RNA polymerase II-mediated transcription at active loci does not require histone H3S10 phosphorylation in *Drosophila*

Weili Cai, Xiaomin Bao, Huai Deng, Ye Jin, Jack Girton, Jørgen Johansen and Kristen M. Johansen*

JIL-1 is the major kinase controlling the phosphorylation state of histone H3S10 at interphase in *Drosophila*. In this study, we used three different commercially available histone H3S10 phosphorylation antibodies, as well as an acid-free polytene chromosome squash protocol that preserves the antigenicity of the histone H3S10 phospho-epitope, to examine the role of histone H3S10 phosphorylation in transcription under both heat shock and non-heat shock conditions. We show that there is no redistribution or upregulation of JIL-1 or histone H3S10 phosphorylation at transcriptionally active puffs in such polytene squash preparations after heat shock treatment. Furthermore, we provide evidence that heat shock-induced puffs in JIL-1 null mutant backgrounds are strongly labeled by antibody to the elongating form of RNA polymerase II (Pol II\(^{\text{ser2}}\)), indicating that Pol II\(^{\text{ser2}}\) is actively involved in heat shock-induced transcription in the absence of histone H3S10 phosphorylation. This is supported by the finding that there is no change in the levels of Pol II\(^{\text{ser2}}\) in JIL-1 null mutant backgrounds compared with wild type. mRNA from the six genes that encode the major heat shock protein in *Drosophila*, Hsp70, is transcribed at robust levels in JIL-1 null mutants, as directly demonstrated by qRT-PCR. Taken together, these data are inconsistent with the model that Pol II-dependent transcription at active loci requires JIL-1-mediated histone H3S10 phosphorylation, and instead support a model in which transcriptional defects in the absence of histone H3S10 phosphorylation are a result of structural alterations of chromatin.

KEY WORDS: Pol II, Histone H3S10 phosphorylation, Transcription, Chromatin structure, JIL-1 kinase

INTRODUCTION

The JIL-1 tandem kinase in *Drosophila* localizes specifically to euchromatic interband regions of polytene chromosomes and is the predominant kinase controlling the phosphorylation state of histone H3S10 at interphase (Wang et al., 2001). JIL-1 is essential for viability and reduced levels of JIL-1 protein lead to a global disruption of chromosome structure (Jin et al., 2000; Wang et al., 2001; Zhang et al., 2003; Deng et al., 2005) as well as to extensive ectopic spreading of heterochromatic factors (Zhang et al., 2006). These findings suggested a model in which maintenance of histone H3S10 phosphorylation levels at euchromatic chromatin regions is necessary to counteract heterochromatization and gene silencing (Wang et al., 2001; Ebert et al., 2004; Zhang et al., 2006; Bao et al., 2007).

Recently, based on analyses of transcriptionally active regions during the heat shock response (Nowak and Corces, 2000; Nowak et al., 2003; Ivaldi et al., 2007), an alternative model was proposed in which JIL-1 is required for transcription by the RNA polymerase II (Pol II) machinery (Ivaldi et al., 2007). According to this model, rather than contributing to global chromosome structure, JIL-1-mediated histone H3S10 phosphorylation maintains a local chromatin environment that serves as a platform for the recruitment of the Positive transcription elongation factor b (P-TEFb; Cdk9 – FlyBase) and the consequent release of Pol II for the recruitment of the Positive transcription elongation factor. JIL-1-mediated histone H3S10 phosphorylation is sufficient to induce a change in higher-order chromatin structure from a condensed heterochromatin-like state to a more open, euchromatic state, and that these changes are not associated with enhanced transcriptional activity (Deng et al., 2008). Thus, these findings are incompatible with the transcriptional elongation model for JIL-1 function and we therefore attempted to repeat the experiments on which it is based using three different histone H3S10 phosphorylation (H3S10ph) antibodies, as well as a newly developed acid-free polytene chromosome squash technique (DiMario et al., 2006) that preserves the antigenicity of the H3S10 phospho-epitope. We show that many of the key findings of Nowak and Corces (Nowak and Corces, 2000), Nowak et al. (Nowak et al., 2003) and Ivaldi et al. (Ivaldi et al., 2007) are likely to be artifacts caused by non-specific antibody cross-reactivity and by fixation procedures that are not suitable for reliable antibody detection of interphase phosphorylated histone H3S10. Taken together, the results of Deng et al. (Deng et al., 2007; Deng et al., 2008) and the findings presented here are inconsistent with the
model of Ivaldi et al. (Ivaldi et al., 2007) that Pol II-dependent transcription at active loci requires JIL-1-mediated histone H3S10 phosphorylation, and instead support a model in which transcriptional defects in the absence of histone H3S10 phosphorylation are the result of structural alterations of chromatin.

