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Phosphorylation of histone H3 at Ser10 facilitates RNA polymerase II release from promoter-proximal pausing in *Drosophila*

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The *Drosophila* JIL-1 kinase is known to phosphorylate histone H3 at Ser10 (H3S10) during interphase. This modification is associated with transcriptional activation, but its function is not well understood. Here we present evidence suggesting that JIl-1-mediated H3S10 phosphorylation is dependent on chromatin remodeling by the brahma complex and is required during early transcription elongation to release RNA polymerase II (Pol II) from promoter-proximal pausing. JIL-1 localizes to transcriptionally active regions and is required for activation of the *E75A* ecdysone-responsive and *hsp70* heat-shock genes. The heat-shock transcription factor, the promoter-paused form of Pol II (Pol IIo^{ser5}), and the pausing factor DSIF (DRB sensitivity-inducing factor) are still present at the *hsp70* loci in *JIL-1*-null mutants, whereas levels of the elongating form of Pol II (Pol IIo^{ser2}) and the P-TEFb kinase are dramatically reduced. These observations suggest that phosphorylation of H3S10 takes place after transcription initiation but prior to recruitment of P-TEFb and productive elongation. Western analyses of global levels of both forms of Pol II further suggest that JIL-1 plays a general role in early elongation of a broad range of genes. Taken together, the results introduce H3S10 phosphorylation by JIL-1 as a hallmark of early transcription elongation in *Drosophila*.

[*Keywords*: Histone; chromatin; transcription; kinase]

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The eukaryotic cell packages its DNA wrapped around histone proteins to form nucleosomes, the basic units of chromatin (Wolffe 1998). These nucleosomes assemble into higher-order chromatin structures through which the transcription machinery must navigate each time it is signaled to transcribe. Mechanisms have consequently evolved to maintain a flexible chromatin state that can readily respond to intrinsic and extrinsic stimuli and accordingly modulate gene expression (Orphanides and Reinberg 2002). Most prominently, histone-modifying enzymes can methylate, acetylate, and phosphorylate various amino acid residues of histone N termini, thereby changing their affinity for different transcriptional regulators (Jenuwein and Allis 2001). ATP-dependent chromatin remodeling complexes can also be recruited to alter the position and accessibility of the nucleosome (Simone 2006). The binding of specific transcription factors triggers a cascade of events during which these diverse chromatin modulators work in concert to allow the RNA polymerase II (Pol II) machinery to bind target genes, initiate transcription, and elongate the messenger RNA (mRNA). These regulators maintain tight control of transcription throughout the elongation process by continuously communicating with the C-terminal domain (CTD) of the largest subunit of Pol II (Orphanides and Reinberg 2002).

The CTD of Pol II consists of a heptad repeat (Tyr–Ser–Pro–Thr–Ser–Pro–Ser) that is conserved from yeast to humans. It integrates transcriptional events by interacting with distinct regulatory proteins that recognize different patterns of CTD phosphorylation (Saunders et al. 2006). When Pol II is first recruited to the promoter as part of the preinitiation complex, its CTD is hypophosphorylated. After Pol II disengages from the promoter, the CTD becomes phosphorylated at Ser5 (Pol IIo^{ser5}) by TFIIH, a general transcription factor that is part of the Pol II machinery (Feaver et al. 1991; Lu et al. 1992; Serizawa et al. 1992). As part of an early elongation complex, Pol II progresses 20–40 base pairs (bp) downstream from the promoter. It then pauses in a process referred to as promoter-proximal pausing to allow for capping of the nascent mRNA. DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) cooperate to repress transcription elongation and maintain this pause.

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Pol II is released once the P-TEFb kinase is recruited to relieve the negative effects of DSIF and NELF and phosphorylate the CTD at Ser2 (Pol IIo^{ser2}), marking the onset of productive elongation (Marshall et al. 1996). The various transcriptional steps are associated with distinct histone modifications and chromatin remodeling complexes. Set1, the enzyme responsible for methylating Lys4 of histone H3 (H3K4) in *Saccharomyces cerevisiae*, is known to physically associate with the CTD of Pol II when it is phosphorylated at Ser5. At the same time, trimethylation of H3K4 has been found concentrated at the 5′ end of transcribed genes (Ng et al. 2003). Methylation of Lys36 of H3 (H3K36), on the other hand, is associated with a later step in elongation; this mark accumulates further downstream from the promoter and associates with the CTD when phosphorylated at Ser2 (Krogan et al. 2003). Other modifications, such as lysine acetylation, arginine methylation, and serine phosphorylation, have also been associated with activation of gene expression (Turner 2000; Kouzarides 2002; Nowak and Corces 2004; Peterson and Laniel 2004). Of interest, phosphorylation of histone H3 at the Ser10 residue (H3S10) has been shown to be important for activation of transcription in yeast, *Drosophila*, and mammalian cells, but its precise role in this process is not well understood.

Several studies have suggested an important role for H3S10 phosphorylation in specific transcriptional responses to signaling stimuli. The yeast Snf1 kinase phosphorylates H3S10 upon activation of the *INO1* gene (Lo et al. 2001). In mammalian fibroblasts, rapid phosphorylation of histone H3 concomitant with activation of immediate-early (IE) response genes takes place when cells are treated with growth factors and various stress-inducing agents (Mahadevan et al. 1991). Further, Coffin-Lowry syndrome is characterized by impaired transcriptional activation of the *c-fos* gene and a loss of EGF-induced phosphorylation of histone H3S10 (Sassone-Corsi et al. 1999). Treatment of immature rat ovarian granulosa cells with follicle-stimulating hormone produces rapid H3S10 phosphorylation in a PKA-dependent manner, suggesting a role for histone phosphorylation in cellular differentiation (DeManno et al. 1999). Additionally, H3S10 phosphorylation follows the stimulation of the suprachiasmatic nucleus of rats with light (Crosio et al. 2000) and activation of hippocampal neurons (Crosio et al. 2003). It further appears to play a central role during cytokine-induced gene expression mediated by IκB kinase α (IKK-α) (Yamamoto et al. 2003). What remains unclear from these studies is whether H3S10 phosphorylation is limited to mediating signal transduction events or whether it plays a more general role in the activation of gene expression in vertebrates.

Studies in *Drosophila* suggest that this modification may be required for the transcription of most genes in this organism. Using the heat-shock response as a model system, we established previously that H3S10 phosphorylation patterns parallel those of active genes. *Drosophila* responds to a rise in temperature by rapidly increasing the transcription of heat-shock genes while repressing genes expressed previously. Before heat shock, phosphorylated H3S10 localizes to euchromatic regions of polytene chromosomes and colocalizes with Pol II. After heat shock, this modification redistributes to the active heat-shock loci and disappears from the rest of the chromosome, where genes are now repressed (Nowak and Corces 2000).

Despite these observations, the precise role of H3 phosphorylation in gene activation remains elusive. The mammalian MSK1 and MSK2 kinases, among others, have been shown to be responsible for H3S10 phosphorylation associated with transcription (Davie 2003; Soloaga et al. 2003). The *Drosophila* homolog of MSK1/2, the JIL-1 threonine/serine kinase, was recently characterized and shown to phosphorylate H3S10 in vitro (Jin et al. 1999). H3S10 phosphorylation levels in vivo are dramatically reduced in *JIL-1*^{z2}-null mutants (Wang et al. 2001). The JIL-1 protein localizes to interband regions of polytene chromosomes and is found up-regulated on the male X chromosome. Furthermore, the *JIL-1*^{z2} allele enhances the phenotype of *trx-G* mutations (Zhang et al. 2003). These data indirectly suggest that JIL-1-mediated H3S10 phosphorylation plays an important role in transcriptional activation.

In this study, we further characterize the role of JIL-1-mediated H3S10 phosphorylation in transcription. We directly establish that JIL-1 is required for the transcription of the majority of, if not all, *Drosophila* genes. Mechanistic analyses place the phosphorylation event subsequent to transcription initiation but prior to productive elongation and show that JIL-1 plays an integral role in the release of Pol II from promoter-proximal pausing. The data therefore highlight H3S10 phosphorylation as a novel hallmark of early productive elongation in *Drosophila*.

