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# P-TEFb kinase recruitment and function at heat shock loci

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**P-TEFb, a heterodimer of the kinase Cdk9 and cyclin T, was isolated as a factor that stimulates formation of productive transcription elongation complexes in vitro. Here, we show that P-TEFb is located at >200 distinct sites on *Drosophila* polytene chromosomes. Upon heat shock, P-TEFb, like the regulatory factor HSF, is rapidly recruited to heat shock loci, and this recruitment is blocked in an HSF mutant. Yet, HSF binding to DNA is not sufficient to recruit P-TEFb in vivo, and HSF and P-TEFb immunostainings within a heat shock locus are not coincident. Insight to the function of P-TEFb is offered by experiments showing that the direct recruitment of a Gal4-binding domain P-TEFb hybrid to an *hsp70* promoter in *Drosophila* cells is sufficient to activate transcription in the absence of heat shock. Analyses of point mutants show this P-TEFb stimulation is dependent on Cdk9 kinase activity and on Cdk9's interaction with cyclin T. These results, coupled with the frequent colocalization of P-TEFb and the hypophosphorylated form of RNA polymerase II (Pol II) found at promoter-pause sites, support a model in which P-TEFb acts to stimulate promoter-paused Pol II to enter into productive elongation.**

[*Key Words*: Transcription regulation; elongation; HSF; cyclin T; Cdk9; Polytene chromosomes]

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The regulation of RNA polymerase II (Pol II) transcription can occur at many discrete steps early in the transcription process. These steps include chromatin remodeling, recruitment of TATA-binding protein or Pol II, DNA melting, initiation of RNA synthesis, early elongation, and finally the escape of a fully competent elongation complex (Lis 1998). Whereas transcription regulation is often assumed to be at the level of Pol II recruitment or transcriptional initiation, the processes of early elongation and escape are frequently rate limiting in vivo. One manifestation of control at these later steps is the accumulation of promoter-paused Pol II at a location 20–40 bp downstream of the transcription start site. This pausing is a feature of *Drosophila* heat-shock genes (Lis 1998), a variety of other *Drosophila* genes (Rougvie and Lis 1990; Law et al. 1998), many human genes including c-*myc* (Krumm et al. 1992; Strobl and Eick 1992), c-*fos* (Plet et al. 1995), and hsp70 (Brown et al. 1996) and viral genes such as those encoded by HIV (Laspia et al. 1993). In these cases, Pol II entry, initiation, and early elongation appear much faster than Pol II's escape from the promoter and entry into productive elongation.

Insight into the regulation of these steps in early elongation would be aided by identifying both the changes that occur in the Pol II complex and the promoter-associated proteins that play roles in these changes. One striking change in Pol II as it progresses from its promoter-entry state to its elongationally competent mode is the phosphorylation of the carboxy-terminal domain (CTD) of its largest subunit (Lu et al. 1991; Dahmus 1994). Pol II that pauses as an early elongation complex is largely in a hypophosphorylated state as demonstrated by both immunofluorescence analysis of polytene chromosomes (Weeks et al. 1993) and at higher resolution by in vivo crosslinking/immunoprecipitation assays (O'Brien et al. 1994) by use of antibodies directed to the hypo- and the hyperphosphorylated CTD of Pol II. The distribution of the hypophosphorylated Pol II (Pol IIa) on *Drosophila* polytene chromosomes shows that in addition to being associated with uninduced native *hsp70* gene and *hsp70* transgenes, it is also present at many additional loci (Weeks et al. 1993). Some fraction of these loci could represent genes that, like the uninduced *hsp70* gene, have more paused than transcribing Pol II. This is consistent with the large fraction of genes seen to have appreciable levels of promoter-paused Pol II (Rougvie and Lis 1990; Law et al. 1998). The hyperphosphorylated Pol II, Pol IIo, shows a distribution on chromosomes that only partially overlaps that of Pol IIa, and Pol IIo is the prominent form of polymerase at sites of highly productive elongation such as large developmental puffs (Weeks et al. 1993).

Two kinases have emerged as major candidates for the activities that phosphorylate the CTD of Pol II. The first, TFIIH, is a nine-polypeptide complex that includes a ki-

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nase (Cdk7) and an associated cyclin (cyclin H) (Svejstrup et al. 1996). This kinase can phosphorylate the CTD efficiently. The CTD-kinase activity of yeast TFIIH has been shown to be stimulated in vitro by a coactivator, the mediator complex (Kim et al. 1994). Although several studies have implicated TFIIH in the very early stages of transcription elongation, the precise functional role of the Cdk7 kinase subunit is less clear (Goodrich and Tjian 1994; Dvir et al. 1997; Tirode et al. 1999).

A second CTD kinase, P-TEFb, has been shown to be critical for allowing Pol II to mature into a elongationally competent complex in vitro (Marshall and Price 1995; Peng et al. 1998b; Price 2000). This kinase contains a kinase subunit, Cdk9 (Zhu et al. 1997), and an associated cyclin T that is required for P-TEFb activity (Peng et al. 1998a). The kinase activity of P-TEFb is needed to overcome the effect of negative elongation factors such as the DSIF (Spt4/Spt5 heterodimer) and NELF complexes (Hartzog et al. 1998; Wada et al. 1998) (Yamaguchi et al. 1999a). The CTD of Pol II is a good substrate for P-TEFb, and the nucleoside analog DRB is a potent inhibitor of P-TEFb's kinase activity and also prevents efficient elongation by Pol II (Marshall et al. 1996). These results suggest a model in which P-TEFb acts to phosphorylate the CTD of Pol II in early elongation complexes, and thereby allow the maturation of Pol II into a competent elongation state. However, whether or not the CTD of Pol II is the critical or the sole target of P-TEFb remains to be determined. Interestingly, a region of Spt5 contains a CTD-like region rich in Ser, Thr, and Tyr, and this region has many phosphorylation sites for several protein kinases and may also be a natural substrate for P-TEFb (Yamaguchi et al. 1999b; D. Renner and D. Price, unpubl.).