MATERIALS AND METHODS

Drosophila melanogaster stocks and heat shock induction

Fly stocks were maintained at 23°C according to standard protocols (Roberts, 1998). Canton-S was used for wild-type preparations. The JIL-1<sup>-2</sup> null allele has been described (Wang et al., 2001; Zhang et al., 2003), as has the recombined JIL-1<sup>-2</sup> Su(var)-3<sup>96</sup> chromosome (Deng et al., 2007). The P-element insertion mutant allele tws<sup>02414</sup> was obtained from the Bloomington Stock Center and the tws<sup>o</sup> allele (Mayer-Jaekel et al., 1993) was the generous gift of Dr D. M. Glover (University of Cambridge, Cambridge, UK). Balanced chromosomes and markers have been described (Lindsley and Zimm, 1992). For heat shock experiments, wandering third instar larvae were subjected to 25 minutes of heat shock treatment at 37°C as described previously (Nowak et al., 2003).

Immunohistochemistry

Salivary gland nuclei smush preparations were made as described (Wang et al., 2001), standard polytene chromosome squash preparations were made (Kelley et al., 1999) using the 5-minute fixation protocol, and acid-free squash preparations were performed the procedure of DiMario et al. (DiMario et al., 2006). Antibody labeling of these preparations was performed as described (Jin et al., 1999; Wang et al., 2001). Primary antibodies used include rabbit anti-H3S10ph (Epitomics, Upstate and Cell Signaling), mouse anti-H3S10ph (Upstate and Cell Signaling), rabbit anti-histone H3 (Cell Signaling), mouse anti-lamin Db (Grunenbaum et al., 1988), rabbit anti-Hsf (gift from Dr C. Wu, National Cancer Institute, Bethesda, MD), mouse anti-Pol II<sup>n</sup> (HS, Covance), rabbit anti-JIL-1 (Jin et al., 1999, chicken anti-JIL-1 (Jin et al., 2000), and anti-JIL-1 mAb SC9 (Jin et al., 2000). DNA was visualized by staining with Hoechst 33258 (Molecular Probes) in PBS. The appropriate species- and isotype-specific Texas Red–, TRITC– and FITC– conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used for experimental use at interphase to avoid artifacts. In this study, we have adapted the ‘smush’ preparation of Drosophila third instar salivary gland nuclei as a rapid and sensitive screening procedure for such antibodies. The smush preparation is a modified whole-mount staining technique in which nuclei from dissected salivary glands are gently compressed beneath a coverslip to flatten them before fixation in a standard paraformaldehyde/PBS solution of physiological pH (Wang et al., 2001). The procedure takes advantage of the finding that JIL-1 is the kinase responsible for interphase histone H3S10 phosphorylation levels and distribution. Consequently, it is important to verify the specificity and suitability of these antibodies for experimental use at interphase to avoid artifacts. In this study, we have adapted the ‘smush’ preparation of Drosophila third instar salivary gland nuclei as a rapid and sensitive screening procedure for such antibodies. The smush preparation is a modified whole-mount staining technique in which nuclei from dissected salivary glands are gently compressed beneath a coverslip to flatten them before fixation in a standard paraformaldehyde/PBS solution of physiological pH. The aim of this study was to examine the role of histone H3S10 phosphorylation in transcriptional regulation in Drosophila. Many of the commercially available antibodies to this histone modification have been mostly used as a marker for mitotic chromosomes (Hendzel et al., 1997; Wei et al., 1998) and, as a result, they are poorly characterized with regard to detection of interphase histone H3S10 phosphorylation levels and distribution. Consequently, it is important to verify the specificity and suitability of these antibodies for experimental use at interphase to avoid artifacts. In this study, we have adapted the ‘smush’ preparation of Drosophila third instar salivary gland nuclei as a rapid and sensitive screening procedure for such antibodies. The smush preparation is a modified whole-mount staining technique in which nuclei from dissected salivary glands are gently compressed beneath a coverslip to flatten them before fixation in a standard paraformaldehyde/PBS solution of physiological pH (Wang et al., 2001). The procedure takes advantage of the finding that JIL-1 is the kinase responsible for interphase histone H3S10 phosphorylation levels and distribution. Consequently, it is important to verify the specificity and suitability of these antibodies for experimental use at interphase to avoid artifacts. In this study, we have adapted the ‘smush’ preparation of Drosophila third instar salivary gland nuclei as a rapid and sensitive screening procedure for such antibodies. The smush preparation is a modified whole-mount staining technique in which nuclei from dissected salivary glands are gently compressed beneath a coverslip to flatten them before fixation in a standard paraformaldehyde/PBS solution of physiological pH (Wang et al., 2001). The procedure takes advantage of the finding that JIL-1 is the kinase responsible for interphase histone H3S10 phosphorylation levels and distribution. Consequently, it is important to verify the specificity and suitability of these antibodies for experimental use at interphase to avoid artifacts. In this study, we have adapted the ‘smush’ preparation of Drosophila third instar salivary gland nuclei as a rapid and sensitive screening procedure for such antibodies. The smush preparation is a modified whole-mount staining technique in which nuclei from dissected salivary glands are gently compressed beneath a coverslip to flatten them before fixation in a standard paraformaldehyde/PBS solution of physiological pH.
In this study, to overcome these difficulties we have adopted the acid-free squash technique of DiMario et al. (DiMario et al., 2006), which was originally developed to preserve the fluorescence of GFP-tagged proteins in fixed preparations. As illustrated in Fig. 2A-C, this technique also preserves the antigenicity of the H3S10ph phospho-epitope as indicated by the robust antibody labeling in both male and female squash preparations by three different H3S10ph antibodies, including upregulation on the male X chromosome. The extensive co-localization of H3S10ph with JIL-1 is particularly evident in the confocal images in Fig. 2A. Furthermore, on immunoblots of protein extracts from third instar larval salivary glands, H3S10ph labeling was greatly reduced in JIL-1 null mutant backgrounds confirming that the antibodies recognized the H3S10ph epitope. However, it should be noted that the Epitomics H3S10ph antibody, in contrast to the other two antibodies, showed strong labeling of the chromocenter (Fig. 2B, asterisks). Although it was more difficult to properly spread the chromosomes and the chromatin structure, as labeled by Hoechst, was slightly less well-preserved in acid-free squashes than in conventional squash preparations, our data strongly suggest that the acid-free squash procedure is the method of choice in all antibody labeling studies of histone H3S10 phosphorylation in polytene squash preparations.