Results

JIL-1 associates with actively transcribed regions on polytene chromosomes

A correlation exists between active transcription in *Drosophila* and H3S10 phosphorylation, and both events take place specifically in most interband regions (less-condensed chromatin) on salivary gland polytene chromosomes. Upon heat-shock treatment, H3 becomes phosphorylated at Ser10 at the highly transcribed heat-shock loci, and it becomes dephosphorylated at genes that were previously active and are now repressed (Nowak and Corces 2000). Since JIL-1 phosphorylates histone H3 in vitro and H3S10 phosphorylation is dramatically reduced in *JIL-1*-null mutants (Wang et al. 2001), we tested whether the JIL-1 kinase is required for transcription. To determine whether JIL-1 associates with actively transcribed regions, we compared its distribution on polytene chromosomes with that of both hypophosphorylated and hyperphosphorylated forms of Pol II. We performed immunofluorescence experiments using polytene chromosomes from third instar larvae, costaining with α-JIL-1 antisera as well as 8WG16, H5, or

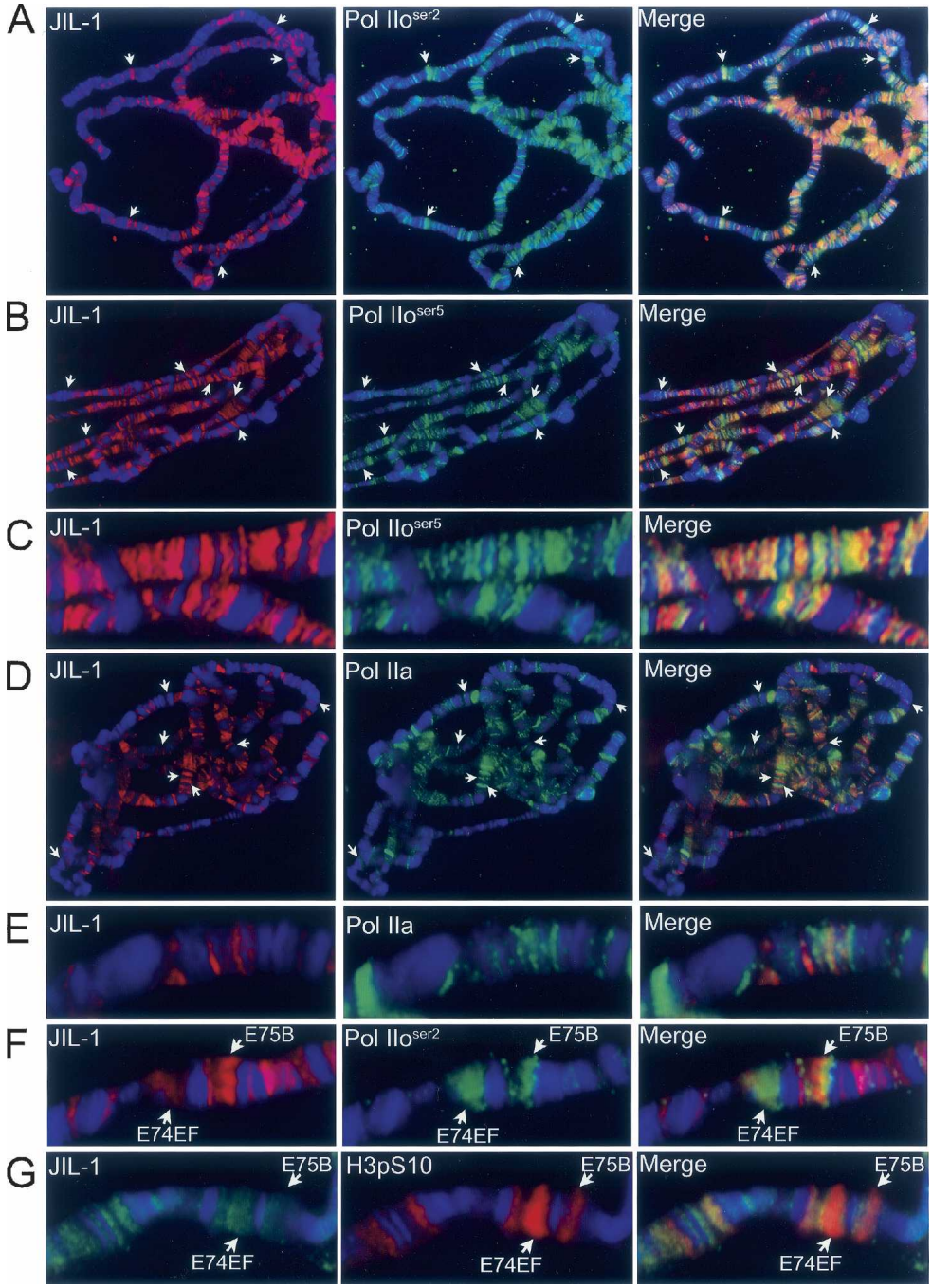

Figure 1. JIL-1 localizes to actively transcribed regions on polytene chromosomes. (*A*) Immunolocalization of JIL-1 (red) and Pol IIo^{ser2} (green) on wild-type (wt) polytene chromosomes; arrows indicate sites of colocalization. (*B*) Immunolocalization of JIL-1 (red) and Pol IIo^{ser5} (green) on wild-type polytene chromosomes; arrows indicate sites of colocalization. (*C*) Detailed view of a section of the chromosomes in *B*. (*D*) Immunolocalization of JIL-1 (red) and Pol IIa (green) on wild-type polytene chromosomes; arrows indicate Pol IIa sites that do not exhibit JIL-1 binding. (*E*) Detailed view of a section of the chromosomes in *D*. (*F*) Immunolocalization of JIL-1 (red) and Pol IIo^{ser2} (green) on ecdysone-induced puffs at E74EF and E75B. (*G*) Immunolocalization of JIL-1 (green) and phosphorylated H3S10 (H3pS10) (red) on ecdysone-induced puffs at E74EF and E75B. The puffs are indicated with arrows. DNA is stained with DAPI (blue) in all images.

H14 antibodies directed against Pol IIa, Pol IIo^{ser2}, and Pol IIo^{ser5}, respectively. The results reflect a broad distribution of JIL-1 throughout euchromatin, localizing to most interband regions, with an extensive overlap between JIL-1 and both phosphorylated forms of Pol II (Fig. 1A,B). Although the relative levels of JIL-1 and Pol II vary from site to site, a close examination shows that JIL-1 is found at ~95% of sites associated with either phosphory-

lated form of Pol II, examples of which are indicated by arrows in Figure 1, A and B; the extent of overlap can be better appreciated in close-up views of the chromosomes (Fig. 1C,F). JIL-1 also overlaps significantly with Pol IIa, albeit to a lesser extent; only ~60% of sites containing Pol IIa also contain JIL-1 (Fig. 1D, arrows indicate sites where there is no overlap, E shows a detailed view).

JIL-1 is required for transcription of ecdysone-induced genes

To further analyze the possible role of JIL-1 in the activation of transcription, we examined the chromosomal localization of JIL-1, Pol IIo^{ser2}, and phosphorylated H3S10 in developmental puffs of third instar larvae polytene chromosomes. The presence of these puffs reflects high levels of transcription regulated by ecdysone hormone. It has been previously demonstrated that Pol IIo^{ser2} localizes to these regions at discrete times during development (Weeks et al. 1993). Later studies also showed phosphorylated H3S10 at these puffs, suggesting that this histone modification may be required for ecdysone-induced transcription (Nowak and Corces 2000). Our results show that JIL-1 colocalizes with Pol IIo^{ser2} and phosphorylated H3S10 at prominent early ecdysone-induced puffs, such as 75B and 74EF (Fig. 1F,G). The presence of JIL-1 in the ecdysone-induced puffs led us to examine whether JIL-1 was necessary for ecdysone-induced transcription. We analyzed the expression levels of *E75A*, one of the isoforms encoded by the early gene *E75*, at the late prepupae stage. At this stage, a high titer of ecdysone triggers the transition from prepupae to pupae, accompanied by a peak in the expression of the early genes (Thummel 1996). Equal amounts of total RNA isolated from wild-type and *JIL-1^{z2}* mutant prepupae were examined by Northern analysis using a probe specific for the *E75A* mRNA. We observed high levels of *E75A* expression in wild-type prepupae and a reduction in *E75A* expression in *JIL-1^{z2}* mutants (Fig. 2A), suggesting that JIL-1 is required for transcription of the ecdysone genes.