HIV transcription provides the most striking demonstration of P-TEFb's role in regulating transcription elongation. P-TEFb has been shown to be critical for the efficient generation of full-length HIV transcripts both in vitro and in vivo (Mancebo et al. 1997; Zhu et al. 1997). The viral Tat protein activates HIV transcription through its interaction with the *cis*-acting TAR region at the start of the HIV transcripts. Tat also interacts directly with cyclin T1, one of the three known cyclin partners for Cdk9, and this interaction is required for its activation (Garber et al. 1998; Peng et al. 1998b). Tat's interactions with both HIV RNA and cyclin T1 recruit the kinase to the promoter and appear to be critical in generating elongationally competent Pol II transcription complexes.

In this work, we explore the broader role of P-TEFb in the transcription of cellular genes of *Drosophila*, and concentrate on its specific role in heat shock gene activation. We begin by examining the distribution of P-TEFb on *Drosophila* polytene chromosomes from both unstressed and heat shock-induced larvae by indirect immunofluorescence analysis. We observe that P-TEFb tracks strongly with transcriptionally active puffs formed either during development or in response to heat shock. P-TEFb is recruited to heat shock loci with an intensity and kinetics similar to the upstream regulatory protein, HSF, which is critical for heat shock gene activation (Jedlicka et al. 1997). However, at highly decondensed heat shock loci and loci containing synthetic polymers of HSF-binding sites, P-TEFb and HSF resolve, indicating that HSF does not act alone to recruit P-TEFb. We then examine the functional significance of P-TEFb's robust and rapid recruitment to heat shock loci and demonstrate that direct recruitment of P-TEFb by promoter tethering of either cyclin T or Cdk9 strongly activates *hsp70* gene expression. Finally, we evaluate P-TEFb's distribution on polytene chromosomes relative to a potential target and a product of its kinase activity, specifically the hypophosphorylated Pol II, which is the form of Pol II present at promoter pause sites (Weeks et al. 1993; O'Brien et al. 1994), and the hyperphosphorylated Pol II, which is the form active in transcription elongation (Lu et al. 1991; Dahmus 1994).

## Results

### *P-TEFb is present at numerous chromosomal sites on salivary gland polytene chromosomes*

P-TEFb is required to produce full-length transcripts from a variety of cellular DNA templates in an in vitro transcription system that accurately recapitulates the normal DRB-sensitive transcription seen in cells (Marshall and Price 1995). These results suggest that P-TEFb may have a role in transcription of many cellular genes. If so, this kinase may localize to chromosomal loci that possess genes that are the target of its activity. We examined the chromosomal distribution of P-TEFb by staining salivary gland polytene chromosomes with a highly specific antibody to the cyclin T regulatory subunit. This cyclin T subunit binds tightly to Cdk9 and is a critical component of the P-TEFb activity (Peng et al. 1998a). Moreover, immunodepletion experiments show that the vast majority of Cdk9 is associated with a cyclin T subunit (Peng et al. 1998a), and probing of phosphocellulose fractions from *Drosophila* Kc cell nuclear extracts indicate that cyclin T is present only where P-TEFb activity is found (J. Peng and D.H. Price, unpubl.). Therefore, the cyclin T antibody provides a good means of tracking the P-TEFb complex. Figure 1A is a Western blot that shows the specificity of the affinity-purified cyclin T antibody. The antibody detects a single major band in *Drosophila* nuclear extracts. The minor bands running below cyclin T are likely to be proteolytic products of cyclin T, as they are recognized by crude and affinity-purified antibody, and bands of the same mobility are found to be generated during some purifications of P-TEFb (unpubl.).

Immunofluorescence staining of formaldehyde-fixed, polytene chromosomes with the antibody to cyclin T provides a global view of its distribution on chromosomes. Figures 1B and 2A, and Table 1 show that cyclin T is present at 60 sites at moderate or high levels and another ~140 sites at lower levels. The most prominent sites include chromosomal puffs and contain genes that are transcriptionally active during this stage of development. Even the highly diffuse early, ecdysone-induced

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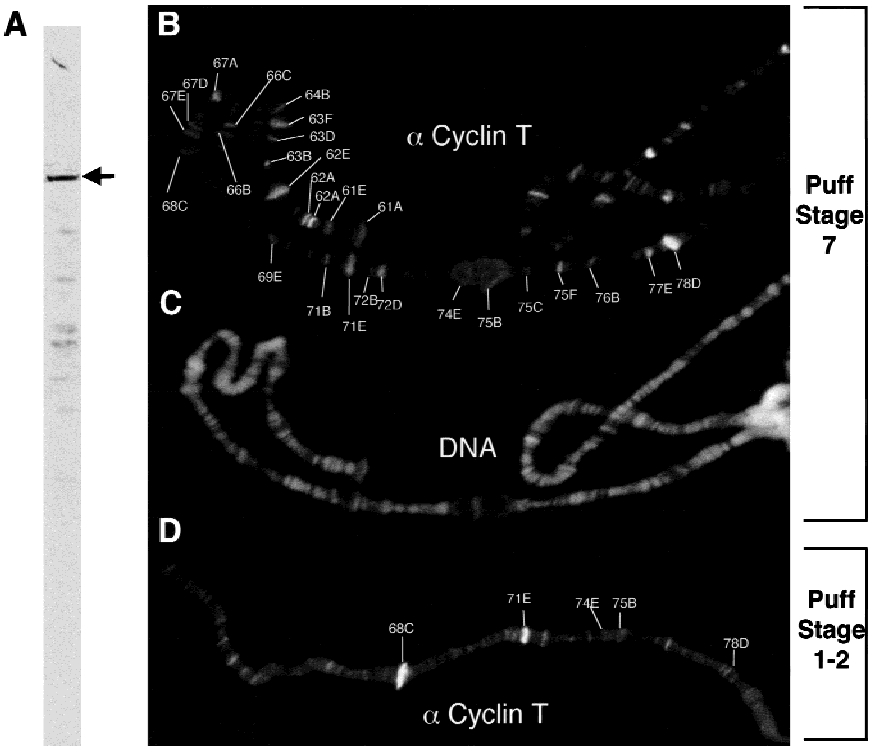


