Coordination of Transcription, RNA Processing, and Surveillance by P-TEFb Kinase on Heat Shock Genes

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Summary
Positive transcription elongation factor b (P-TEFb) is a kinase that phosphorylates the carboxyl-terminal domain (CTD) of RNA Polymerase II (Pol II). Here, we show that flavopiridol, a highly specific P-TEFb kinase inhibitor, dramatically reduces the global levels of Ser2—but not Ser5—phosphorylated CTD at actively transcribed loci on Drosophila polytene chromosomes under both normal and heat shocked conditions. Brief treatment of Drosophila cells with flavopiridol leads to a reduction in the accumulation of induced hsp70 and hsp26 RNAs. Surprisingly, the density of transcribing Pol II and Pol II progression through hsp70 in vivo are nearly normal in flavopiridol-treated cells. The major defect in expression is at the level of 3' end processing. A similar but more modest 3' processing defect was also observed for hsp26. We propose that P-TEFb phosphorylation of Pol II CTD coordinates transcription elongation with 3' end processing, and failure to do so leads to rapid RNA degradation.

Introduction
The largest subunit of eukaryotic RNA Pol II has a CTD consisting of many tandem repeats of the heptad consensus YSPTSPS. This CTD becomes phosphorylated at Ser2 and Ser5 of the heptad as Pol II progresses through the transcription cycle (Dahmus, 1996). Several kinases are capable of phosphorylating Pol II CTD (Kobor and Greenblatt, 2002); however, only two of them have emerged as being particularly important for stimulating transcription. One is the kinase subunit of the general transcription factor TFIIH, CdK7 (Akuolitchev et al., 1993). TFIIH prefers to phosphorylate Ser5 of the Pol II CTD within promoter regions in vivo (Komaritsky et al., 2000; Ramanathan et al., 2001). The second CTD kinase P-TEFb was first identified from Drosophila Kc cell nuclear extracts as a CdK9/CyclinT complex that could stimulate run-off transcript formation in vitro (Marshall et al., 1996; Marshall and Price, 1995). P-TEFb preferentially phosphorylates Ser2 of the Pol II CTD, but its substrate specificity can be modulated to target both Ser2 and Ser5 by the HIV transactivator Tat (Zhou et al., 2000).

The Pol II CTD also has a role in coupling transcription with RNA processing, and this coupling appears to be regulated by CTD phosphorylation (Proudfoot et al., 2002). For example, a CTD peptide with phosphorylated Ser5 (Ser5-P) is able to stimulate one of the capping enzymes, the guanylyltransferase, to form enzyme-GMP intermediates (Ho and Shuman, 1999). In vitro studies showed that some 3' end processing factors can associate with phosphorylated CTD (Barilla et al., 2001; Licata-losi et al., 2002), and both 3' cleavage and polyadenylation are stimulated by both hypophosphorylated Pol II (Ila) and hyperphosphorylated Pol II (Ilo) (Hirose and Manley, 1998; Proudfoot and O'Sullivan, 2002); however, the role of CTD phosphorylation of particular residues and the responsible kinases involved in 3' end formation in vivo are less well defined.

The Drosophila heat shock genes are highly inducible and provide a well-established system to study transcription and RNA processing. Recent studies showed that Drosophila P-TEFb kinase localizes to many actively transcribing loci including developmental and heat-induced loci (Lis et al., 2000). Kinetic studies indicate that P-TEFb is rapidly recruited to heat shock promoters and tracks along with RNA Pol II through the heat shock genes (Andrulis et al., 2000; Boehm et al., 2003). These results indicate that P-TEFb is likely to be a player in hsp70 RNA production; however, the mechanisms by which P-TEFb acts and its role at specific other steps in the activation of heat shock gene expression are not clear.

In this report, we examine the function of P-TEFb in vivo. We find that native Drosophila P-TEFb is important for maintaining the phosphorylation status of Pol II and for the accumulation of both hsp70 and hsp26 mRNAs upon heat induction. Surprisingly, upon flavopiridol (FP) treatment, Pol II transcription on the hsp70 gene is not significantly affected and the decrease in Pol II density is too small to account for the dramatic reduction in hsp70 RNA levels. However, the 3' end cleavage of hsp70 RNA is compromised in the presence of flavopiridol and the improperly processed RNAs appear to be rapidly degraded. A similar but modest 3' processing defect accounts for part of the decrease of hsp26 RNA level as well.

Results
P-TEFb RNAi Impairs the Accumulation of Heat-Induced hsp70 RNA
To investigate the function of native P-TEFb in heat-inducible transcription of the hsp70 gene, we measured the amount of hsp70 RNA after reducing the level of P-TEFb in Kc cells by RNAi. The protein levels of CdK9, CyclinT, and the upstream activator of heat shock genes HSF were specifically reduced by their cognate RNAi (Figure 1A). We used the transcript of the ribosomal protein (Rpl32) gene r49 as an internal control (O’Connell and Rosbash, 1984), because the r49 gene locus shows no detectable staining by P-TEFb antibody on
Polytene chromosomes (Lis et al., 2000) and the rp49 mRNA levels are constant through all the conditions tested. HSF RNAi decreases the level of induced hsp70 RNA by a factor of 20, while the β-galactosidase RNAi control has no effect (Figure 1B, compare lanes 7 and 10 with lane 6). Treatment of cells with either Cdk9 or CyclinT RNAi elicits a 4- to 5-fold decrease in heat-induced hsp70 RNA accumulation (Figure 1B, lanes 8 and 9). A similar result following RNAi depletion of P-TEFb was also observed in C. elegans embryos (Shim et al., 2002). Combination of Cdk9 and CyclinT RNAi shows no further decrease in hsp70 RNA levels (data not shown). These data and previous studies of P-TEFb recruitment to activated heat shock loci (Lis et al., 2000) indicate that P-TEFb plays a direct and positive role in heat shock gene expression in vivo and that P-TEFb is a limiting factor for inducible gene expression.