JIL-1 is required for transcription of the heat-shock genes

Histone H3 becomes phosphorylated at Ser10 concomitantly with transcription activation of the heat-shock genes. We therefore asked whether JIL-1 is involved in the transcription activation of the heat-shock genes by examining whether JIL-1 localizes to the heat-shock puffs of polytene chromosomes upon thermal induction. Using α-JIL-1 antisera, we performed immunofluorescence microscopy on polytene chromosomes from wild-type third instar larvae salivary glands subjected to 25 min of heat shock. JIL-1 localization at the heat-shock loci was revealed by a robust staining at several heat-shock puffs, most prominently at the *hsp70* genes (chromosomal subdivisions 87A/C) and at the 93D and 95D puffs (Fig. 3A). However, unlike phosphorylated H3S10, JIL-1 can still be detected at the previously transcribed

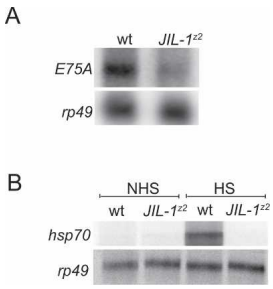

Figure 2. JIL-1 is required for ecdysone and heat-shock-induced gene expression. (*A*) Expression analysis of the ecdysone-induced gene *E75A*. Total RNA isolated from wild-type (wt) and *JIL-1^{z2}* mutant prepupae was analyzed by Northern blot hybridization to detect *E75A* mRNA; *rp49* was used as loading control. (*B*) Expression analysis of *hsp70* gene expression. Total RNA isolated from heat-shocked (HS) and non-heat-shocked (NHS) wild-type and *JIL-1^{z2}* mutant larvae was analyzed by Northern blot hybridization to detect *hsp70* mRNA; *rp49* was used as loading control.

genes that are repressed after heat shock. To confirm that JIL-1 recruitment is transcription dependent, we examined whether JIL-1 is present at the *hsp70* genes under non-heat-shock conditions. *Drosophila* has six nearly identical genes that encode Hsp70, the major heat-shock protein. Two are located at division 87A and four are located at 87C on the right arm of chromosome 3 (Gong and Golic 2004). Anti-JIL-1 staining combined with in situ hybridization to a portion of the *hsp70* transcription unit shows no colocalization between JIL-1 and the *hsp70* genes at the 87C locus in the absence of heat shock (Fig. 3B). Nevertheless, JIL-1 is present at very low levels at the 87A locus. This could be explained by the fact that the *hsp70* genes at 87A are surrounded by actively transcribed genes that cannot be distinguished from *hsp70* at the level of resolution provided by polytene chromosomes. This is supported by the presence of low levels of Pol IIo^{ser2} at 87A but not 87C under non-heat-shock conditions (Fig. 3C). These results suggest that JIL-1 is recruited to the heat-shock loci in a transcription-dependent manner.

To confirm that JIL-1 is required for the phosphorylation of H3S10 at the *hsp70* loci upon heat shock, we tested whether this modification is JIL-1 dependent. Antibodies to phosphorylated H3S10 were used for immunofluorescence microscopy combined with in situ hybridization on polytene chromosomes. Before heat shock in wild-type chromosomes, phosphorylated H3S10 cannot be detected at the 87C locus while low levels are observed at 87A (Fig. 3D), consistent with the localization of both JIL-1 and Pol IIo^{ser2}. After heat shock, an intense signal can be detected at both loci in wild-type (Fig. 3E) but is severely reduced in chromosomes from *JIL-1*-null mutants (Fig. 3F). To further confirm this conclusion, we examined the expression of the *hsp70* genes in wild-type and *JIL-1^{z2}* mutants before and after heat shock by Northern analysis. A robust increase in the levels of *hsp70* mRNA is detected in wild-type larvae after heat shock, while no *hsp70* transcripts are detected

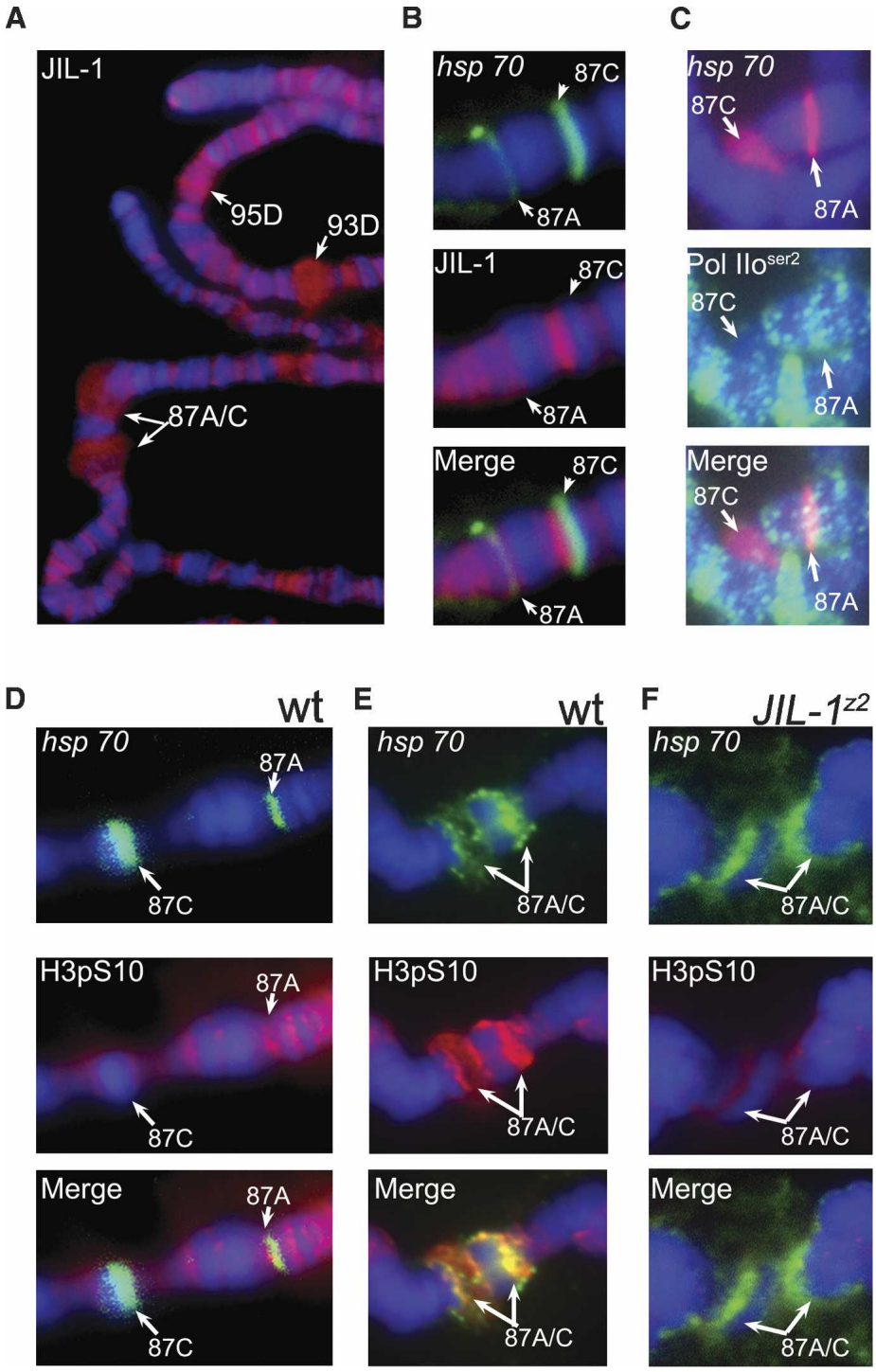

Figure 3. JIL-1 is recruited to the *hsp70* loci after heat-shock treatment. (*A*) Immunolocalization of JIL-1 (red) on wild-type (wt) heat-shock-induced polytene chromosomes. The major heat-shock-induced puffs at 87A, 87C, 93D, and 95D are indicated with arrows. (*B*) JIL-1 (red) combined with in situ hybridization to detect *hsp70* (green) on wild-type non-heat-shocked polytene chromosomes. (*C*) Pol IIo^{ser2} (green) combined with in situ hybridization to detect *hsp70* (red) on wild-type non-heat-shocked polytene chromosomes. (*D*) H3pS10 (red) combined with in situ hybridization to detect *hsp70* (green) on wild-type non-heat-shock polytene chromosomes. The cytological sites 87A and 87C are indicated with arrows. (*E*,*F*) H3pS10 (red) combined with in situ hybridization to detect *hsp70* (green) on wild-type and *JIL-1*^{z2} heat-shock-induced 87A and 87C puffs.

in *JIL-1*-null mutants under the same conditions (Fig. 2B). These results suggest that JIL-1-mediated H3 phosphorylation is required for transcription of the *hsp70* genes.