A limitation of the smush procedure is that the visualization of chromatin structure and bands is inferior to the normal squash technique. However, as previously reported (Wang et al., 2001), the highly acidic fixation conditions of conventional squash protocols (Zink and Paro, 1989; Kelley et al., 1999) prevent reliable antibody labeling of the histone H3S10 phospho-epitope. In such preparations, H3S10ph antibody labeling is extremely weak and, except for rare cases, the upregulation of H3S10ph phosphorylation on the male X chromosome (Wang et al., 2001) (data not shown) cannot be detected, indicating incomplete or defective antibody recognition. In this study, to overcome these difficulties we have adopted the acid-free squash technique of DiMario et al. (DiMario et al., 2006), properties. Of the suitable antibodies, the rabbit mAb from Epitomics, the rabbit pAb (lots 2 and 3) from Cell Signaling, and the rabbit pAb (lot 32219) from Upstate, were selected for further use in the present studies.

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Table 1. Properties of histone H3S10ph antibodies

<table>
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<tr>
<th>Antibody</th>
<th>Lot No.</th>
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<th>Smush</th>
<th>Squash</th>
<th>Note</th>
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<tr>
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<td>C03143</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Centromere labeling</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>Labels heat shock puffs</td>
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<tr>
<td>Upstate Rb pAb</td>
<td>32219</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Labels heat shock puffs</td>
</tr>
<tr>
<td>Upstate Mo mAb</td>
<td>26436</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Cell Signaling Rb pAb</td>
<td>Lot 1</td>
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<td>+/–</td>
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<tr>
<td>Cell Signaling Rb pAb</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>Cell Signaling Rb pAb</td>
<td>Lot 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Cell Signaling Mo mAb</td>
<td>Lot 5</td>
<td>–</td>
<td>+/–</td>
<td>ND</td>
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</tbody>
</table>

Rb, rabbit; Mo, mouse. +, positive; +/-, weakly positive; –, negative; ND, not determined.