**Flavopiridol Inhibits the Kinase Activity of Drosophila P-TEFb In Vitro**

The RNAi approach is a specific method for reducing levels of P-TEFb, but it has limitations, including incomplete depletion of P-TEFb and the long treatments, which create concern over possible secondary effects. To study the consequences of rapid and complete inactivation of P-TEFb on the kinetics of heat shock gene induction, we utilized a very specific, potent, and fast-acting human P-TEFb kinase inhibitor, FP (Chao et al., 2000). Because Drosophila and human Cdk9/CyclinT complexes share high sequence homology and the Drosophila P-TEFb complex has a unique domain of Spt5 (Figure 2), a transcription elongation factor that has been reported to act both positively and negatively on transcription (Bourgeois et al., 2002; Hartzog et al., 1998; Yamaguchi et al., 1999), we hypothesized that FP would efficiently inhibit Drosophila P-TEFb as well. Like its human homolog (Ivanov et al., 2000), Drosophila P-TEFb phosphorylates in vitro the Pol II CTD and the C-terminal domain of Spt5 (Figure 2), a transcription elongation factor that has been reported to act both positively and negatively on transcription (Bourgeois et al., 2002; Hartzog et al., 1998; Yamaguchi et al., 1999). In the presence of increasing amount of FP, the phosphorylation of both substrates is inhibited in a similar fashion. The IC50 of FP in the presence of 25 nM P-TEFb is about 20–30 nM, which is similar to what has been observed for human P-TEFb at this kinase concentration (Chao and Price, 2001).

**Flavopiridol Inhibits the Kinase Activity of Drosophila P-TEFb In Vivo**

To study the effects of FP inhibition of P-TEFb in vivo, we first examined HSF and phosphorylated forms of Pol II at multiple heat shock gene loci on salivary gland polytene chromosomes (Figure 3A). As expected, the intensity of HSF staining is not significantly affected by FP-treated glands, because HSF binding to the promoter is an early step of heat shock gene activation (Lis, 1998). The most dramatic intensity change in response to FP treatment is the decrease of the Ser2-P form of Pol II at all major heat shock gene loci as well as at an hsp70 transgene inserted at the 59D locus (Simon and Lis, 1987). In contrast, there is little change in the intensity of Ser5-P (Figure 3A). These results agree with previous studies showing that Ser2 is the preferred substrate of P-TEFb (Ramanathan et al., 2001; Shim et al., 2002). Moreover, because the kinase most closely related to P-TEFb is Cdk7, the major kinase responsible for Ser5 phosphorylation, the dramatic decrease in Ser2 but not Ser5 phosphorylation indicates that FP is acting specifically on P-TEFb in vivo. To investigate this further, we performed the same experiments using a cdk7 strain at the nonpermissive temperature to abolish cdk7 activity (Schwartz et al., 2003a). Combining cdk7 inactivation with FP treatment results in a near-complete disappearance of Ser2-P and Ser5-P at hsp70 loci 87A and 87C (Figure 3B). This indicates that Cdk7 of TFIIH and Cdk9 of P-TEFb are the two major serine kinases that phosphorylate Pol II CTD.

By polytene immunofluorescence using FP-treated, non-heat shocked, salivary glands (Figure 3C), we also observed a striking decrease in Ser2-P staining when compared to the intensity of the control, trithorax-like protein GAF (Benyajati et al., 1997). The residual Ser2-P staining in FP-treated samples is localized mainly to a single site 75B. Interestingly, this is a locus containing
Figure 3. FP Inhibits the Kinase Activity of Drosophila P-TEFb In Vivo

Salivary glands dissected from fly third instar larvae (Z243 strain for [A] and [C] and cdk7<sup>ts</sup> line for [B]) were incubated in insect cell media without (-FP) or with 500 nM of FP (+FP) followed by 10 min heat shock (HS, [A] and [B]) or none (NHS, [C]). The epitopes detected by indirect immunofluorescence with specific antibodies are labeled on each panel. Exposure times for each -FP/+FP pair were the same except for "Ser2-P" of the -FP experiment in (A), which is a 10× longer exposure than the corresponding +FP image. The chromosomes shown are representative examples of a large set of nuclei examined. From 8 to 36 nuclei under each condition were photographed at multiple exposures, and the variation for at least 90% of nuclei was estimated visually to be less than a factor of two.

(A) Ser2-P but not Ser5-P forms of Pol II decreases dramatically on heat-induced loci. Major heat shock loci are indicated with thin arrows; only hsp70 loci (87A and C), hsp26 locus (67B), and the hsp70 transgene-inserted locus (59D) are labeled.