JIL-1 is not required for the recruitment of the HSF transcription factor

Having established that JIL-1 is required for transcription of the *hsp70* genes, we sought to place H3S10 phosphorylation in the cascade of events leading from transcription factor recruitment to transcription elongation. It is conceivable that the overall disruption of chromosome structure in *JIL-1* mutants (Wang et al. 2001) prevents the binding of the heat-shock transcription factor HSF to its response element upon gene induction. We therefore examined the recruitment of HSF to polytene chromosomes after heat shock in wild-type and *JIL-1*^{z2} mutant larvae. When inactive, HSF is diffusely distributed throughout the chromosomes at very low levels, including at the heat-shock genes (Fig. 4A). After 25 min of heat shock, HSF redistributes to discrete sites, most prominently at the heat-shock loci, as can be visualized by immunofluorescence microscopy of polytene chromosomes using anti-HSF antibodies (Fig. 4A; Westwood et al. 1991). Mutation of *JIL-1* has no effect on this relocalization, as HSF can be observed to bind polytene chromosomes of *JIL-1*^{z2} mutants despite their poorly organized chromatin (Fig. 4B). This binding appears to be very specific, as suggested by the strong signal at the 87A and 87C loci and by the lack of nonspecific binding before heat shock (Fig. 4B). These results indicate that the disrupted chromatin organization of chromosomes in *JIL-1* mutant larvae does not preclude transcription factor binding, suggesting that JIL-1 acts to promote a transcription step downstream from transcription factor recruitment.

Transcription initiation takes place at the hsp70 *genes in* JIL-1 *mutants, but elongation is impaired*

The heat-shock genes accumulate a hypophosphorylated form of promoter-paused Pol II at a location 20–40 bp downstream from their transcription start sites prior to induction. Immediately after induction and recruitment of HSF, levels of Pol IIo^{ser5} rapidly increase, especially at the promoter-proximal region of the gene, indicating promoter clearance. Soon thereafter, Pol IIo^{ser2} can be detected at the transcription start site and throughout the reading frame, marking transcription elongation (Boehm et al. 2003). In order to determine the stage of the transcription process at which JIL-1 acts, we first examined the localization of Pol IIo^{ser5} at the *hsp70* loci of wild-type and *JIL-1*^{z2} mutants using antibody H14, which is specific for this form of Pol II. Anti-HSF antibodies were also used to mark the heat-shock puffs and as an internal control of signal level. Pol IIo^{ser5} localizes to the 87A and 87C loci of wild-type chromosomes after 25 min of heat-shock treatment (Fig. 4C). Similarly, *JIL-1*^{z2} mutants display high levels of Pol IIo^{ser5}, suggesting that transcription initiation is not affected and that elongation might be defective in these mutants (Fig. 4D). To test this possibility, we carried out immunofluorescence analysis using the H5 antibody, which recognizes Pol IIo^{ser2}. In contrast to HSF and Pol IIo^{ser5}, levels of Pol IIo^{ser2} are dramatically reduced in *JIL*^{z2} mutants (Fig. 4F) as compared with wild type (Fig. 4E).

JIL-1 is required for P-TEFb recruitment during promoter-proximal pausing

The results so far suggest a requirement for H3S10 phosphorylation by JIL-1 during transcription elongation of the *hsp70* genes. The low levels of Pol IIo^{ser2} observed at 87A/C could be explained by two scenarios. It is possible that H3S10 phosphorylation is necessary for the recruitment of the kinase responsible for phosphorylation of the CTD at Ser2. If this is the case, the polymerase should not become phosphorylated in *JIL-1*-null mutants and would not be released from its pause. A second possibility is that the chromatin structure in *JIL-1* mutants interferes with elongation. In this case, the polymerase could become phosphorylated, but disengages shortly after being released.

The P-TEFb complex, a heterodimer of the CDK9 kinase and cyclin T (CycT), is responsible for the phosphorylation of the CTD at Ser2. This complex is rapidly recruited upon heat shock and acts to release Pol II from promoter-proximal pausing by antagonizing the effects of the pausing factors DSIF and NELF. Immunofluorescence analysis using anti-CycT antibodies has previously detected high levels of P-TEFb at 87A/C after heat shock (Lis et al. 2000). To obtain a better understanding of the role of JIL-1 and to narrow down the window during which it acts, we performed similar analyses and compared levels of CycT at 87A/C in wild-type and *JIL-1*^{z2} mutant larvae. Antibodies against Pol IIo^{ser5} were used to mark the puffs and as an internal control for signal intensity. A dramatic decrease in CycT binding at heat-shock genes was observed in *JIL-1* mutants, as can be seen by comparing Figure 5, B and A. Quantitative RT–PCR analyses revealed normal transcript levels of CycT in these larvae as compared with wild type, suggesting that this observation is not due to indirect effects on *CycT* expression (data not shown). We then performed the same comparative analyses using antibodies against SPT5, a component of the pausing factor DSIF. Unlike the case with P-TEFb, we observed no changes in SPT5 binding in *JIL-1*^{z2} mutants (Fig. 5D) when compared with wild type (Fig. 5C). These data suggest that JIL-1 acts prior to P-TEFb recruitment.

JIL-1 is required for transcription of most Drosophila *genes*

JIL-1 localizes to all interbands on polytene chromosomes and colocalizes extensively with RNA polymerase throughout the genome, suggesting that JIL-1 may

