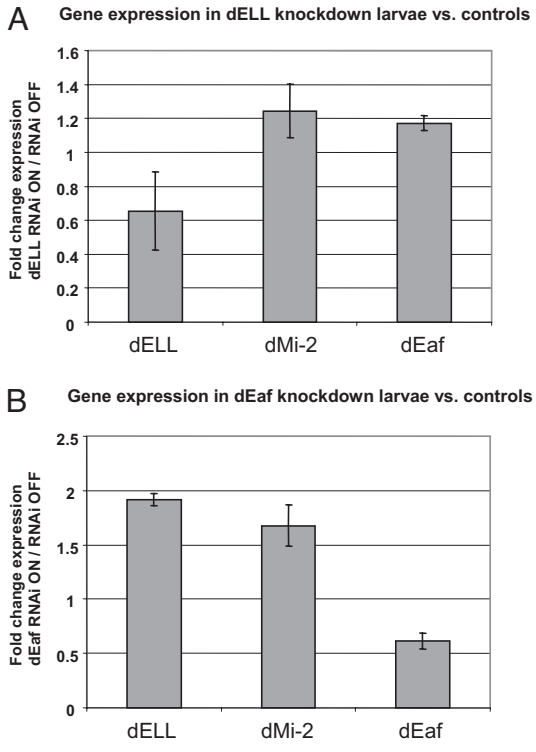

Fig. 2. dELL and dEaf mRNA levels are reduced by RNAi in transgenic flies. (*A*) dELL mRNA levels are reduced in dELL knockdown larvae relative to control larvae. RNA was extracted from dELL knockdown larvae or their control siblings and used for real-time RT-PCR using primers to dELL. For comparison, expression levels of dMi-2, the gene whose large intron contains the dELL gene, and expression levels of dEaf, an interacting partner of dELL, were measured. Values are normalized to rp49 and represent the average of three collections of control and knockdown larvae. Error bars indicate the standard error. (*B*) dEaf mRNA levels are reduced in dEaf knockdown larvae. RT-PCR was performed as in *A*, but with dEaf knockdown larvae and their control siblings.