(B) Ser2-P and Ser5-P decrease dramatically on heat-induced hsp70 loci (87A and 87C as labeled) on polytene chromosomes from cdk7<sup>ts</sup> larvae at nonpermissive temperature. The lack of a detectable decrease in Ser5-P staining in the -FP larvae may be a consequence of P-TEFb partially compensating for cdk7 activity at the nonpermissive temperature (Schwartz et al., 2003a; Zhou et al., 2000).

(C) Ser2-P form but not total Pol II decreases dramatically on non-heat-induced chromosomes. One of the ecdysone-induced puffs 75B is labeled in the +FP images.

an exceptionally long ecdysone-induced transcript, which is greater than 100 kb and takes well over an hour to transcribe (Thummel et al., 1990). The short duration of our FP treatment may not be sufficient to eliminate the engaged Ser2-P form of Pol II on this gene. To our surprise, we did not see a significant reduction in the intensity of total Pol II staining at actively transcribing loci in FP-treated glands under either non-heat shock or heat shock conditions (Figure 3C and data not shown, also Figure 4).

Flavopiridol Decreases the Accumulation of Induced hsp70 and hsp26 RNA

The dramatic reduction in Ser2-P at heat shock puffs upon FP treatment provoked us to examine the effect of FP on the induction of heat shock RNA. We treated
Figure 4. Effects of FP on Heat Shock Gene RNA Accumulation and the Distribution of Selected Transcription Factors and Pol II on hsp70 and hsp26 Genes

(A) Experimental scheme: the concentration of P-TEFb in Drosophila Kc cell nuclei was estimated to be about 500 nM by Western blots (data not shown), and we also empirically titrated the FP concentration for Kc cell treatment and found 500 nM FP is sufficient for effective inhibition of hsp70 RNA production and for maintaining specificity (Figure 3). Drosophila Kc cell cultures were treated with 500 nM FP or mock treated at 23°C and then heat shocked at 37°C for 5 min. About 1 × 10⁶ cells were used for RNA measurements (B), 1 × 10⁸ cells for run-on analysis (D), and the rest for ChIP analysis (C).

(B) RNA analyses of hsp70 and hsp26 RNA using rp49 RNA as an internal control. On the left is the PERT analysis (four experiments) of hsp70 RNA as described (Figure 1B). On the right are the S1 protection assays using probes complementary to 3′ coding region of hsp70 (four experiments) and hsp26 RNA (five experiments). Assays on decreasing amounts (Amt.) of untreated samples show that the detection is within the linear range. Protected probes corresponding to specific RNAs are indicated by black dots, and the undigested probes are labeled with arrowheads. Controls of yeast RNA (yRNA) with or without S1 digestion are shown on the right of each panel.

(C) ChIP analysis showing the distribution of different phosphorylation states of Pol II and selected transcription factors on induced hsp70 (top panel) and hsp26 (bottom panel) genes in the absence (solid bar) or presence (hatched bar) of FP. The diagrams of hsp70 and hsp26 genes are shown on the right of each chart with the arrow indicating the location of the transcription unit relative to PCR fragments shown underneath each gene with the same grayscale as in the histogram. For the hsp70 gene, upstream promoter “Up” (∼200/−108), 5′ (−4/−112), and 3′ (+1649/+1754) fragments are shown in gray, white, and black, respectively. For hsp26 gene, the 5′ promoter (−22/+63) and the 3′ region (+580/+669) are shown in white and black. Immunoprecipitation was done with antibodies as labeled on the x axes. The y axes represent the percentage of input material. The right hand y axes are for Spt5 and HSF, and the left hand axes are for the others. The two groups are separated by a vertical black line. Each ChIP result is shown as an average of four experiments with the standard error of mean (SEM).

(D) Nuclear run-on analysis of the density of transcribing Pol II on hsp70 and hsp26 genes. The arrow on the schematic gene diagrams indicate...
Drosophila Kc cells with FP for 15 min prior to a 5 min heat shock and then examined the level of heat-induced hsp70 and hsp26 RNA (Figure 4A). We observed 14- and 20-fold less hsp70 RNA from FP-treated cells by S1 and PERT, respectively, and a 5-fold decrease of hsp26 RNA, whereas rp49 RNA levels are unchanged (Figure 4B).