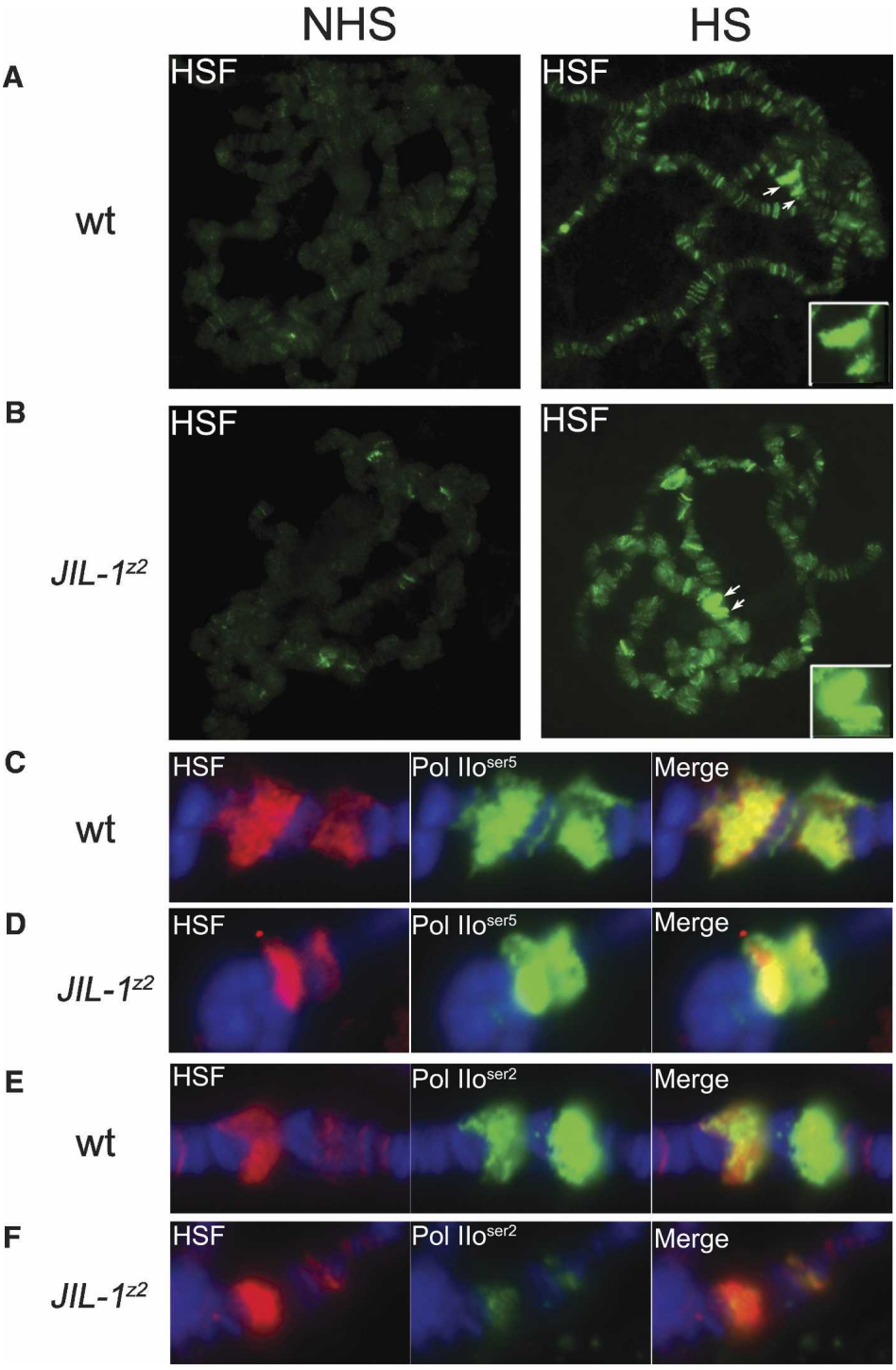

Figure 4. Transcription factor recruitment and transcription initiation occur in *JIL-1* mutants, but transcription elongation is impeded. Immunolocalization of HSF on polytene chromosomes of wild-type (wt) (*A*) or *JIL-1*^{z2} (*B*) larvae that have been either heat-shocked (HS) or non-heat-shocked (NHS). *Insets* indicate the 87A/C heat-shock puffs in both panels. Detailed view of the heat-shock puffs at 87A/C stained with Pol IIo^{ser5} (green) (*C*,*D*) or Pol IIo^{ser2} (green) (*E*,*F*) from wild type (*C*,*E*) or *JIL-1*^{z2} (*D*,*F*). HSF (red) was used as control, and DNA is stained with DAPI (blue) in *C–F*.

play a role in the transcription of the vast majority of *Drosophila* genes (Fig. 1A). The results from the previous experiments suggest that JIL-1 is required for proper elongation but not initiation of the *hsp70* genes. To examine whether this role is conserved throughout the genome, we performed Western analyses of protein extracts from wild-type and *JIL-1*^{z2} mutant larvae and compared total levels of the two phosphorylated forms of Pol

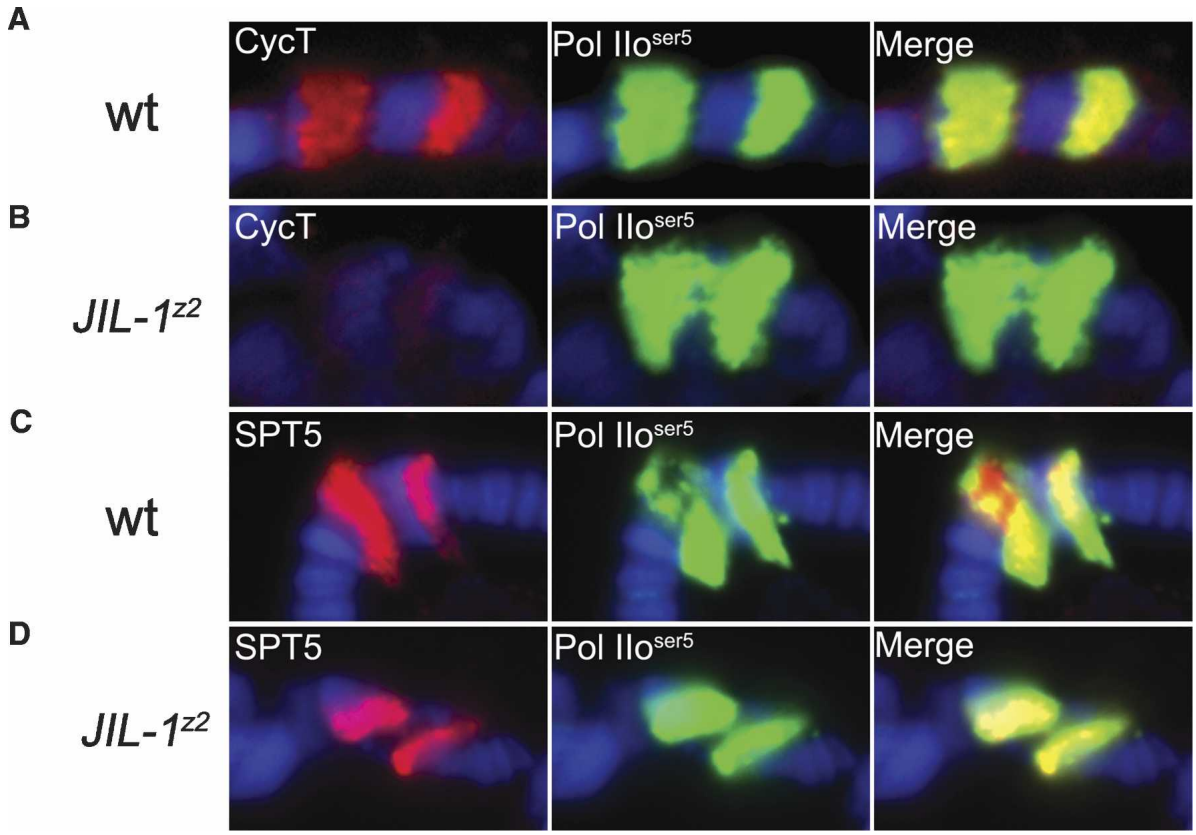

Figure 5. JIL-1 is required for P-TEFb recruitment but not DSIF binding after heat shock in *JIL-1*-null mutants. Detail view of the heat-shock puffs at 87A/C stained with CycT (red) (*A*,*B*) and SPT5 (red) (*C*,*D*) antibodies from wild-type (*A*,*C*) and *JIL-1*^{z2} (*B*,*D*) polytene chromosomes under heat-shock conditions. Pol IIo ^{ser5} (green) was used as control, and DNA is stained with DAPI (blue) in *A–D*.

II. Total Pol IIo^{ser2} levels are significantly reduced in *JIL-1*^{z2} mutants as compared with wild type (Fig. 6A). In contrast, total Pol IIo^{ser5} levels are maintained in these mutants at wild-type levels (Fig. 6B). Furthermore, levels of trimethylated H3K4—a marker of a chromatin environment that promotes transcription initiation—are also maintained in *JIL-1*^{z2} mutants (Fig. 6C). Taken together, these results suggest a general role for JIL-1 in early elongation of transcription.

Histone phosphorylation requires the Brahma chromatin remodeling complex

To more precisely place JIL-1-mediated H3S10 phosphorylation into currently understood steps of the transcription process, we examined the presence of phosphorylated H3S10 in mutants that abrogate transcription at two distinct steps by disrupting key components of chromatin remodeling complexes. The *brahma* (*brm*) gene encodes an ATPase that is part of the SWI/SNF remodeling complex. When a dominant-negative form of BRM (UAS*brm*^{K804R}) is expressed in salivary glands, levels of Pol IIo^{ser2} and Pol IIa are dramatically reduced, suggesting that chromatin remodeling by the BRM complex is required at an early step in transcription initiation (Armstrong et al. 2002). A synergistic genetic interaction between *JIL-1*^{z2} and mutations in the *trx-G* genes *brm* and *trithorax* (*trx*) has been reported previously (Zhang et al. 2003), suggesting that JIL-1 functions in the same pathway. We compared phosphorylated H3S10 levels on polytene chromosomes derived from wild-type larvae (Fig. 7A) with those derived from salivary glands that express *brm*^{K804R} (Fig. 7B). The loss of normal BRM function dramatically reduces levels of phosphorylated H3S10 while levels of Su(Hw), a chromatin-associated protein used as a control, are maintained. Western blot analysis performed on protein samples derived from wild-type versus *brm*^{K804R} salivary glands using anti-H3pS10 antibodies further shows that overall levels of phosphorylated H3S10 are reduced in *brm* mutants (Fig. 6D). These results are consistent with a role for JIL-1-mediated H3S10 phosphorylation at a later step in transcription and suggest that the modification is dependent on chromatin remodeling by the BRM complex.