**Figure 1.** P-TEFb shows a specific distribution on *Drosophila* polytene chromosomes. (*A*) Western Blot analysis of the affinity-purified cyclin T (P-TEFb) antibody. Both antiserum and affinity-purified cyclin T antibodies were used to probe a Western blot of *Drosophila* Kc nuclear extracts. Nuclear extracts were prepared as described previously (Price et al. 1987). An aliquot of 13 μg of extract was analyzed in each lane of a 9% SDS–polyacrylamide gel. (*B*,*C*) Staining of polytene chromosomes at puff stage 7 (Ashburner 1972) with cyclin T antibody (*B*) or the DNA stain Hoechst (*C*). Binding of cyclin T antibody was then detected with an anti-rabbit, Texas red-labeled, secondary antibody. The labeled sites on chromosome 3L are identified by their number and letter division. These sites show reproducible labeling in other nuclei of this and other salivary gland spreads of animals at the same developmental stage, but differ from those sites labeled at different stages, e.g., puff stage 1–2 in *D*. The data for all chromosomes are compiled in Table 1. Note that several very minor sites of labeling seen in this spread have not been assigned a band designation. (*D*) Staining of polytene chromosome 3L at puff stage 1–2 (Ashburner 1972) with cyclin T antibody.

puffs show prominent labeling, although the signal is spread over a large area. Table 1 contains the complete listing of sites that show major and moderate labeling (as well as some sites that are more weakly labeled) at this third instar larval stage—specifically at a time when early ecdysone puffs at 74E and 75B are near the end of their maximally active phase [puff stage 7 (Ashburner 1972)]. These large puffs show high levels of P-TEFb (Fig. 1B) as do many of the other major early ecdysone-inducible puffs including 2B5-6, 2B13-17, 62E, 71E, 72D, 88D, 89B, and 93D. At an earlier developmental stage (puff stage 1–2) the 68C puff is near maximal activity and is a major site of P-TEFb localization (Fig. 1D), as are other intermolt puffs at 3C, 71E, and 90BC, which all encode one or more abundantly expressed salivary gland secretion proteins (Crowley et al. 1983; Guild and Shore 1984; Guay and Guild 1991). These changes in the distribution of P-TEFb during development indicate that P-TEFb is directed to a large number of genes when they become active. Although these experiments do not address whether or not cyclin T is providing a critical function at every site it occupies, this pattern of labeling is consistent with its participation in the transcriptional regulation of numerous, but not necessarily all, active *Drosophila* genes.

### *Heat shock triggers the rapid recruitment of P-TEFb to heat shock puff sites*

Heat shock causes a rapid and dramatic activation of transcription of heat shock genes and a concomitant reduction in transcription of many normally expressed genes (Lis et al. 1981b). Immunofluorescence analysis of polytene chromosomes revealed that Pol II relocates to heat shock loci after a brief heat shock (Greenleaf et al. 1978). P-TEFb distribution also changes dramatically following heat shock as seen in Figure 2. In uninduced larvae, P-TEFb is undetectable at major heat shock loci 87A and 87C, which contain the five *hsp70* genes (Artavanis-Tsakonas et al. 1979), or at 59B, which, in this strain, contains an *hsp70–lacZ* transgene (Simon and Lis 1987). After a 20-min heat shock, these and all the other major heat shock loci at 63B, 67B, 93D, and 95D are the prominent sites of labeling (Fig. 2A). Loci that had high levels of P-TEFb before heat shock now have a reduced level. Therefore, P-TEFb redistributes to heat shock loci following heat shock.

The Pol II level on the 5′ end of the *hsp70* gene begins to be elevated in as little as 70 sec following a very rapid heat shock induction (mixing cells with warm medium), and Pol II is detected beyond the pause region and in the middle of the gene in as little as 2 min (O'Brien and Lis 1993). This rapid transcriptional activation leads to a very high density of hyperphosphorylated Pol II on these genes (Weeks et al. 1993; O'Brien et al. 1994). Could P-TEFb be playing a role in the transition of Pol II to its hyperphosphorylated, elongationally competent mode? If so, then one might expect P-TEFb to be recruited as rapidly as Pol II to these newly activated heat shock sites.

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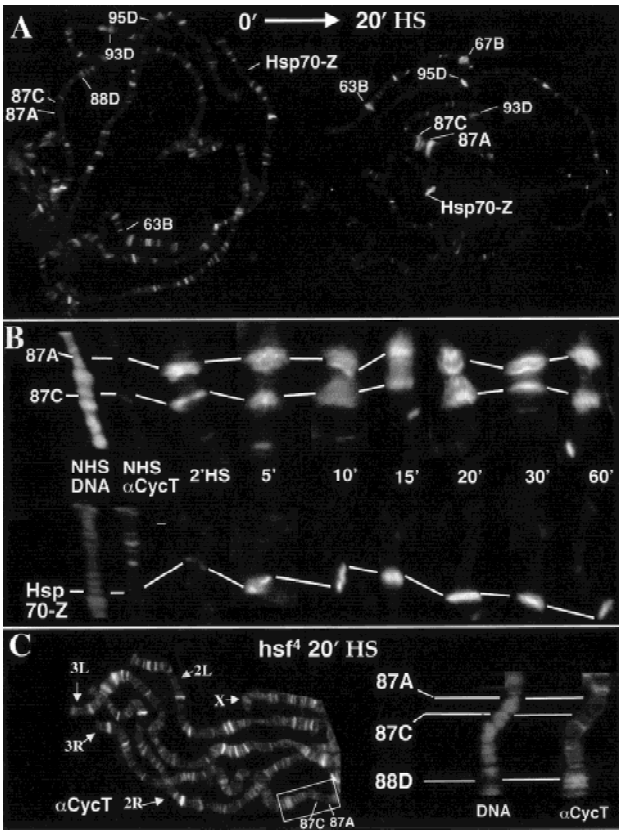