JIL-1 and histone H3S10 phosphorylation are not upregulated at transcriptionally activated loci during heat shock

To determine the distribution of JIL-1 before and after heat shock, we double labeled polytene chromosome squash preparations with the JIL-1 mAb 5C9 and with antibody to the elongating form of RNA polymerase II (Pol II$_{ser2}$), which is phosphorylated at serine 2 in the C-terminal domain and which serves as a marker for active transcription (Weeks et al., 1993; Boehm et al., 2003; Ivaldi et al., 2007). In non-heat shock preparations, both JIL-1 and Pol II$_{ser2}$ were localized to a large number of euchromatic interband regions (Fig. 3A, left panel). The composite image in Fig. 3A (left panel) further shows that although there may be some co-localization between JIL-1 and Pol II$_{ser2}$ as previously reported (Ivaldi et al., 2007), relatively low levels of JIL-1 were observed at many sites where there were especially high levels of Pol II$_{ser2}$ staining, such as at developmental puffs. After 25 minutes of heat shock treatment, there was a striking change in the distribution of Pol II$_{ser2}$ labeling, whereas, by contrast, there was no appreciable redistribution of JIL-1. The Pol II$_{ser2}$ labeling was reduced at most sites, while being upregulated at heat shock puffs where transcription of heat shock-activated genes was occurring. This was especially prominent at the heat shock loci 87A/C and 93D (Fig. 3B). Notably, there were no indications of a concomitant upregulation of JIL-1 at these sites (Fig. 3B). This result was confirmed using two other JIL-1 antibodies (a chicken pAb and a rabbit pAb; data not shown).
We next used the acid-free polytene squash technique to determine the distribution of H3S10ph before and after heat shock using three different H3S10ph antibodies (from Epitomics, Cell Signaling and Upstate). To mark heat shock puffs and other regions of enhanced transcription, the preparations were double labeled with antibody to Pol IIoser2. As illustrated in Fig. 4A-D, there was no obvious change in H3S10ph distribution before and after heat shock as detected by the Epitomics and Cell Signaling H3S10ph antibodies. Importantly, as also observed for JIL-1 (Fig. 3B), there was no upregulation at the 87A/C heat shock puffs, although they were robustly labeled by the Pol IIoser2 antibody (Fig. 4B,D). By contrast, we found that the Upstate H3S10ph antibody strongly labeled the 87A/C puffs after heat shock (Fig. 4G,F). However, contrary to the Cell Signaling and Epitomics H3S10ph antibodies, the Upstate pAb also labeled heat shock puffs in polytene chromosome squashes from JIL-1 null mutant larvae, which are devoid of histone H3S10ph phosphorylation (Fig. 5). Because similar results were obtained with two different lots of the Upstate H3S10ph pAb (Table 1), we conclude that the labeling of heat shock puffs by this antibody is due to non-specific cross-reactivity, possibly with proteins involved in the heat shock response. Furthermore, immunoblot analysis with all three H3S10ph antibodies of protein extracts from salivary glands before and after heat shock confirmed that there was no change in the overall level of histone H3S10 phosphorylation (Fig. 4G) as indicated by the polytene squash labelings (Fig. 4A,C,E). By contrast, there was a clear downregulation in the levels of Pol IIoser2 in response to heat shock (Fig. 4H).

Previously, evidence has been presented that Protein phosphatase 2A [PP2A; Twins (Tws) – FlyBase] activity might regulate histone H3S10 phosphorylation at interphase (Nowak et al., 2003). Using a P-element insertion mutation into the regulatory subunit of PP2A, twsP, that causes reduced catalytic activity (Mayer-Jaekel et al., 1993), Nowak et al. showed that on immunoblots of extracts from twsP mutant larvae, there is a higher level of H3S10 phosphorylation than in wild-type larvae (Nowak et al., 2003). This difference was attributed to reduced PP2A phosphatase activity, indicating that PP2A might function as a H3S10ph phosphatase at interphase (Nowak et al., 2003) in addition to its role as a mitotic H3S10ph phosphatase (Mayer-Jaekel et al., 1993). However, because whole larval extracts were used it remained a possibility that the increased upregulation of H3S10ph levels was due solely to decreased dephosphorylation of mitotic H3S10ph. Using a likely null PP2A regulatory subunit P-element mutation, tws02414, we confirmed a higher level of H3S10 phosphorylation in extracts from homozygous tws02414 mutant larvae as compared with wild-type larvae (Fig. 6A). However, when extracts were compared from salivary glands, which do not contain mitotic cells, there was no difference (Fig. 6B). Furthermore, in extracts from salivary glands of homozygous twsP mutant larvae with or without heat shock there
was no difference in H3S10 phosphorylation levels as detected by the Epitomics H3S10ph mAb (Fig. 6C). Taken together, these results indicate that the PP2A phosphatase might play a role in H3S10 dephosphorylation only at mitosis and not at interphase.