Histone H3S10 phosphorylation is not affected in kismet *mutants*

Kismet-L (KIS-L) is another transcriptional regulator that exhibits *trx-G* phenotypes. It contains an ATPase domain that is highly related to that of BRM and has been proposed to act as the catalytic subunit of a novel chro-

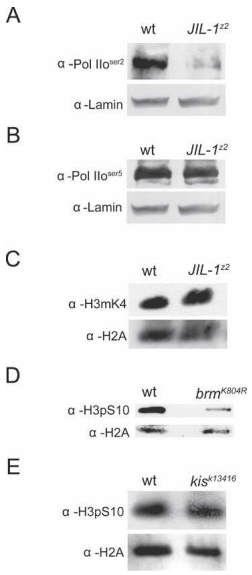

Figure 6. Analysis of Pol II and histone modifications in wild-type (wt) and mutant larvae. Western analysis of the following: (*A*) Pol IIo^{ser2} levels in wild-type and *JIL-1*^{z2} larvae; lamin was used as loading control. (*B*) Pol IIo^{ser5} levels in wild-type and *JIL-1*^{z2} larvae; lamin was used as loading control. (*C*) H3pS10 levels in wild-type and *brm*^{K804R} larvae; H2A was used as loading control. (*D*) H3 trimethylated K4 (H3mK4) levels in wild-type and *JIL-1*^{z2} larvae; H2A was used as loading control. (*E*) H3pS10 levels in wild-type and *kis*^{k13416} larvae; H2A was used as loading control.

matin remodeling complex required downstream from BRM. Disruption of the *kismet* gene (*kis*^{k13416}) leads to reduction in the elongating form of Pol II but not that of the initiating form, suggesting that KIS-L is required in an early step in transcription elongation (Srinivasan et al. 2005). To further characterize the step at which JIL-1 acts during transcription elongation, we examined the levels of phosphorylated H3S10 in *kis*^{k13416} mutants. Unlike the *brm*^{K804R} mutant, *kis*^{k13416} had no effect on H3S10 phosphorylation as visualized by immunofluorescence microscopy (Fig. 7C) when compared with wild type (Fig. 7A). These results were confirmed by Western blot analysis (Fig. 6E). These observations suggest either that H3S10 phosphorylation by JIL-1 acts upstream of KIS function or that the two chromatin regulators function in independent pathways.

Discussion

Eukaryotic transcription is a dynamic process that is regulated at many different levels. Intricate interactions between RNA polymerase and its chromatin environment are essential for proper progression through transcription initiation, elongation, and termination. Previous findings suggest a correlation between histone H3S10 phosphorylation and transcription activation of most genes in *Drosophila* (Nowak and Corces 2000; Labrador and Corces 2003). Results presented here establish H3S10 phosphorylation by JIL-1 as a key event during early elongation of transcription in *Drosophila*. JIL-1 appears to interact with the transcription machinery at most or all actively transcribed regions on *Drosophila* polytene chromosomes, including active ecdysone and heat-shock genes. At the same time, expression levels of the *hsp70* and *E75A* genes are decreased in *JIL-1*-null mutants. Importantly, when *JIL-1* is mutated, a global decrease in the phosphorylation levels of elongating RNA polymerase II is observed, suggesting that JIL-1 is required for transcription of the majority of genes.

The results further elucidate the timing of H3S10 phosphorylation within the framework of the cascade of events that lead to activation of transcription in eukaryotes (Fig. 8). Phosphorylation of H3S10 is not required for transcription factor recruitment, since loss of JIL-1 does not affect binding of HSF at the *hsp70* genes after heat shock. Also, H3S10 phosphorylation is dependent on BRM chromatin remodeling, which is required genome-wide prior to the recruitment of Pol II. Transcription initiation can take place independently of JIL-1, as shown by the normal levels of Pol IIo^{ser5} and H3K4 methylation in *JIL-1*^{z2} mutants, indicating that the chromatin environment in the absence of JIL-1 is still suitable for transcription initiation. However, productive elongation is impaired in these mutants, as is evident by the decrease in Pol IIo^{ser2} levels. These findings introduce H3S10 phosphorylation as a new component of an increasingly complex chromatin environment that is required at the onset of transcription elongation in *Drosophila*, suggesting a role for JIL-1 in the release of Pol II from promoter-proximal pausing and facilitation of early elongation. Specifically, in *JIL-1* mutants, P-TEFb is not detected at the induced *hsp70* genes while levels of DSIF are maintained. In the absence of P-TEFb, neither DSIF nor Pol II can be phosphorylated, which is sufficient to block productive elongation. It is likely that Pol II arrests in a paused state and cannot elongate in these mutants. It is also possible that Pol II continues to elongate but is unable to communicate with the proper mRNA processing machinery, which is normally contingent on Ser2 phosphorylation of its CTD (Orphanides and Reinberg 2002). In this case, the mRNA would be produced but quickly degraded, leading to the transcription defects observed in the Northern analyses. Further work is needed to distinguish between these two possibilities.

Although JIL-1 is required for transcription, its presence is not sufficient to ensure gene activation, since JIL-1 is present at all previously transcribed genes that are silenced after heat shock, whereas phosphorylated H3S10 is found exclusively at the transcriptionally active heat-shock genes (Nowak and Corces 2000). Nevertheless, recruitment of JIL-1 to the *hsp70* gene is transcription dependent. One possibility is that JIL-1 can exist in both active and inactive states. Once recruited to activate a gene, it may eventually be repressed by inactivation rather than disassociation. Alternatively, the net levels of phosphorylated H3S10 could result from a delicate balance between kinase and phosphatase activities. It has been proposed previously that phosphatase 2A