**Figure 2.** Kinetics of recruitment of P-TEFb to heat shock loci. Antibody to cyclin T (αCycT) was used to stain polytene chromosomes, fixed, and spread at various times following heat shock. (*A*) Entire nuclei before and after a 20-min heat shock treatment. Major heat shock loci are identified as well as the control locus 88D that contains a moderate level of P-TEFb before heat shock and is used for comparison *C*. (*B*) The *top* series shows the staining at native heat shock loci 87A and 87C that contain *hsp70* genes as a function of time following heat shock; the *bottom* series shows the 59B locus containing an *hsp70–lacZ* transgene. (*C*) The pattern of cyclin T antibody staining at the 87A and 87C following a 20-min heat shock in the HSF temperature-sensitive mutant (*cn bw hsf4*$^{ts}$) (Jedlicka et al. 1997). An enlargement of a portion (boxed) of a nuclear spread is shown with the corresponding DNA-stained image. The average diameter of these polytene chromosome is ~3 µm.

Figure 2B shows the kinetics of localization to heat shock loci at 87A and 87C and to 59B, which in this strain contains an *Hsp70–lacZ* transgene (Simon and Lis 1987). No P-TEFb is detected at the native or the transgenic sites before heat shock. However, within 2 min, staining is apparent at 87A and 87C, each of which contain multiple copies of *hsp70*. Some staining is also detectable at the transgenic copy of *HSP70–lacZ*. By 5 min of heat shock, staining at all heat shock loci is strong and this high level persists and may even increase in the 10- and 15-min time points. The level remains high during heat shock measured out to 60 min. A shift back to normal fly culture temperature (e.g., a 60-min recovery) reduces heat shock gene transcription and the normal pattern transcription is largely re-established (data not shown).

The recruitment of P-TEFb to heat shock loci is completely dependent on HSF. A *Drosophila* temperature-sensitive mutant HSF strain, *hsf*$^{4}$, shows a much reduced induction of heat shock gene transcription and chromosome puffing (Jedlicka et al. 1997). In this strain, heat shock fails to concentrate P-TEFb at heat shock loci (Fig. 2C). Additionally, heat shock does not lead to a dramatic loss of P-TEFb at the normally active chromosomal sites in the HSF mutant strain as exemplified at 88D (Fig. 2, cf. C to A, left).

### *HSF is not sufficient to recruit P-TEFb to heat shock loci*

Heat shock rapidly stimulates the trimerization and binding of HSF to the heat shock elements (HSEs) located upstream of every heat shock gene. HSF acquires strong DNA-binding activity and localizes to heat shock loci on polytene chromosomes within 2 min following heat shock (Westwood et al. 1991; Weeks et al. 1993). Therefore, the rapid induction of HSF binding is similar to the rapid recruitment of P-TEFb seen here. Could HSF

**Table 1.** *Chromosomal sites of cyclin T*

| X | | 2L | | 2R | | 3L | | 3R | |
|---|---|---|---|---|---|---|---|---|---|
| 1C | + | 21B | + | 42A | + | 61A | ++ | 82D | + |
| 2B5–6 | ++ | 21C | + | 42C | + | 61E | ++ | 82F | +++ |
| 2B13–17 | ++ | 22B | ++ | 43E | ++ | 62A | +++ | 83C | ++ |
| 2F | ++ | 22CD | ++ | 46A | ++ | 62B | ++ | 83F | + |
| 3F | ++ | 23E | + | 47C | ++ | 62E | ++++ | 84F | + |
| 5C | ++ | 23F | ++ | 48B | + | 63DE | ++ | 85C | + |
| 5F | ++ | 25C | + | 50C | ++ | 63F | +++ | 85D | + |
| 8D | + | 26A | ++ | 51C | ++ | 64B | + | 85F | + |
| 9D | + | 26C | + | 52C | + | 66B | ++ | 88D | ++ |
| 10F | + | 26D | + | 52E | + | 66C | ++ | 88F | ++ |
| 12D | ++ | 26F | + | 55B | ++ | 67A | ++ | 89B | ++ |
| 16A | + | 27D | + | 55C | ++ | 67D | ++ | 90B | ++ |
| 16F | ++ | 28B | ++ | 56D | + | 67E | ++ | 91B | + |
| 17C | ++ | 28C | + | 57A | +++ | 68C | + | 91E | ++ |
| | | 29C | ++ | 57F | + | 71B | + | 93B | +++ |
| | | 33B | +++ | 58B | ++ | 71E | +++ | 93D | ++ |
| | | 37F | ++ | 58D | + | 72B | + | 93F | + |
| | | 39A | ++ | 60A | ++ | 72D | ++ | 95F | ++ |
| | | 39C | ++ | 60C | + | 74E | +++ | 96F | ++ |
| | | | | 60F | + | 75B | +++ | 98F | ++ |
| | | | | | | 75C | + | 100E | + |
| | | | | | | 75F | ++ | | |
| | | | | | | 78B | + | | |
| | | | | | | 77E | ++ | | |
| | | | | | | 78D | ++++ | | |

Sites as detected in Fig. 1.
The chromosomes analyzed are from puff stage 7 when early ecdysone-inducible loci are maximally puffed. (+) The relative levels of anti-cyclin T staining of each locus as estimated by eye, represented by an average value from analysis of at least three independent nuclei. Staining: (+++ and ++++) High; (++) moderate; (+) weak. The sites listed are reproducibly detected on different nuclei from this stage. Additional weak and very weak sites were detected; and the total number of sites detected with the cyclin T antibody is ~200.

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itself be sufficient to recruit P-TEFb through a stable interaction? We tested this hypothesis in vivo using a transgenic line containing a polymer of native HSF-binding sites that are unlinked to the rest of the *hsp70* promoter (Shopland and Lis 1996). The structure of the DNA polymer at the transgenic site is illustrated in Figure 3A. Following heat shock, HSF is known to localize to sites on polytene chromosomes containing this polymer (Fig. 3B). This anti-HSF staining is more than an order of magnitude stronger than that seen at the regulatory region of a single *hsp70* gene (Shopland and Lis 1996), and here can be compared with the 87A and 87C loci that contain two and three copies of native *hsp70*, respectively (Mirault et al. 1979). The 87C signal is considerably stronger than 87A, because additional copies to the *hsp70* regulatory region are associated with heat-inducible αβ transcription units present at 87C (Lis et al. 1981a).