**JIL-1 and H3S10 phosphorylation are not required for transcription at active loci during heat shock**

Deng et al. have recently provided evidence that the lethality as well as some of the chromosome defects associated with the JIL-1 null phenotype can be substantially rescued by reducing the dose of the Su(var)3-9 gene (Deng et al., 2007). This suggests that the Pol II transcriptional machinery has the capacity to function more or less normally in the complete absence of JIL-1-mediated interphase histone H3S10 phosphorylation. We therefore investigated the distribution of Pol II\(\text{ser}^{2}\) labeling and heat shock-induced transcription in JIL-1\(^{+/+}\)/JIL-1\(^{w}w^{+}\) null as well as in JIL-1\(^{+/+}\)/JIL-1\(^{w}w^{+}\) Su(var)3-9\(^{06}\) double mutant backgrounds. In JIL-1\(^{+/+}\)/JIL-1\(^{w}w^{+}\) Su(var)3-9\(^{06}\) larvae, the adult eclosion rate increases to 60% of that of wild-type larvae as compared with 0% for JIL-1\(^{+/+}\)/JIL-1\(^{w}w^{+}\) null larvae (Deng et al., 2007). Fig. 7A shows robust antibody labeling of Pol II\(\text{ser}^{2}\) in polytene chromosome squashes from both genotypes, even though the chromatin structure was greatly perturbed. This included the characteristic ‘puffed’ male X chromosome in JIL-1 null larvae (Fig. 7A, upper panel). Furthermore, on immunoblots of extracts from wild-type, JIL-1\(^{+/+}\)/JIL-1\(^{w}w^{+}\), and JIL-1\(^{+/+}\)/JIL-1\(^{w}w^{+}\) Su(var)3-9\(^{06}\) salivary glands, there was no detectable difference in Pol II\(\text{ser}^{2}\) levels (Fig. 7B). This indicates that transcript elongation by the Pol II machinery is likely to be functional in JIL-1 null mutant backgrounds. To further investigate this possibility, we double labeled JIL-1 mutant polytene chromosome squashes, after they were heat shocked, with Pol I\(\text{I}^{\text{ser}^{2}}\) antibody and with antibody to the heat shock transcription factor Hsf (Fig. 8A). When inactive, Hsf is diffusely distributed at very low levels; however, following heat shock, Hsf redistributes very prominently to heat shock-induced puffs (Westwood et al., 1991; Ivaldi et al., 2007). As shown in Fig. 8A, although the chromosome morphology was greatly disrupted, puffed regions could be clearly identified in JIL-1 null mutant backgrounds as defined by the presence of decondensed chromatin and strong Hsf antibody labeling. Importantly, these heat shock-induced puffs were also strongly labeled by Pol II\(\text{ser}^{2}\) antibody in a pattern coincident with that of the Hsf antibody (Fig. 8A, arrows). Furthermore, as also confirmed by immunoblot analysis (Fig. 8B), Pol II\(\text{ser}^{2}\) levels were greatly reduced at non-heat shock sites (Fig. 8A). This suggests that Pol II\(\text{ser}^{2}\) is actively involved in heat shock-induced transcription in JIL-1 null mutants. To test this directly, we used qRT-PCR to measure the transcription of the six, nearly identical, genes that encode Hsp70, the major heat shock protein in *Drosophila* (Gong and Golic, 2004), under heat shock and non-heat shock conditions. Primers were designed that would amplify transcripts from all six Hsp70 genes, and primers specific to the gene encoding the ribosomal non-heat-shock-sensitive protein Rp49 [Ribosomal protein L32 (RpL32) – FlyBase] were used for normalization. We performed two independent experiments in which total RNA was isolated from wild-type, JIL-1\(^{+/+}\)/JIL-1\(^{w}w^{+}\), and JIL-1\(^{+/+}\)/JIL-1\(^{w}w^{+}\) Su(var)3-9\(^{06}\) third instar larvae, and in which qRT-PCR determination of transcript levels was performed in duplicate. As illustrated in Fig. 8C, very low levels of Hsp70 mRNA transcripts were detected in both wild-type and JIL-1 mutant backgrounds under non-heat
shock conditions. However, a robust increase in Hsp70 mRNA transcript levels, relative to rp49 transcript levels, was detected in response to heat shock treatment in all three genotypes (Fig. 8C). The increase in JIL-1^{f2}/JIL-1^{f2} null mutant larvae was at least two orders of magnitude greater than under non-heat shock conditions, although this response was only about one-third that observed in wild-type larvae. Interestingly, the heat shock-induced increase in Hsp70 mRNA levels was enhanced considerably, to almost two-thirds of wild-type levels, in larvae in which Su(var)3-9 levels were reduced by half (e.g. JIL-1^{f2}/JIL-1^{f2} Su(var)3-9^{06} larvae).