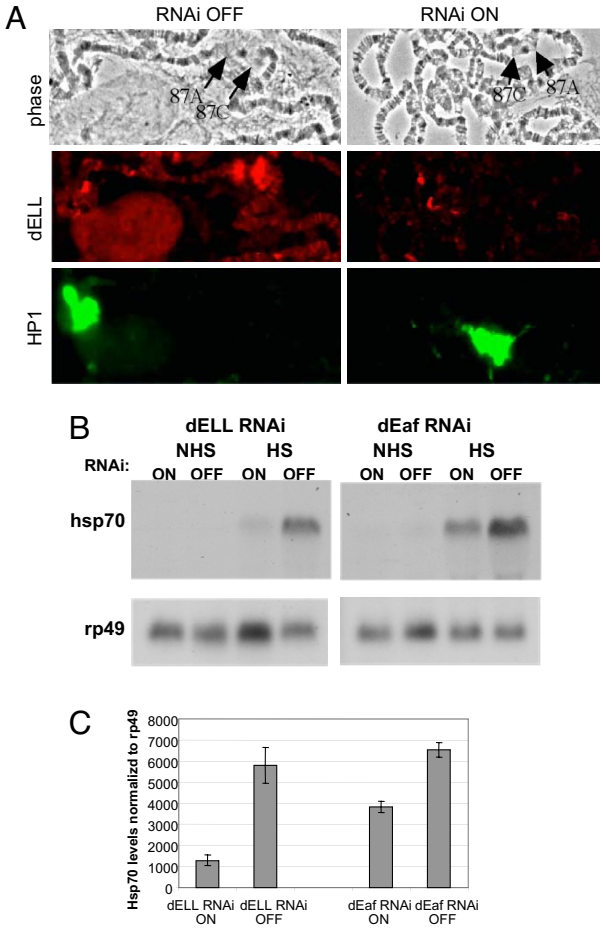

Fig. 3. *Hsp70* induction is reduced in dELL and dEaf RNAi knockdown larvae. (*A*) Knockdown dELL RNAi larvae (RNAi ON) or control siblings (RNAi OFF) were heat-shocked and stained with antibodies to dELL. Although dELL normally goes to the major heat-shock loci, it cannot be detected at the 87A/C loci in the knockdown larvae. HP1 levels are comparable in the knockdown and control larvae. Phase contrast images are shown. (*B*) Heat-shock response is lower in dELL and dEaf knockdown larvae. (*Upper*) Hsp70 mRNA levels were measured by Northern blotting in dELL and dEaf knockdown and control larvae, before heat shock [no heat shock (NHS)], and after heat shock (HS). (*Lower*) rp49 was probed as a loading control. (*C*) Similar reductions in *Hsp70* mRNA levels after heat shock induction are seen by RT-PCR. Error bars indicate the standard error.

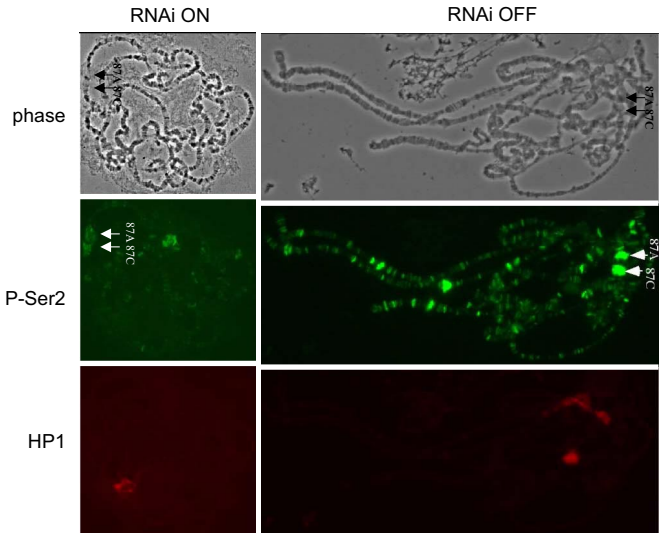

Fig. 4. dELL knockdown results in the reduction of the Ser-2-phosphorylated form of Pol II. Polytene chromosome preparations from dELL knockdown (RNAi ON) and control larvae (RNAi OFF) were probed with the H5 monoclonal antibody to the Ser-2-phosphorylated, elongating form of Pol II. Representative images of the average staining level of Ser-2-phosphorylation levels are shown for the dELL knockdown and control. HP1 levels were unchanged.

domain (CTD) kinase PTEF-B (19). To determine whether dELL knockdown affects the recruitment of Pol II to the *Hsp70* genes, dELL knockdown and control polytene chromosomes were probed with antibodies to the Ser-2-phosphorylated, elongating form of Pol II. We consistently observed lower levels of Ser-2-phosphorylated Pol II at the *Hsp70* heat-shock loci in dELL-knockdown larvae (Fig. 4), suggesting a close link between dELL function and phosphorylation of the Pol II CTD.

dELL and dEloA Are Not Redundant *in Vivo*. ELL belongs to a class of transcription elongation factors that have been shown to stimulate the K_m and/or V_{max} of RNA Pol II *in vitro* by alleviating pausing on a purified DNA template. Another member of this class is Elongin A and its *Drosophila* ortholog dEloA (1, 3). From the present and previous studies, it is clear that both dELL and dEloA localize to the *Hsp70* gene upon heat shock, and each is required for full levels of heat-shock gene expression, suggesting that the *in vivo* roles of these elongation factors in *Hsp70* gene transcription are not redundant (8). Similarly, we have observed that the knockdown phenotypes of these two proteins can be unique, such as the enhanced male lethality in dELL RNAi larvae (Table 1). How could both elongation factors be redundant *in vitro*, yet nonredundant *in vivo*? The *in vitro* studies were performed on naked DNA templates, whereas the chromatin environment of RNA Pol II-transcribed genes can provide additional challenges to the polymerase. Each of these elongation factors has its own interaction partners and may be recruited to distinct states of the polymerase, such as initiating, elongating, or stalled polymerase. Consistent with this view, knockdown of dELL, but not dEloA, results in decreased levels of Ser-2-phosphorylated Pol II at the *Hsp70* and other loci (Fig. 4) (8). Interestingly, the chromosomal targeting of dELL, but not dEloA, is dramatically reduced by the knockdown of CDK9, the Pol II CTD kinase (19), suggesting that dELL and dEloA are recruited to genes by distinct mechanisms. Fine mapping of dELL and dEloA on the well characterized *Hsp70* gene at different time points after activation could clarify the distinct roles for these enzymes.