If HSF is sufficient to recruit P-TEFb to heat shock loci in vivo, then one would expect to see high levels of P-TEFb at the polymer site. Figure 3C shows that there is detectable P-TEFb at the polymer site, but the level is less than at the native heat shock loci 87A and 87C. Moreover, the ratio of P-TEFb to HSF staining is much higher at heat shock genes than at the polymer site. These results indicate that HSF does not on its own recruit P-TEFb, and other features of the heat shock promoters are required to provide P-TEFb's strong recruitment to heat shock genes.

Figure 3D shows that P-TEFb appears to resolve from HSF at the 87A locus. We observe that in most extended chromosomes examined, the P-TEFb label separates into a doublet with HSF overlapping and falling between the peaks of the P-TEFb doublet. This can be interpreted in terms of the known arrangement of *hsp70* genes at 87A (Ish-Horowicz and Pinchin 1980). The *hsp70* genes are divergently transcribed and the regulatory DNA containing the binding sites for HSEs resides in this region between the genes (Fig. 3E). HSF binds these regulatory regions as seen in the band of fluorescence in the middle of the puff. In contrast, the centers of P-TEFb staining appear to reside downstream of the HSEs on both copies of the *hsp70* gene. The partial separation of P-TEFb and HSF is also consistent with the idea that P-TEFb does not derive its stable association with heat shock genes solely through interaction with HSF.

A biochemical assay of the interaction of HSF and P-TEFb adds further support to the conclusion that these proteins do not interact strongly. We cotransfected plasmids that express HSF, Cdk9–Flag, and cyclin T–6His into *Drosophila* cells. Following a standard heat shock treatment, cleared lysates were prepared from these cells, and the lysates were then chromatographed over nickel–NTA beads, which bind the 6His-tag. Portions of the lysates and nickel-bound fractions were then examined by Western blotting using HSF or Flag antibodies. Figure 3F shows that whereas Cdk9 is efficiently recovered in the Ni-bound fraction, HSF is not recovered at

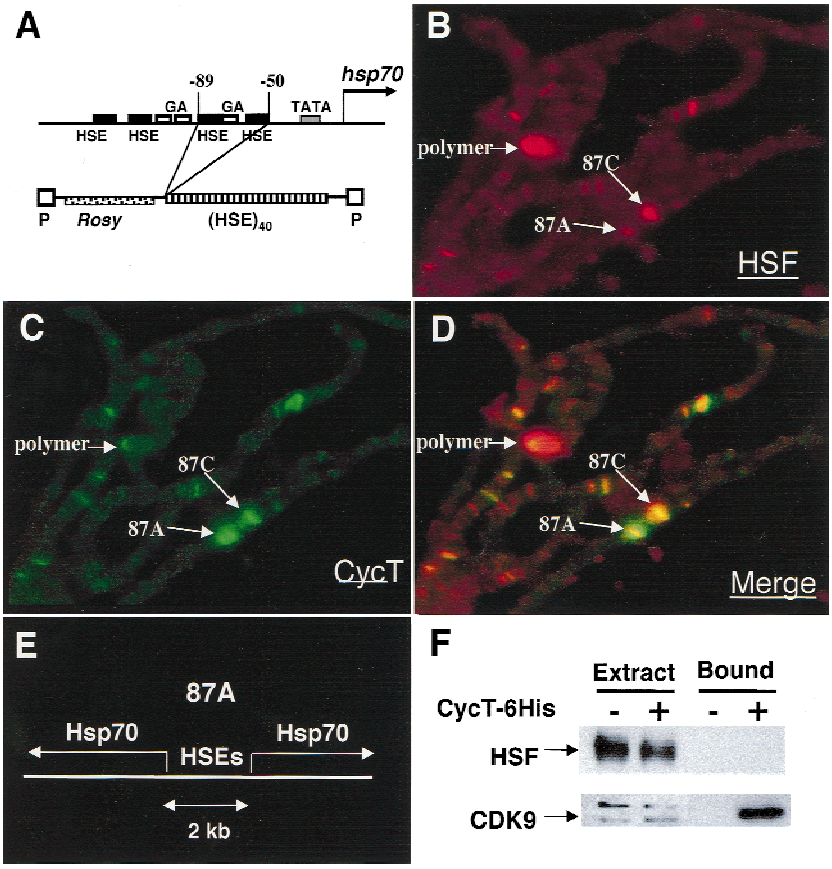


**Figure 3.** The P-TEFb localization on polytene chromosomes does not simply track with HSF. Chromosomes shown in *B–D* are from larvae subjected to a 20-min heat shock treatment. (*A*) Diagram of the transgenic site containing a polymer of 40 copies of the –89 to –50 region of *hsp70* stably transformed into *Drosophila* (Shopland and Lis 1996). (HSE) The heat shock element to which HSF binds; (GA) a GAGA element to which GAGA factor binds (Wilkins and Lis 1997). The *Rosy* gene and P represent components of the transformation vector (Rubin and Spradling 1982). (*B*) Antibody staining of HSF in the transgenic line containing polymers of HSEs. (*C*) The anti-cyclin T (P-TEFb) staining of the same chromosomal spread. (*D*) A merged image of *B* and *C* with HSF as red and cyclin T (P-TEFb) as green. (*E*) Diagram of the 87A locus. (*F*) Absence of a detectable interaction between HSF and P-TEFb in *Drosophila* cell lysates. Total lysates or nickel-bound fractions from cells transfected with HSF, Cdk9-Flag, and 6His–CycT were examined by Western blotting with HSF or Flag antibodies. The arrows indicate the expected mobilities of HSF and Cdl9–Flag, and the upper band in extracts (*bottom*) is a cross-reactive band found also in non-transfected cells.