**DISCUSSION**

A number of studies have suggested that the regulation of early stages of transcriptional elongation might be a relatively common phenomenon in higher eukaryotes (reviewed by Hartzog and Tamkun, 2007), and that histone H3S10 phosphorylation might play an important role in specific transcriptional responses to signaling stimuli (Mahadevan et al., 1991; Lo et al., 2001; Ivaldi et al., 2007). In this study, we characterized three commercially available histone H3S10ph antibodies and used an acid-free squash protocol to revisit the role of histone H3S10ph in response to heat shock treatment. (A-F) Acid-free polytene chromosome squash preparations from female third instar Drosophila larvae triple labeled with antibodies to Pol IIopser2 (green), H3S10ph (red), and with Hoechst (DNA, blue/grey). H3S10ph labeling with antibodies from Epitomics (A), Cell Signaling (C) or Upstate (E) with (+HS) and without (–HS) heat shock treatment. (B,D,F) Higher magnification images of the heat shock-induced puffs 87A/C (boxed regions) labeled by H3S10ph antibodies from Epitomics (B), Cell Signaling (D) or Upstate (F). (G) Immunoblots of protein extracts from salivary glands from wild-type larvae without heat shock treatment (wt) and with heat shock treatment [wt (HS)] labeled with H3S10ph antibody from Cell Signaling, Epitomics or Upstate. Labeling with histone H3 (H3) antibody was used as a loading control. (H) Immunoblots of protein extracts from salivary glands from wild-type larvae without heat shock treatment (wt) and with heat shock treatment [wt (HS)] labeled with Pol IIopser2 antibody. Labeling with lamin antibody was used as a loading control.
phosphorylation in transcription in Drosophila under both heat shock and non-heat shock conditions. We show that there is no change in the levels of the elongating form of RNA polymerase II in larvae from JIL-1 null mutant backgrounds as compared with wild type. Furthermore, we provide evidence that heat shock-induced puffs in JIL-1 null mutant backgrounds are strongly labeled by Pol II\textsuperscript{ser2} antibody in a pattern coincident with that of Hsf antibody, indicating that Pol II\textsuperscript{ser2} is actively involved in heat shock-induced transcription in the absence of H3S10 phosphorylation. That mRNA of the six genes that encode Hsp70, the major heat shock protein in Drosophila, is transcribed at robust levels in JIL-1 null mutants was directly demonstrated by qRT-PCR. Thus, these data strongly suggest that histone H3S10 phosphorylation by JIL-1 is not involved in transcriptional elongation in Drosophila. The finding that there is no redistribution of JIL-1 or H3S10 phosphorylation to transcriptionally active puffs in wild-type polytene squash preparations during the heat shock response further supports this conclusion. These results are contrary to those reported previously (Nowak and Corces, 2000; Nowak et al., 2003; Ivaldi et al., 2007). However, the discrepancies might largely be due to the reliance in these studies on the Upstate H3S10\textsuperscript{ph} pAb, which our study indicates is unsuitable for analysis of heat shock-induced transcription owing to non-specific cross-reactivity at heat shock-induced puffs. Nonetheless, it should be noted that the present study confirms the findings of Ivaldi et al. (Ivaldi et al., 2007) that the chromatin remodeling associated with heat shock puff formation still occurs in JIL-1 null mutants, despite the disruption of chromatin structure and the absence of H3S10 phosphorylation.