The Effects of Flavopiridol on the Distribution of Pol II and Specific Transcription Factors on hsp70 and hsp26 Genes
To examine the FP effect on transcription factor and Pol II recruitment to heat shock genes both quantitatively and at a high resolution, we used chromatin immunoprecipitation (ChiP) assays. We quantified the coprecipitated DNA by real-time PCR, which was normalized to the percentage of input material run in parallel. Previous studies using this method showed that most transcription factors tested reach their maximum levels on the hsp70 gene after a 5 min heat shock (Boehm et al., 2003). Thus, we used a 5 min heat shock for our ChiP analysis (Figure 4A). Figure 4C (top panel) shows a dramatic decrease of Ser2-P on the hsp70 gene in FP-treated cells, with the greatest decrease at the 3′ end of the gene (11.6-fold). FP treatment causes no change in Ser5-P in the upstream promoter or 5′ region, but approximately a 3.1-fold decrease at the 3′ end of the gene. While the amount and distribution of Pol II epitopes change dramatically, there is no significant change in total Pol II or 8WG16-recognized Pol II epitopes (preferentially unphosphorylated Pol II; Cho et al., 2001). The distributions of Spt5, P-TEFb, and HSF in FP-treated cells are not significantly changed either, indicating that their recruitment to hsp70 does not require P-TEFb kinase activity. Figure 4C (bottom panel) also shows a dramatic decrease of Ser2-P on hsp26 gene (about 10-fold at the 3′ region), while the Ser5-P on the promoter is not altered and only decreases 2.4-fold on the 3′ region. There is no significant change of total Pol II or 8WG16-recognized Pol II form associated with the hsp26 gene, nor is the HSF association altered. A 2-fold decrease of P-TEFb and a 2-fold increase of Spt5 at the hsp26 5′ promoter region, effects which were not observed for hsp70, may reflect subtle differences in regulatory mechanisms of the two genes. In conclusion, inhibition of P-TEFb kinase activity dramatically reduced Ser2 phosphorylation of Pol II (and had smaller effects on Ser5 phosphorylation) on heat shock genes but appeared not to significantly change the distribution of Pol II itself or other transcription factors tested.

Flavopiridol Has Only a Modest Effect on the Density of Transcribing Pol II on hsp70 as Assayed by Nuclear Run-On
The uninduced heat shock genes have a paused/stalled Pol II in the promoter-proximal region and the escape of this paused polymerase into productive elongation appears to be a rate-limiting step for heat shock gene induction (Lis, 1998). Moreover, the escape of Pol II correlates with an increase in the level of Ser2 phosphorylation (O’Brien et al., 1994). We were surprised to find that the total polymerase density, as measured in the ChiP assay, does not change significantly when P-TEFb kinase activity is inhibited (Figure 4C), yet the accumulation of heat shock RNAs, especially hsp70 RNA, is severely affected (Figure 4B). The possibility remained that some of these RNA polymerases measured by ChiP assays may be transcriptionally inactive; therefore, we used the nuclear run-on assay as an independent method to estimate the transcribing polymerase density on hsp70 and hsp26 genes (Figure 4D). The results are similar with or without sarkosyl, a detergent that allows transcription elongation by stalled/paused polymerases (Rougvie and Lis, 1988). For hsp70, the run-on results show no detectable change in polymerase density at the 5′ end of the gene from FP-treated cells. We detect only a 30% and 40% decrease at the 3′ regions of the gene in reactions with and without sarkosyl. Nuclear run-on assays with P-TEFb RNAI-treated cells did not reveal any decrease in polymerase density on hsp70 gene (data not shown). The nuclear run-on assay supports the conclusions from the ChiP analysis that inhibition of P-TEFb kinase activity only modestly decreases the RNA polymerase density on the hsp70 gene, which indicates that FP may have a mild effect on the escape of paused polymerase.

For hsp26, we noted a 3-fold decrease of Pol II density in FP-treated cells in both run-on conditions. The decrease in Pol II density is greater than that observed by the ChiP assay and may reflect the subtle differences in what is measured in the two assays. For example, Pol II in FP-treated cells may be more prone to arrest on hsp26 gene, thereby preventing detection by nuclear run-on. The 3-fold decrease of transcribing Pol II on hsp26 gene could partially account for the observed 5-fold decrease of RNA (Figure 4B).

Loss of P-TEFb Kinase Activity Does Not Affect the Entry or Progression of Pol II on hsp70 In Vivo
How is the substantial decrease in hsp70 RNA accumulation upon FP treatment reconciled with only a small decrease in density of RNA polymerase on the gene? We consider two hypotheses. First, the overall transcription process may be slower leading to a slower rate of transcript production even though the density of elongating Pol II is largely unchanged. This hypothesis derives from the findings that hyperphosphorylation of Pol II correlates with its transition to productive elongation (O’Brien et al., 1994) and that P-TEFb can function as a positive regulator in production of full-length transcripts (Price, 2000). Second, because phosphorylated Pol II CTD has the location of transcription units relative to DNA fragments used for hybridization: 5′ (−5′/−412), “mid” (+1003/+1420), and 3′ (+1758/+2162) fragments for hsp70, and (+485/+910) fragment for hsp26. We used 1% of the run-on transcripts to probe an rDNA gene, thereby providing a normalization standard, and the rest was used to probe heat shock gene fragments. Background (BG) from an untranscribed region is subtracted from each signal. Reactions with or without 0.6% sarkosyl (Sark) and from the control or FP-treated cells (FP) are labeled. The results were plotted as a bar graph with the y axis representing the geometric mean of the ratio of polymerase density from FP-treated cells to that of mock-treated cells; a ratio of 1 indicates no change. The error bars are drawn as calculated from the geometric SEM of four (no sarkosyl) or three (0.8% sarkosyl) independent experiments.
Figure 5. FP Inhibition of P-TEFb Kinase Activity Does Not Affect Pol II Entry or Progression on the hsp70 Gene

Drosophila Kc cells mock treated (−FP, gray bar) or treated with 500 nM FP (+FP, hatched gray bar) were subject to 2 min heat shock at 37°C before ChIP analysis. A schematic diagram of hsp70 gene is shown on the upper left with the midpoint of each real-time PCR fragment shown underneath (approximately drawn to scale). The antibodies used for immunoprecipitation are labeled on each panel. The x axes show the midpoint of each PCR fragment along hsp70 gene and the y axes represent the percentage of input (SEM of three experiments is shown).