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levels exceeding the background from cells lacking cyclin T–6His. These results and the in vivo results above indicate that the high levels of P-TEFb association with heat shock loci cannot be explained by an interaction of HSF with P-TEFb.

### *Direct recruitment of cyclin T or Cdk9 to the* hsp70 *promoter stimulates transcription*

Does the redistribution of P-TEFb to heat shock loci influence transcription of the heat shock genes? We tested the effects of directly recruiting P-TEFb subunits, Cdk9 or cyclin T, to the *hsp70* promoter. A pair of Gal4-binding sites (UASgal) was introduced upstream of a *Drosophila hsp70-M* reporter gene (Fig. 4A). The expression of this hybrid reporter gene can be distinguished from native *hsp70* genes as it is marked by fusion to a bacterial DNA sequence. This reporter construct and copper-inducible expression vectors, which express the Gal4 DNA-binding alone (G4) or G4 fused to Cdk9, cyclin T and a variety of controls, were cotransfected into *Drosophila* cells. The inserted UASgal sequences are upstream of the regions critical for heat shock expression, so, as anticipated, transcription of this reporter gene is heat inducible, albeit at about a twofold lower level than the control containing no UASgal insert (Fig. 4B, lanes 2,6, and C). The reporter gene containing UASgal sites is strongly activated without heat shock when cells are cotransfected with G4 fused to the activation domain of HSF (G4–HSF, Fig. 4B, lane 11). The reporter gene carrying the UASgal sites is also strongly activated without heat shock when cells are cotransfected with plasmids expressing G4–Cdk9 or G4-cyclin T (Fig. 4B, lanes 7,9). A point mutation that disrupts the activity of the kinase subunit, Cdk9/D199N (Peng et al. 1998a), also disrupts the ability of the G4–Cdk9 hybrid protein to activate transcription from the *hsp70* reporter (Fig. 4B, lane 8). The levels of expression of wild-type and mutant G4–Cdk9 are similar (Fig. 4D). Also, a pair of mutations in cyclin T that disrupt its ability to interact with Cdk9, the double point mutant CycT/2XMut (Bieniasz et al. 1999), greatly impairs the ability of G4–cyclin T to activate transcription (Fig. 4B, lane 10). These results demonstrate that artificially recruiting P-TEFb to the promoter by directly recruiting either of its two subunits is sufficient to strongly activate an *hsp70* gene. A similar activation by G4–HSF, G4–Cdk9, and G4–cyclin T was observed with UASgal sequences inserted further upstream at –256, although the level of activation was reduced two to threefold (data not shown).

To determine whether this transcriptional activation shows specificity for the P-TEFb kinase, we also examined the effect of artificially recruiting related kinases Cdk2 and Cdk7 to the *hsp70* promoter. Both the G4–Cdk2 or G4–Cdk7 fail to stimulate transcription of the *hsp70* promoter at the level seen for G4–Cdk9 (Fig. 4E). All of these proteins are expressed at comparable levels as shown by Western blotting (Fig. 4F). Additionally, the cotransfection of plasmids expressing G4–Cdk7 with G4–Cdk9 does not stimulate transcription over that pro-

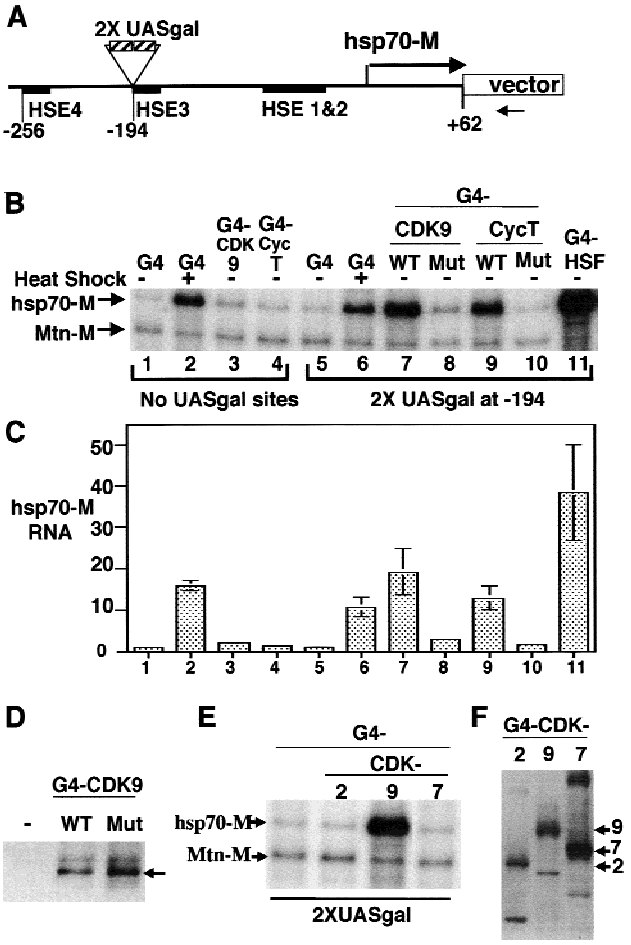


**Figure 4.** Transcription of *hsp70* is stimulated by direct recruitment of cyclin T or Cdk9. (*A*) Diagram showing the *hsp70-M* gene reporter. The 2XGal4 UAS designates that two copies of Gal4-binding site was inserted at –194 of the *Drosophila hsp70* gene. The *hsp70-M* reporter gene is marked at position +62 by fusion to the bacterial *malE* gene. The location of HSEs and start of transcription (base of arrow) are marked, as is the primer (small arrow) used in the primer extension assays. (*B*) The *hsp70-M* reporter RNA expression in *Drosophila* Kc cells transfected with this reporter and various expression vectors. Expression vectors all contain the metallothionein promoter driving the expression of GAl4 DNA-binding domain, amino acids 1–147 (G4), or the G4 fused to full-length Cdk9 and cyclin T proteins and their respective point mutant derivatives (Mut) Cdk9/D199N and CycT/2xMut, or G4 fused to the HSF activation domain (amino acids 462–691). A reporter lacking the UAS insert is used in lanes *1–4*; lanes *5–11* show expression from the UAS-containing reporter diagramed in *A*. RNA was isolated from transfected cells and assayed by the primer extension method (see Materials and Methods). (*C*) Plot showing the average levels of activation and standard deviations from three independent experiments of results obtained as in *B*. (*D*) A Western blot of extracts from transfected cells in *B*. Antibody to the common Gal4-binding domain was used, and the arrow indicates the expected mobility of Cdk9. (*E*) The effect of direct recruitment of related kinases on *hsp70* reporter transcription. The fusions are to the full-length CDKs. (*F*) Western blot of extracts from transfected cells in *E*. Antibody to the common Gal4 DNA-binding domain was used, and the arrows indicate the expected mobilities of the three kinases.