Previous studies indicated that the lethality of JIL-1 null mutants might be due to ectopic Su(var)3-9 activity and the disruption of chromatin structure (Zhang et al., 2006; Deng et al., 2007). At interphase, JIL-1 phosphorylates the histone H3S10 phosphorylation in transcription in Drosophila under both heat shock and non-heat shock conditions. We show that there is no change in the levels of the elongating form of RNA polymerase II in larvae from JIL-1 null mutant backgrounds as compared with wild type. Furthermore, we provide evidence that heat shock-induced puffs in JIL-1 null mutant backgrounds are strongly labeled by Pol II\textsuperscript{ser2} antibody in a pattern coincident with that of Hsf antibody, indicating that Pol II\textsuperscript{ser2} is actively involved in heat shock-induced transcription in the absence of H3S10 phosphorylation. That mRNA of the six genes that encode Hsp70, the major heat shock protein in Drosophila, is transcribed at robust levels in JIL-1 null mutants was directly demonstrated by qRT-PCR. Thus, these data strongly suggest that histone H3S10 phosphorylation by JIL-1 is not involved in transcriptional elongation in Drosophila. The finding that there is no redistribution of JIL-1 or H3S10 phosphorylation to transcriptionally active puffs in wild-type polytene squash preparations during the heat shock response further supports this conclusion. These results are contrary to those reported previously (Nowak and Corces, 2000; Nowak et al., 2003; Ivaldi et al., 2007). However, the discrepancies might largely be due to the reliance in these studies on the Upstate H3S10\textsuperscript{ph} pAb, which our study indicates is unsuitable for analysis of heat shock-induced transcription owing to non-specific cross-reactivity at heat shock-induced puffs. Nonetheless, it should be noted that the present study confirms the findings of Ivaldi et al. (Ivaldi et al., 2007) that the chromatin remodeling associated with heat shock puff formation still occurs in JIL-1 null mutants, despite the disruption of chromatin structure and the absence of H3S10 phosphorylation.

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transcription of heat shock-induced genes in the Pol II machinery is functional, as indicated by the robust results clearly indicate that such repression is not global and that (Zhang et al., 2006; Deng et al., 2007). However, the present this phosphorylation during interphase prevents Su(var)3-9- (Zhang et al., 1999; Wang et al., 2001), suggesting as a plausible model that JIL-1Su(var)3-1 might be attributable to JIL-1 kinase activity at ectopic locations leading to misregulated localization of the phosphorylated histone H3S10 mark and thereby counteracting the spreading and gene repression of Su(var)3-9. This is supported by the finding of Deng et al. that ectopic H3S10 phosphorylation at interphase can function as a causative regulator of higher-order chromatin structure in vivo (Deng et al., 2008). Furthermore, studies of PEV of the white allele have indicated that loss of JIL-1 function can also cause a change in the levels of heterochromatic factors at the chromocenter that can indirectly affect gene expression at nearby loci (Lerach et al., 2006) (reviewed by Girton and Johansen, 2005). Thus, the lethality might instead be caused by a severe repression of a few essential genes and/or a more graded decrease in the expression of a larger number of genes owing to the altered chromatin structure. The latter scenario is supported by the finding that the expression of heat shock-induced genes in JIL-1 null mutants is partially rescued by reducing the levels of the heterochromatic factor Su(var)3-9 (Deng et al., 2007) (this study). That JIL-1 levels can directly affect gene expression was recently demonstrated by experiments that showed that loss-of-function JIL-1 alleles act as enhancers of position-effect variegation (PEV), whereas the gain-of-function JIL-1Su(var)3-1 allele acts as a suppressor of PEV at pericentric sites (Bao et al., 2007). The JIL-1Su(var)3-1 allele is one of the strongest suppressors of PEV so far described (Ebert et al., 2004) and it generates truncated proteins with C-terminal deletions that mislocalize to ectopic chromosome sites (Ebert et al., 2004; Zhang et al., 2006). Thus, the dominant gain-of-function effect of the JIL-1Su(var)3-1 alleles might be attributable to JIL-1 kinase activity at ectopic locations leading to misregulated localization of the phosphorylated histone H3S10 mark and thereby counteracting the spreading and gene repression of Su(var)3-9.

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