Also been implicated in the coupling of transcription and RNA processing (Proudfoot et al., 2002), an alternative hypothesis is that newly transcribed hsp70 pre-mRNA may undergo defective processing when P-TEFb is inhibited. This could lead to rapid turnover of the hsp70 RNA and cause the dramatic decrease in mRNA accumulation.

To test the first hypothesis, we examined the FP effect on Pol II distribution along the hsp70 gene 2 min after heat induction. The transcription rate of Pol II on hsp70 gene is about 1.2 kb/min in vivo and polymerases are first detectable on the 3’ end of the gene after 2 min of heat shock activation (O’Brien and Lis, 1993). The 2 min time point captures the first wave of Pol II escaping from the promoter pause sites and elongating through hsp70 as well as the loading and progression of the following polymerases. Therefore, comparing the Pol II distributions in the presence and absence of FP at this early phase of gene activation should reveal defects in the flow of Pol II through the gene (Boehm et al., 2003). Figure 5 shows that the distribution of total Pol II is not significantly different in untreated versus FP-treated cells. Similarly, the distribution of the Ser5-P form of Pol II, the 8WG16-recognized form (preferentially Pol IIa), and other factors tested on the hsp70 gene is not significantly affected by FP treatment. As expected, the Ser2-P form of Pol II decreases significantly across the gene in FP-treated cells. We conclude that loss of P-TEFb kinase activity causes little effect on the entry and progression of Pol II on the induced hsp70 gene.

Loss of P-TEFb Kinase Activity Leads to Inefficient 3’ Processing of hsp70 and hsp26 Pre-mRNA

Because transcription by Pol II on the hsp70 gene is not significantly affected by FP, we tested our second hypothesis that the inhibition of P-TEFb kinase activity results in inefficient RNA processing, which leads to rapid degradation of transcripts. The end products of 3’ processing are polyadenylated transcripts. Figure 6A shows that the ratio of unadenylated-to-polyadenylated RNA (PolyA−/A+) after FP treatment increases 8.5- and 3.4-fold for hsp70 RNAs detected with 5’ and 3’ probes, respectively. The larger effect on the PolyA−/A+ ratio seen with the 5’ probe may be a consequence of 3’ to 5’ degradation of unprocessed transcripts (see Discussion below) and, in part, due to the small decrease in Pol II on the 3’ end of the gene caused by FP. Consistent with these two possibilities, we observed an underrepresen-
the cleaved and uncleaved RNA using DNA probes com-

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P-TEFb Coordinates Transcription and Processing

Only when FP treatment is combined with cdk7 inactiva-

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Figure 6. Effects of FP on the Accumulation of Total and Processed Heat Shock RNA

RNAs from mock-treated (-FP) and 500 nM

FP-treated (+FP) cells after a 5 min heat shock at 37°C were used for S1 nuclease protection assay.

(A) Poly (A)’ and poly (A)’ RNA from -FP (5 

μg total RNA) and +FP (25 μg total RNA) cells were analyzed using S1 probes (see Supplemental Data available on Molecular Cell’s website) corresponding to both 5’ and 3’ se-

quences of hsp70 and hsp26 RNAs. The probes and the distance between the 5’ and 3’ probes relative to each gene coding region are shown on the top. The y axes show the ratio of Poly A’ / A’ RNA with the left hand one for the hsp70-5’ and the right hand one for the others as separated by a thick black line. The geometric mean of each PolyA / A’ ratio is shown with the SEM from four (hsp70) or five (hsp26) independent experiments.

(B) Relative amounts of 5’ and 3’ RNA se-

quences of hsp70 and hsp26 in total RNA (10 μg) from -FP and +FP cells are measured with S1 probes as in (A). RNA amount from untreated samples are set at 100 and the amount from FP-treated samples relative to the control are shown as the geometric mean with the SEM from six (hsp70) or four (hsp26) independent experiments. The range of each effect can be obtained with >95% confidence by dividing and multiplying the geometric mean with the SEM.

tation of 3’ hsp70 RNA sequences in FP-treated cells (Figure 6B). A similar, albeit more modest effect, was seen on the PolyA / A’ ratio for hsp26 RNA (Figure 6A).

Before polyadenylation can take place, the primary transcript must first be cleaved at the 3’ end. To test the cleavage efficiency in FP-treated cells, we measured the cleaved and uncleaved RNA using DNA probes complementary to the 3’ untranslated region (3′UTR) flanking the normal cleavage site of both hsp70 and hsp26 genes (Figure 6C). We observed that the ratio of cleaved to uncleaved hsp70 RNA (C/U) in the control cells is 7-fold greater than that in FP-treated cells. Additionally, a moderate depletion of P-TEFb by RNAi results in a modest 3’ cleavage defect of hsp70 RNA (1.8-fold effect, data not shown), confirming this 3’ processing defect is specific to loss of P-TEFb activity. The C/U ratio decrease for hsp26 RNA from FP-treated cells is 1.4-fold, but statistically significant (Figure 6C). In summary, the results above show that loss of P-TEFb kinase activity alters proper 3’ end processing of both hsp70 and hsp26 RNA, resulting in a clear defect at the level of RNA cleavage. This failure in processing may lead to its instability and rapid turnover.