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duced by G4–Cdk9 alone [actually, the level of transcription is slightly reduced relative to G4–Cdk9 alone as would be expected because the inactive G4–Cdk7 will compete with G4–Cdk9 for the G4-binding site on the reporter (data not shown)]. Thus, this stimulation of the *hsp70-M* reporter by direct recruitment of a cyclin-dependent kinase shows specificity for P-TEFb.

### *P-TEFb colocalizes with the hypophosphorylated form of Pol II*

We propose a model in which P-TEFb acts on promoter-paused Pol II complexes to stimulate their escape into productive elongation. If P-TEFb is a major kinase that acts on the promoter-paused Pol II complex, its distribution should overlap at least some of the chromosomal sites that accumulate Pol IIa. However, the correlation need not be perfect, as the rate of formation of a promoter-paused Pol IIa is likely to be governed by mechanisms distinct from those that are responsible for recruiting P-TEFb. These mechanisms appear to be quite independent in an extreme case of heat shock genes, in which Pol IIa is present at full occupancy on the uninduced *hsp70* promoter (Lis 1998), and heat shock is needed to trigger both high levels of transcription (Lis et al. 1981) and recruitment of P-TEFb (Fig. 2). However, when a gene is active, Pol IIa is being continuously recruited to the promoter and maturing into a productive Pol IIo elonagation complex. In the case of heat shock genes, the entry is fast enough to keep the pause region fully occupied with Pol II even when the gene is fully induced (Giardina et al. 1992). Therefore, both the kinase responsible for phosphorylation and the Pol IIa would be expected to be present on active promoters, and their respective levels would be dictated by the relative rates of Pol entry and its maturation into a productive elongation complex.

Figure 5A shows the results of chromosome staining with antibodies to P-TEFb and Pol IIa. Most chromosomal sites in unstressed larvae that are labeled strongly by the P-TEFb (cyclin T) antibody are also labeled to various extents by the Pol IIa antibody; however, the ratio of labeling by these two antibodies at different sites varies. Therefore, the level of Pol IIa must be governed by factors that act at least somewhat independently from factors that govern the level of P-TEFb at specific sites. Nonetheless, the strong tendency of these proteins to colocalize is consistent with a model in which Pol IIa is a substrate for P-TEFb, and this phosphorylation serves to convert Pol II into a productive elongation complex.

The hyperphosphorylated form, PoI IIo, labels many more sites than does P-TEFb (Fig. 5B). Numerous sites are strongly labeled with antibody to Pol IIo, but not detectably labeled with antibody to P-TEFb. This pattern does not easily fit a model in which P-TEFb has a universal role in all Pol II transcription elongation. Presumably, there are distinct mechanisms (and other kinases) for producing Pol IIo that do not require the stable and continuous association of P-TEFb with a locus.

In contrast, there are few chromosomal sites that have P-TEFb, but no Pol IIo. A simple interpretation of this result, which is consistent with the known properties of P-TEFb, is that the recruitment of P-TEFb to a locus generally leads to efficient formation of transcription elongation complexes. These results also indicate that there is little recruitment of P-TEFb to sites that are not transcriptionally active.

## Discussion

P-TEFb was identified as a kinase/cyclin heterodimer that was critical for overcoming an early block to transcriptional elongation (Marshall and Price 1995). Interestingly, the short transcripts of 20–40 nucleotides that

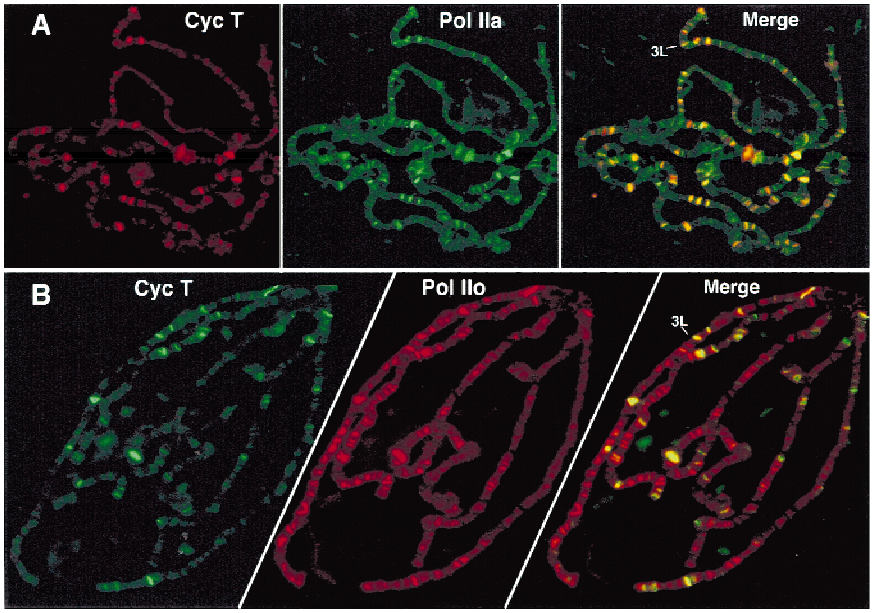


**Figure 5.** (*A*) Colocalization of cyclin T and Pol IIa. Antibody staining of cyclin T on polytene chromosomes from non-heat-shocked larvae (*left*); staining of Pol IIa (*middle*); merged image (*right*). (*B*) Colocalization of cyclin T and Pol IIo. Antibody staining of cyclin T on polytene chromosomes from non-heat-shocked larvae (*left*); staining of Pol IIo (*middle*); merged image (*right*). We use the pseudocolor scheme of Weeks et al. (1993), in which Pol IIa is green and Pol IIo is red. Cyclin T is red in *A* and green in *B*.