The lesser effect of dEaf knockdown on *Hsp70* gene induction could be indicative of a requirement of dEaf for optimal function of dELL, whereas dELL can partially function without dEaf. Indeed, *in vitro* transcription studies have demonstrated that human Eaf proteins, in combination with ELL, stimulate transcription elongation by Pol II above the levels obtained with ELL alone (17). In dEaf RNAi larvae, we observed that dELL levels are increased, conceivably as a cellular response to increased pausing resulting from lower dEaf levels.

Males Are More Sensitive to dELL Knockdown. Previous work on the function of dELL made use of alleles of the *Su*(*Tpl*) locus, which encodes dELL (2). All known *Su*(*Tpl*) alleles are embryonic lethal. In contrast, RNAi of dELL allows survival to the larval or adult stages depending on the insertion line of the dsRNA construct. Interestingly, the few "escaper" dELL RNAi adults are overwhelmingly female. As seen with the heat-shock defect, the difference in male and female viability is less in dEaf RNAi flies than in dELL RNAi flies, consistent with dEaf enhancing, but not being absolutely required for, dELL function. A previous study showed that males express much higher levels of a dELL transcript than females, although the functional significance of this difference has not been investigated (7). One hypothesis is that dELL is needed in males as

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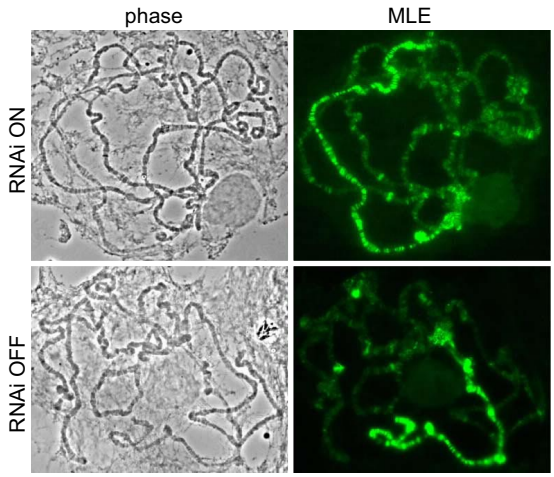

Fig. 5. X chromosome morphology and male-specific lethal protein localization are not apparently affected by reduction of dELL. Polytene chromosome preparations from dELL knockdown males (RNAi ON) or their control brothers (RNAi OFF) were probed with antibodies to MLE, an RNA helicase that is required for dosage compensation in *Drosophila* and spreading of the complex along the X chromosome (20). MLE staining patterns appear similar in knockdown and control brothers.

part of the process of X chromosome dosage compensation; *Drosophila* dosage compensation factors are thought to enhance transcription elongation of X-linked genes in males, and loss of any of these factors leads to male-specific lethality (20). In addition, reduced levels of several global chromatin regulators, including the supercoiling factor, Jil-1 H3 kinase, heterochromatin protein HP1, and the chromatin remodeler ISWI, have been reported to differentially affect the survival of males and/or the morphology of the X chromosome (21–24). However, in dELL knockdowns, MSL localization and the male polytene X chromosome morphology appears similar in dELL knockdown male larvae and their control brothers (Fig. 5). Whether there are specific defects in dosage compensation of X-linked genes may be an interesting avenue for future investigations. Alternative explanations for a male-enhanced lethality also should be considered. For example, *Drosophila* males differ from females not just in having one less X chromosome, but also in carrying a Y chromosome, which comprises ≈12% of the male genome. A number of genes are male-lethal due to the presence of the mostly heterochromatic Y chromosome, including modulators of position effect variegation, such as the *Su*(*var*)*3-3* gene that encodes the histone demethylase LSD1, the uncharacterized *Su*(*var*)*2-1*, as well as the HP1-interacting protein Bonus (dTIF1), an enhancer and suppressor of position-effect variegation (reviewed in ref. 25). For *Su*(*var*)*2-1* and *Bonus*, the Y-lethal effect is not Y-specific but can be phenocopied by other sources of heterochromatin (25). A role for dELL in the regulation of heterochromatin is unknown but could conceivably be required for the expression of heterochromatin components.