Discussion

P-TEFb is rapidly recruited to heat shock loci following gene activation (Lis et al., 2000). Here, we demonstrated that P-TEFb functionally contributes to Drosophila heat shock gene expression at multiple levels. Inhibiting P-TEFb kinase activity in vivo with a highly potent, fast-acting, and specific drug, FP, leads to the prominent decrease in Ser2 phosphorylation of Pol II that is associated with induced heat shock genes, and globally, with loci normally active during development. In contrast, the effect on Ser5 phosphorylation is in general small and is negligible at the promoter region of heat shock genes. Only when FP treatment is combined with cdk7 inactiva-

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/tion in complete loss of both Ser2 and Ser5 phosphorylation on chromosomes. FP also re-

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duces heat shock gene RNA levels. A dramatic effectgenes (Figure 6C). We observed that the ratio of cleaved 

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/hsp70 

/hsp26

end processing. A similar but more modest effect, was observed for hsp70 RNA (1.8-fold effect, data not shown), confirming this 3’ processing defect is specific to loss of P-TEFb activity. The C/U ratio decrease for hsp26 RNA from FP-treated cells is 1.4-fold, but statistically significant (Figure 6C). In summary, the results above show that loss of P-TEFb kinase activity alters proper 3’ end processing of both hsp70 and hsp26 RNA, resulting in a clear defect at the level of RNA cleavage. This failure in processing may lead to its instability and rapid turnover.

P-TEFb and Transcription

Productive transcription is concomitant with hyper-

phosphorylation of Pol II CTD (Dahmus, 1996; O’Brien et al., 1994), but the contributions of CTD phosphoryla-

/tion to the numerous steps of mRNA production need to


Pol II on induced hsp26 shown that, in a prokaryotic system, cooperation between processing events on a high density of transcribing Pol II, and hsp70/H11032 the differences between the two genes and between the more efficient 3' elongation rate of RNA Pol II (Chao et al., 2000). This is processing factors have been shown to be regulated by hsp70/H11032 in RNA accumulation, the large decrease in Ser2-P level cruitment of 3' gene expression in terms of the large decrease hsp70/P-TEFb for transcription and RNA processing in vivo, tion of Ser2 by P-TEFb is critical for efficiently recruiting "X" indicates the loss of P-TEFb kinase activity and the 3' relative amounts of these species in control and FP-treated cells. (Skaar and Greenleaf, 2002). In addition, a CTD peptide are shown in the figure as labeled. The black and gray "P" symbols genes (Figure 6). This connection to 3' RNA expression in the absence (FP) of FP. Only 5' and 3' regions of the gene are shown and are indicated by the thick black lines. The transcription start site is represented as a right turn arrow and the polyadenylation signal "AATAAA" as an arrowhead. Only factors related to this study are shown in the figure as labeled. The black and gray "P" symbols represent phosphorylation of Pol II CTD on Ser2 and Ser5, respectively. The 3' symbol represents the 3' processing machinery. The number of P labels on the Pol II CTD, the height of the polymerase symbol, and the number of capped RNA transcripts represent the relative amounts of these species in control and FP-treated cells. "X" indicates the loss of P-TEFb kinase activity and the 3' processing machinery activity on hsp70 gene.

be better understood. We found that inhibition of P-TEFb kinase activity has similar effects on both hsp26 and hsp70 gene expression in terms of the large decrease in RNA accumulation, the large decrease in Ser2-P level of Pol II CTD, the small decrease in Pol II density, and the defects in 3' RNA processing. The quantitative differences may reflect different degrees of reliance on P-TEFb for transcription and RNA processing in vivo, with hsp26 more dependent on P-TEFb for generating a high density of transcribing Pol II, and hsp70 more dependent on P-TEFb for RNA processing. Previous in vitro transcription experiments using a mammalian CMV-driven DNA template indicated that FP slowed the elongation rate of RNA Pol II (Chao et al., 2000). This is different from what we saw for hsp70, which may reflect the differences between the two genes and between the in vitro and in vivo systems. Recently, it has been shown that, in a prokaryotic system, cooperation between the leading RNA polymerase and the following polymerases can physically stimulate transcription elongation (Epshtein and Nudler, 2003). The high density of Pol II on induced hsp70 gene (O'Brien and Lis, 1993) may make transcription less dependent on CTD phosphorylation than other less densely transcribed genes. In addition, FP-treated cells still have residual levels of Ser2 phosphorylated Pol II CTD (Figures 3 and 4). This, along with the nearly normal levels of Ser5 phosphorylation, may be sufficient for Pol II progression on hsp70. Also, other transcription factors could be acting redundantly with P-TEFb in vivo.