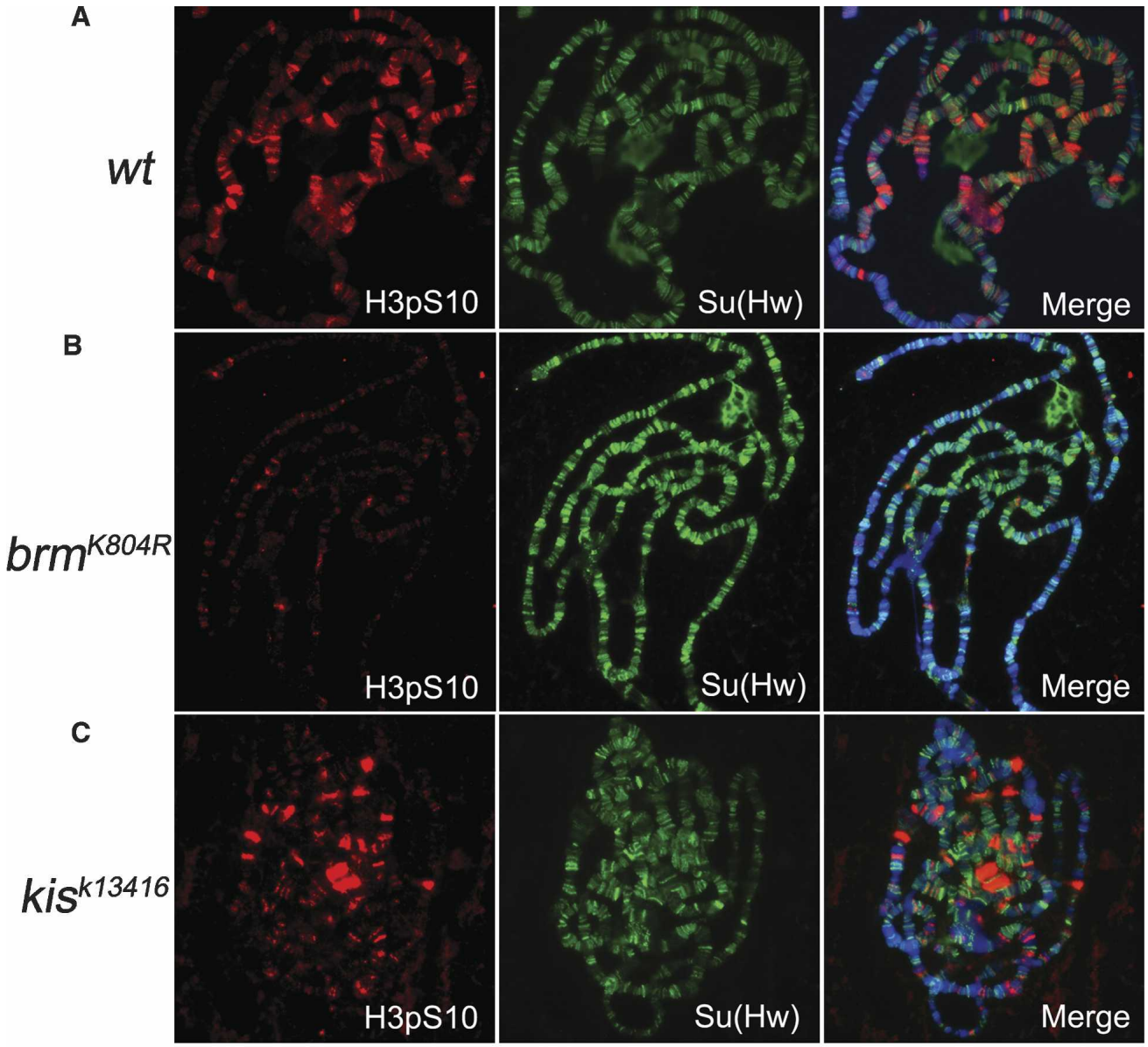

Figure 7. Histone phosphorylation requires BRM but not KIS chromatin remodeling factors. Immunolocalization of H3pS10 (red) and Su(Hw) (green) on polytene chromosomes from wild-type (*A*), brm^{K804R} (*B*), or kis^{k13416} (*C*) third instar larvae. DNA is stained with DAPI (blue) in all merge panels.

(PP2A) plays a major role in transcription-dependent H3S10 phosphorylation (Nowak et al. 2003). Therefore, even if JIL-1 is actively maintained at silent genes, its action may be counterbalanced by PP2A. Further studies are required to shed light on how JIL-1 activity can be regulated to affect transcription.

In vertebrates, phosphorylation of H3S10 seems to be limited to transcription activation of specific genes in the context of particular signal transduction pathways (Mahadevan et al. 1991; DeManno et al. 1999; Sassone-Corsi et al. 1999; Crosio et al. 2000, 2003; Yamamoto et al. 2003; Vicent et al. 2006). In fact, activation of the *hsp70* genes by different stressors in mammalian cells is associated with distinct signaling pathways that are not always linked to H3S10 phosphorylation. Contrary to the *Drosophila* response, heat shock elicits histone H4 acetylation instead of H3S10 phosphorylation at the *hsp70* loci in mouse fibroblasts. On the other hand, both H3S10 phosphorylation and H4 acetylation are detected at the *hsp70* genes upon arsenite treatment of the same cells (Thomson et al. 2004). Therefore, mammals appear to have more diverse mechanisms of transcription activation and may partially rely on H3S10 phosphorylation in a context-dependent manner. In yeast, substituting the H3 Ser10 for an Ala prevents the recruitment of the TATA-binding protein to the *INO1* and *GAL1* gene pro-

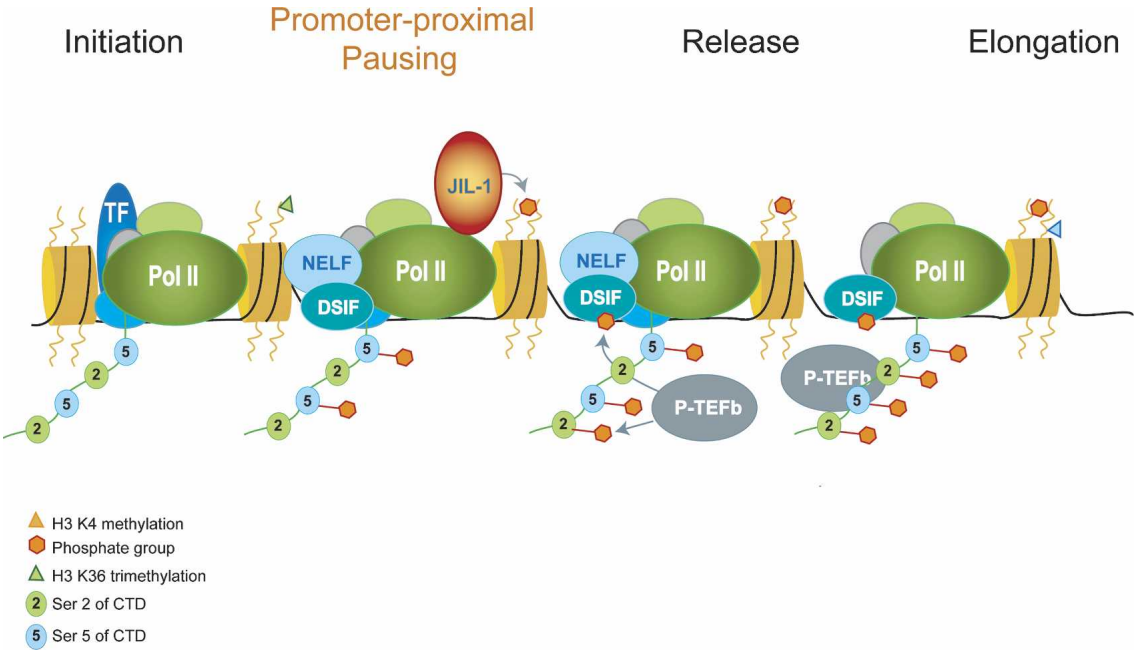

Figure 8. The role of JIL-1 in transcription elongation. The diagram indicates the time during the transcription process at which JIL-1 and various other factors are thought to act. JIL-1 acts during promoter-proximal pausing after phosphorylation of Ser5 in the CTD of Pol II and recruitment of the complex NELF–DSIF. Phosphorylation of the CTD on Ser2 by P-TEFb and release of Pol II from the halt are dependent on JIL-1. See the text for details.

moters, suggesting that H3S10 phosphorylation is required for the assembly of the preinitiation complex (Lo et al. 2005). It would be interesting to explore the significance of this apparent diversity across species.

The results presented here shed light on the mechanism of transcription regulation by H3S10 phosphorylation. It has been recently shown that H3S10 phosphorylation antagonizes the binding of the heterochromatin protein HP1 to histone H3 methylated in Lys9 (H3K9) during mitosis in mammalian cells (Fischle et al. 2005; Hirota et al. 2005). It was consequently proposed that JIL-1 maintains chromosome structure in *Drosophila* by counteracting heterochromatin formation and preventing its spreading into euchromatin (Zhang et al. 2006). This model for JIL-1 activity could explain a lack of transcription in *JIL-1*^{z2} mutants, since any ectopic heterochromatin would make the DNA inaccessible to the Pol II machinery. However, contrary to such a prediction, our results show that heat-shock puffs are still formed in *JIL-1*^{z2}mutants, and transcription factors and the Pol II machinery retain the ability to bind despite the disruption of chromatin structure. Furthermore, transcription can be initiated, as is evident by the phosphorylation of Pol II at Ser5. This requires several components of the core transcription machinery and the procession of Pol II a few bases downstream from the promoter. These results suggest that, rather than contribute to global chromosome structure, JIL-1-mediated H3S10 phosphorylation may be required to maintain a local chromatin environment that serves as a platform for the recruitment of P-TEFb and the consequent release of Pol II from promoter-proximal pausing.

It has become increasingly evident that transcription elongation is a rate-limiting step of gene expression that requires tight regulation (Saunders et al. 2006). It was reported recently that the majority of gene promoters in human embryonic stem cells are occupied by a promoter-proximally paused Pol II, poised for productive elongation (Guenther et al. 2007). This suggests that the expression of these genes is predominantly regulated at the level of Pol II release rather than during preinitiation. The exact mechanism of P-TEFb recruitment, a key step in this process, remains to be determined. Several transcription regulators have been shown to recruit P-TEFb (Jang et al. 2005; Yang et al. 2005; Guiguen et al. 2007), but this is the first evidence of a histone modification required precisely at the timing of recruitment.