The finding that a large number of developmentally regulated genes have Pol II poised at their promoters in the absence of detectable full-length transcripts suggests that regulated Pol II elongation is an important transcription regulatory mechanism in eukaryotes. Consistent with that view, the *Drosophila* ELL is an essential protein that, as shown here, is required for the full induction of *Hsp70*, one of the earliest examples of a gene regulated by Pol II elongation. Our current model is that dELL is a Pol II elongation factor that controls the expression levels of diverse genes. Importantly, our studies in *Drosophila* are beginning to define distinct regulatory roles for different Pol II elongation factors that otherwise behave similarly *in vitro*. A biochemical description of how the binding of dELL (and its associated factor dEaf) enhances Pol II elongation is essential to understanding gene regulation during development.

Materials and Methods

Fly Stocks and Crosses. A 600-bp region (1,200–1,800 bp) of the dELL ORF or a 600-bp region (200–800 bp) of the dEaf ORF was cloned into *Sym-pUAST-w* P element vector (26) vector and injected into yw embryos to generate transgenic flies. To induce dsRNA, *yw*; *P(Sym-pUAST-dELL)* or *yw*; *P(Sym-pUAST-dEaf)*, males were crossed to *yw*; *Act5C-Gal4/CyO*, y^+ females.

Antibodies. dELL rabbit polyclonal serum was raised against full-length recombinant His_6-tagged dELL and was described previously (27). Anti-HP1 monoclonal antibody C1A9 was a gift from Sarah Elgin (Washington University, St. Louis). Anti-Ser-2-phosphorylated CTD of Pol II H5 monoclonal was purchased from Covance. MLE rabbit polyclonal serum was a gift from John Lucchesi (Emory University, Atlanta). Polytene chromosome staining was performed as described in ref. 28. Briefly, salivary glands were fixed for 30 s in 2% paraformaldehyde and then in 45% acetic acid/2% formaldehyde for 3 min. RNAi knockdown and control larvae were chosen based on the presence of yellow or black mouth hooks, respectively.

Northern Blot Analysis and Real-Time PCR. Northern blotting was done as described previously (8). Briefly, third-instar larvae, sorted by the color of the mouthooks as described above, were untreated or heat shocked for 30 min. Then 15 μg of total RNA was separated on 1% formaldehyde-agarose gel, blotted to Biotrans nylon membrane (ICN), and hybridized with a riboprobe in 50% formamide at 65°C.

For real-time PCR, total RNA from larvae was treated with DNase and repurified by RNeasy columns (Qiagen). Finally, 50 ng of total RNA was used in a 25-μl reaction with the iScript one-step RT-PCR kit with SYBR Green in a Bio-Rad MyCycler.

Primers used were: *rp49*, AGAGTTCTTGTAACGTGGTCGGAATA and CAATGGTGCTGCTATCCCAATC; *Hsp70*, GGGTGTGCCCCAGATAGAAG and TGTCGTTCTTGATCGTGATGTTC (29); *dELL*, TTCCAGGACTTATCCGAACG and CAATGGTGCGATCGTTATTG; *dEaf*, CAGGGAGCATGAACTGACCT and TCTGTGCTGTCATCCGAGTC; and *dMi-2*, TTCAGCGACACAAGGACAAG and TGCCGTACGATGGTTGATTA.

ACKNOWLEDGMENTS. We thank Tyler Marquart for technical assistance and Sarah Elgin (Washington University, St. Louis, MO) and John Lucchesi (Emory University, Atlanta, GA) for providing reagents. This work was supported by National Science Foundation Grants MCB 0131414 and MCB 0615831 (to J.C.E.) and National Institutes of Health Grant CA089455 (to A.S.).

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