In vitro transcription assays showed that Tat activator can stimulate human P-TEFb phosphorylation of both Pol II and Spt5 (Ping and Rana, 2001). Interestingly, when P-TEFb activity is inhibited, the amount of Spt5 on hsp70 is not significantly affected, which is consistent with the fact that Spt5 can interact with both Pol IIa and ilo (Lindstrom and Hartzog, 2001; Lindstrom et al., 2003; Wen and Shatkin, 1999). Although our antibody does not distinguish phosphorylated and unphosphorylated forms of Spt5, we know that FP equally inhibits P-TEFb phosphorylation of Pol II and Spt5 in vitro (Figure 2) and we expect that the level of Spt5 phosphorylation, like Ser2-P of Pol II, is compromised in FP-treated cells (Lavoie et al., 2001). The fact that the overall Pol II tran-
scription is nearly normal on the hsp70 gene suggests that underphosphorylated Spt5 does not strongly affect transcription of this gene.

P-TEFb and RNA Processing

Over the last several years, emerging evidence shows that RNA Pol II, especially its CTD, participates in RNA processing (Proudfoot et al., 2002). Our results show further that specific inhibition of P-TEFb kinase activity causes a reduction in Ser2 phosphorylation of Pol II CTD and a defect in 3' RNA processing of two heat shock genes (Figure 6). This connection to 3' processing may be related to recent studies showing that Ctk1, the ki-

nase subunit of the potential yeast homolog of P-TEFb, genetically interacts with a 3' end processing factor (Skaar and Greenleaf, 2002). In addition, a CTD peptide with phosphorylated Ser2 was found to directly interact with a yeast 3' processing factor in vitro (Licatalosi et al., 2002). Consistent with our results in Drosophila, Ahn et al. (this issue of Molecular Cell) demonstrate in the accompanying article that a ctk1a mutant causes a re-
duction of Ser2-P and defects in 3' processing and re-
cruitment of 3' processing factors, but has no effect on the recruitment of Pol II and elongation factors in yeast (Ahn et al., 2004). We propose that during hsp70, and to some extent hsp26, gene expression, phosphoryla-
tion of Ser2 by P-TEFb is critical for efficiently recruiting 3' processing machinery and/or directly participating in the 3' cleavage and polyadenylation reactions (Figure 7). While this coupling model is attractive, we can not rule out that P-TEFb directly regulates the recruitment and activity of processing machinery, since some of the processing factors have been shown to be regulated by phosphorylation (Bond et al., 2000).

The more efficient 3' end processing of hsp26 than hsp70 RNA in P-TEFb-inhibited cells implies that processing events on hsp26 gene are less dependent on the activity of the kinase. The reason for this is unclear. Perhaps this coupling of the CTD phosphorylation to 3' end processing is less critical for short genes, like hsp26; or the sequences that specify 3' processing may be inherently stronger for hsp26, making it less dependent
on this coupling; or the decrease of Pol II transcription on hsp26 suppresses inefficient 3' end processing.

**P-TEFb and Nuclear RNA Turnover**

Higher eukaryotes turn over more than half of the newly transcribed RNA in the nucleus, including normally processed RNA (e.g., introns) and improperly processed RNAs (Moore, 2002). Here, we show that loss of P-TEFb kinase activity produces a higher fraction of unadenylated hsp70 and hsp26 RNA than normal. Studies in yeast showed that a mutation in the exosome complex, a multisubunit complex of 3' to 5' exoribonuclease, can suppress a mutation in the polyadenylation polymerase, indicating that the exosome complex serves as a checkpoint mechanism to degrade improperly 3' end-processed RNA (Burkard and Butler, 2000; Hilleren et al., 2001). Recently, the *Drosophila* exosome complex was found to localize to the whole hsp70 and hsp26 genes upon activation (Andrulis et al., 2002), making it a good candidate to degrade the improperly processed hsp70 and hsp26 RNA caused by loss of P-TEFb kinase activity (Figure 7). In our model, P-TEFb phosphorylation of Pol II CTD ensures the proper 3' end processing and generation of stable mRNA.

**Experimental Procedures**

Fly Stocks and Reagents

Z243-2-1 is a *Drosophila melanogaster* transgenic fly containing a hsp70 transgene at the 59D locus (Simon and Lis, 1987). cdk7" flies (line #15) (Larochelle et al., 2001) were propagated at 18°C on standard yeast/glucose media. To inactivate cdk7, third instar larvae were incubated at 29°C for 24 hr prior to dissection. Antibodies are as described (Boehm et al., 2003), except for the rabbit antibody against Cdk9 (P. Mason and J.T.L., unpublished data) and the rabbit antibody against GAF (O'Brien et al., 1995). The DNA plasmids and oligonucleotides are described in the Supplemental Data (available online at http://www.molecule.org/cgi/content/full/13/1/55/DC1). FP (Aventis, Inc., Bridgewater, NJ) was dissolved in water to make 10 mM stock, stored at -70°C, and diluted in water before use.

**Protein Expression and Purification**

The pBAC-Dmp-P-TEFb construct was used to transfect Sf9 cells to make recombinant baculovirus using the BacVector-1000 Transfection Kit (Novagen). P-TEFb was purified as described (Peng et al., 1998) except without the Mono Q purification step. The MBP-dSpt5 C-terminal domain fusion protein and MBP-dCTD were expressed and purified as described (Andrulis et al., 2000).