The exact contribution of H3S10 phosphorylation to P-TEFb recruitment remains open to further investigation. Recent reports have shown that the ubiquitous protein 14–3–3 binds to H3 only when phosphorylated at Ser10, and this interaction could provide a mechanistic link between H3S10 phosphorylation and P-TEFb (Macdonald et al. 2005). It is possible that 14–3–3 interacts with P-TEFb directly or indirectly through other transcription regulators that are known to recruit it. Alternatively, 14–3–3 is known to interact with many chromatin-related proteins (Tzivion et al. 2001), thus providing another avenue to manipulate the local chromatin environment to support P-TEFb recruitment and early elongation. Further analyses will be necessary to test these hypotheses and clarify the role and mechanism of regulation of JIL-1 and H3S10 phosphorylation in gene expression.

Materials and methods

Drosophila *stocks*

Stocks were maintained in standard medium at 23°C. Oregon R larvae were used for wild-type controls in all experiments. The *JIL-1^{z2}* stock was a gift from Dr. K. Johansen (Iowa State University, Ames, IA). The *brm^{K804R}* and *kis^{k13416}* stocks were a gift from Dr. J. Tamkun (University of California at Santa Cruz, Santa Cruz, CA).

Induction of the heat-shock response and analysis of polytene chromosomes

Salivary gland polytene chromosome squashes were prepared from wandering third instar larvae maintained at 23°C. For heat-shock experiments, third instar wild-type and *JIL-1^{z2}* mutant larvae were subjected to heat-shock treatment as described previously (Nowak et al. 2003). Salivary glands were dissected in 0.7% NaCl and fixed for 2 min in 45% acetic acid and 1.85% formaldehyde. Fixed salivary glands were subsequently squashed in 45% acetic acid on subbed slides. The slides were frozen in liquid nitrogen and stored dry at –70°C. For immunostaining of H3S10 phosphorylation, salivary glands were fixed for 2 min in 15% acetic acid and 1.85% formaldehyde. Slides were incubated overnight at 4°C in antibody dilution buffer (PBS, 0.1% Triton X-100, 1% BSA) containing primary antibodies at concentrations of 1:30 chicken α-JIL-1 (gift from Dr. K. Johansen), 1:20 rabbit α-H3pS10 (Upstate Biotechnology), 1:30 mouse α-Pol IIo^{ser2} (H5, Covance), 1:30 mouse α-Pol IIo^{ser5} (H14, Covance), 1:50 rabbit α-HSF (gift from Dr. C. Wu, National Cancer Institute, Bethesda, MD), 1:50 rabbit α-SPT5 (gift from Dr. F. Winston, Harvard Medical School, Boston, MA), 1:20 rabbit α-CycT (gift from Dr. D. Price, University of Iowa, Iowa City, IA; affinity-purified as described in Lis et al. 2000), and 1:150 rat α-Su(Hw). Following incubation, slides were washed three times in PBS and 0.1% Triton X-100 and incubated for 1 h at 37°C in the appropriate secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:200 in antibody dilution buffer. Slides were washed three times as described above, stained with 0.5 µg/mL 4′,6-diaminidine-2-phenylindole (DAPI), and mounted in Vectashield mounting medium (Vector Laboratories) for viewing.

Preparation of Drosophila *protein extracts and Western analysis*

Protein extracts from third instar larvae were prepared by lysing the anterior tissues, containing salivary glands, brains, and imaginal discs, in sodium dodecyl sulfate (SDS) sample buffer (0.175 M Tris-HCl, 5% SDS, 15% glycerol, 0.0005% bromophenol blue, 4.95 mM β-mercaptoethanol) using a Dounce homogenizer. Following homogenization, protein samples were immediately run on SDS–polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes for immunodetection. Membranes were incubated overnight at 4°C in antibody dilution buffer (PBS, 0.05% Tween, 5% milk) containing primary antibodies at concentrations of 1:100 mouse α-DDA2.7 EcR common (Developmental Studies Hybridoma Bank), 1:1000 mouse α-Pol IIo^{ser2} (H5, Covance), 1:500 mouse α-Pol IIo^{ser5} (H14, Covance), 1:1000 rabbit α-H3pS10 (Upstate Biotechnology), 1:1000 rabbit α-Lys4 trimethylated histone H3 (Upstate Biotechnology), 1:1000 rabbit α-histone H2A (Upstate Biotechnology), and 1:1000 mouse α-lamin (Risau et al. 1981). After incubation, the membranes were washed twice with PBS and 0.25% Tween, incubated for 1 h at room temperature in the appropriate HRP secondary antibody (Jackson ImmunoResearch Laboratories), and washed twice with PBS and 0.25% Tween. Antibody signal was visualized using chemiluminescence detection methods (SuperSignal West Pico kit, Pierce).

Preparation of Drosophila *RNA and Northern analysis*

Total RNA was isolated from 20 wild-type or *JIL-1^{z2}* third instar larvae and prepupae using RNA-Bee (Tel-Test, Inc.) according to the manufacturer's instructions. RNA samples (20 µg per lane) were denatured in MOPS/formaldehyde/formamide buffer and separated by electrophoresis on a 1% formaldehyde/agarose gel at low voltage. Following electrophoresis, RNA was transferred to a Nytran supercharge membrane (Scheleicher and Schuell) by capillary blotting overnight in 10× SSC. Resolved RNAs were cross-linked to the membrane by UV irradiation and incubated in hybridization solution (Ultrahyb, Ambion) for 1 h at 45°C before addition of the probe. Radiolabeled DNA probes were prepared using the Prime-A-Gene random priming kit (Promega). Labeled probes were purified using MicroSpin G-50 columns (Amersham), added to the hybridization mixture, and incubated overnight at 45°C. The blots were then washed using solutions containing decreasing concentrations of SDS and SSC (1× SSC is 0.15 M NaCl, 0.015M sodium citrate). Transcripts were visualized by autoradiography or PhosphorImager. All probes were generated from amplified genomic *Drosophila* DNA. A probe specific for *hsp70* mRNA was generated using the following pair of primers: Hsp70 IIIA (5′-TGGTGCTGAC CAAGATGAAG-3′) and Hsp70 IIIB (5′-TAGTCTGCTTGCAC GGAATG-3′). A probe specific for the *E75A* mRNA isoform was generated using the following pair of primers: E75A-1 (5′-CATTGACTAACTGCCACTCGCA-3′) and E75A-2 (5′-CAA CACTGCAGTGGGACCATCG-3′). A probe against *rp49* mRNA was synthesized using primers rp49 I (5′-CCCAAGGGTATC GACAACAG-3′) and rp49 II (5′-ATGGTGCTGCTATCCC AATC-3′). Membranes used to analyze expression of the *E75A* and *hsp70* genes were stripped in 1% SDS boiling solution for 30 min and rehybridized with *rp49* probe as a loading control.

DNA in situ hybridization

Digoxigenin-labeled DNA probes were prepared using the Prime-A-Gene random priming kit using a mixture of deoxyribonucleotides containing DIG-11-dUTP (Roche). The reactions were incubated overnight at room temperature. The template used to generate the probe was synthesized as described above. Labeled probes were precipitated and stored in hybridization buffer (4× SSC, 1× Denhardt's, 0.4 mg/mL sonicated salmon sperm, 50% formamide) until ready for use. Salivary glands were dissected in 0.7% NaCl, transferred for 30 sec to 45% acetic acid, and then fixed for 2 min in 1 vol lactic acid/2 vol water/3 vol acetic acid. Fixed salivary glands were squashed as described above, and the slides were left overnight at room temperature. Slides were then frozen in liquid nitrogen, the coverslips were removed, and the slides were dehydrated in 70% ethanol for 5 min and incubated twice 5 min in 95% ethanol. The slides were stored at room temperature until ready for use. Slides were incubated in 2× SSC for 60 min at 65°C, dehydrated through an ethanol series, air-dried, and denatured for 2 min in 70 mM NaOH. Following denaturation, the slides were dehydrated again as described above and air-dried at room temperature prior to hybridization. For hybridization, boiled probes were added to the dried, denatured polytene chromosome squashes and covered with a coverslip. The slides and coverslips were sealed with rubber cement and incubated overnight at 37°C in a humidified chamber. Following hybridization, cover-

slips were removed and the slides were washed twice for 10 min at 37°C in 2× SSC, twice for 5 min at room temperature in 1× PBS, 2 min in PBS/0.1% Triton X-100, and 5 min in 1× PBS. After washing, immunocytochemical treatment was carried out as described above.

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