**In Vitro Kinase Assay**

Kinase assays were performed as described (Marshall et al., 1996) with some modifications. P-TEFb (25 nM) was mixed with no or various amounts of FP in kinase reaction buffer (20 mM HEPES [pH 7.6]/5 mM MgCl2/55 mM KCl) and incubated at room temperature for 10 min, and then protein substrate (240 nM) was added. The reaction was initiated by adding ATP (10 μM cold ATP mixed with 10 μM of [γ-32P]ATP, AEN Life Science Products) at room temperature for 10 min. The reactions were stopped by adding SDS loading buffer and analyzed on 10% SDS-PAGE. Signals were detected by a Storm 840 Phospholmager and analyzed with ImageQuant software (Molecular Dynamics).

**RNA Interference**

The dsRNA production for Cdk9, CyclinT, HSF, and β-galactosidase was done as described (Clemens et al., 2000). *Drosophila* Kc cells (1 × 10^6) maintained in 2 ml of serum-free HyQ-CCM media (HyClone Laboratories, Inc.) were added to each well of 6 well cell culture dish (Corning) and 30 μg of dsRNA was added. The cell-RNA mixture was incubated at room temperature for a total of 3 days. Another 30 μg of each dsRNA was added to corresponding wells on each of the 2nd and 3rd days. The RNAi-treated cells have the same viability (trypan blue staining) as before the RNAi treatment. Cells were subject to heat shock at 37°C for 30 min or kept at 23°C before being harvested for Western blot and RNA preparation.

**RNA Isolation and Analysis**

Total RNA was isolated using RNaseasy Mini Kit (Qiagen) or Trizol reagent (Invitrogen) according to the manufacturers' manuals. Polyadenylated and unadenylated RNA was separated as described (Licatolasi et al., 2002). Total RNA was spiked with gel-purified and end-labeled poly(A) RNA (Roche Applied Science) as an indicator and fractionated using BioMag Oligo(dT)15 (Polysciences, Warrington, PA). More than 90% of the labeled poly(A) RNA was in the poly (A)+ fraction.

For PERT assays, 25–30 μg of total RNA was used to reverse-transcribe hsp70 and rp49 cDNA in the same reaction using SuperScript™ II RNaseH-Reverse Transcriptase (Invitrogen). The RNA template was digested with RNaseH and RNaseA/T1 Cocktail (Ambion). The second strand DNA was synthesized by extending the 5' end-labeled forward primers. There are no rounds of amplification in our PERT analysis, and the detection is within the linear range. The products were chloroform extracted and ethanol precipitated before being analyzed on a 4% denaturing acrylamide gel.

For S1 nuclease protection assays, RNA was coprecipitated with end-labeled DNA probes complementary to 5’, 3’, or 3'-UTR regions of hsp70 or hsp26 RNA. The mixture was denatured at 90°C and hybridized in the hybridization buffer (60% deionized formamide/100 mM sodium citrate [pH 6.4]/300 mM sodium acetate [pH 6.4]/1 mM EDTA) at 25°C for 12–16 hr. S1 nuclease (Invitrogen) (200 U for the 5’ and 3’ probes and 100 U for the 3'-UTR probe) in 200 μl of S1 digestion buffer (5% glycerol/1 mM MnSO4/30 mM NaOAc [pH 5.2]/50 mM NaCl) was added to each reaction and incubated at 25°C for 30 min. The reaction was stopped by adding 40 μl of stop buffer (4 μl of 500 mM EDTA/33 μl of 3 M NaOAc/15 μl of linear acrylamide). The products were precipitated and analyzed on a 12% or 6% denaturing acrylamide gel.

**Indirect Immunofluorescence**

For experiments involving FP inactivation of P-TEFb, salivary glands from the third instar larvae were dissected in 50% Grace’s insect cell media (Invitrogen) and incubated in the media containing 500 nM FP for 20 min. Glands were then heat shocked for 10 min at 36.5°C in saran wrap. The next, the glands were processed as described (Schwartz et al., 2003b). Primary antibodies were used at 1:50 dilutions for H14 (Ser5-P), H4 (Ser2-P), HSF, and GAF and 1:20 dilution for total Pol II. Appropriate secondary donkey antibodies conjugated to either Rhodamine Red X or Cy2 labels (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1:500, and images were collected as described (Schwartz et al., 2003b).

**Chromatin Immunoprecipitation**

Kc cell culture was treated with FP at the final concentration of 500 nM or the same volume of water for control at room temperature for 15 min with gentle stirring. Both control and FP-treated cells were subject to various periods of heat shock at 37°C in parallel. For the 5 min heat shock, the flask were placed in a 42°C water bath to bring the temperature up to 37°C within 1 to 2 min, which was monitored by thermometers in the cultures. Then, the flask were immediately moved to a 37°C water bath for 5 min. The culture was cooled down quickly on ice to room temperature (23°C) for crosslinking, which was done immediately thereafter. The 2 min heat shock was done similarly as described (Boehm et al., 2003). Equal volume of hot media prewarmed at 51°C was added to the room temperature cultures (23°C) to bring the temperature up to 37°C, and the flasks were put in 37°C water bath for 2 min. Crosslinking, immunoprecipitation, and real-time PCR were done as described (Boehm et al., 2003).

**Nuclear Run-On Analysis**

The nuclear run-on assays were done as described (Schwartz et al., 2003a).
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References


O’Connell, P.O., and Rosbash, M. (1984). Sequence, structure, and