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are produced in the absence of P-TEFb were remarkably similar in size to those measured in vivo at genes that show promoter-associated pausing (Rasmussen and Lis 1993). Such pausing has been observed at a variety of genes; however, the heat shock promoters of *Drosophila* are perhaps the most thoroughly studied in eukaryotes (Lis 1998). That P-TEFb stimulates production of full-length transcripts in vitro (Marshall and Price 1995), and also from HIV templates in vivo (Mancebo et al. 1997), provoked us to investigate whether the chromosomal distribution of P-TEFb is consistent with a general role in stimulating transcription elongation on genes that contain paused RNA polymerase. Notably, the heat shock genes of *Drosophila* provide a system in which this can be examined on polytene chromosomes at moderate spatial and temporal resolution.

P-TEFb is normally located at >200 loci, but upon heat shock, it redistributes to native and transgenic heat shock loci with a robustness and rapidity that make it a good candidate for playing a critical role in the activation of heat shock gene transcription. The normally broad distribution of P-TEFb is simplified during heat shock, where the bulk of P-TEFb concentrates at all the major heat shock genes. Whereas P-TEFb is recruited with kinetics that parallel the recruitment of the upstream regulatory factor HSF, and functional HSF is required in vivo for its recruitment, P-TEFb's association with heat shock loci is not simply a consequence of a stable P-TEFb interaction with HSF. A transgenic locus containing a polymer of the binding sites for HSF is capable of very strong recruitment of HSF, but is very ineffective in recruiting P-TEFb, at least relative to P-TEFb's strong recruitment to heat shock genes that have a fully functional promoter. Moreover, HSF and P-TEFb can be seen to resolve spatially at an *hsp70* gene 87A locus, indicating that if HSF has a role in recruiting P-TEFb, it is clearly not the only regulatory or promoter factor involved in maintaining P-TEFb's association with the locus. P-TEFb's recruitment may be stimulated by HSF activity, and some of its strong association with heat shock genes may depend on direct interaction with HSF. However, other macromolecules at the promoter must play a role in recruiting P-TEFb and, together with HSF, could provide the specificity and binding energy for P-TEFb binding to a locus. Alternatively, HSF may not provide any binding specificity for P-TEFb, but instead, it may alter the promoter architecture, and this in turn allows promoter interactions with P-TEFb. Our experiments to date do not have the resolution to distinguish conclusively between these possibilities, although the partial spatial resolution of HSF and P-TEFb at the 87A locus supports the latter view.

The resolution of HSF and P-TEFb staining within the 87A locus is consistent with the long-held view that the DNA within this activated heat shock locus is in a very extended configuration. The two divergently transcribed *hsp70* genes at this locus are separated by only 2 kb (Ish-Horowicz and Pinchin 1980), and yet the P-TEFb staining resolves as two distinct bands. The major HSF staining resides between the two P-TEFb bands (Fig. 3). HSF binding sites are known to reside in the region between the start sites of these genes (Xiao and Lis 1988). If the DNA in a highly decondensed puff approximates B-form DNA, that is, it has a chromatin packing ratio similar to that of highly transcribed ribosomal DNA, then the distance between the start sites of the two *hsp70* genes would be ~0.7 μm. The centers of the two bands of staining are approximately twice that distance, implying that P-TEFb may be distributed over a region that extends downstream of the *hsp70* start sites. Higher resolution biochemical methods will be required to precisely define the limits of the P-TEFb distribution. Nonetheless, the partial resolution of HSF and P-TEFb staining supports a view that these two components act at distinct points in the process of activating heat shock genes.

P-TEFb is not simply recruited by the hypophosphorylated Pol IIa. Pol IIa is the form of Pol II that is at the promoter pause region of *hsp70* and other heat shock genes (Weeks et al. 1993; O'Brien et al. 1994). Yet, very little or no P-TEFb is detected at these sites prior the heat shock. We speculate that a separate event must occur at these promoters to cause the association of P-TEFb, for example, another protein or proteins could recruit P-TEFb to these promoters. In the case of HIV, the Tat protein interacts with cyclin T to recruit the P-TEFb complex (Garber et al. 1998). For normal cellular genes, other host transcription factors may also play such a role; P-TEFb has been shown recently to be functionally recruited to MHC class II gene promoters by the CIITA activator (Kanazawa et al. 2000). Alternatively, transcription activation may normally allow a paused Pol IIa, a likely in vivo substrate of P-TEFb, to undergo a change or unmasking that now allows its association with P-TEFb.

P-TEFb is normally located at many chromosomal sites that are transcriptionally active. The chromosomal loci scored as positive with the cyclin T antibody may represent only a fraction of the genes that could be regulated by P-TEFb, owing to the dynamic developmental regulation of the *Drosophila* genome. Also, the existence of additional cyclin subunits that can couple with Cdk9 may produce a P-TEFb activity lacking cyclin T (Edwards et al. 1998). Although we cannot evaluate whether P-TEFb activates transcription at all of the loci containing cyclin T, in the case of heat shock genes, the direct recruitment (via a Gal4 DNA-binding domain) of P-TEFb to an *hsp70* promoter leads to an activation of this gene in the absence of heat shock. Although this activation is less than the very high level of activation caused by directly recruiting the activation domain of HSF, it is nonetheless clearly dependent on P-TEFb kinase activity. Interestingly, related kinases, Cdk2 and Cdk7, fail to activate this promoter and critical point mutations in the P-TEFb kinase or cyclin T disrupt the activation. The fact that Cdk7, the kinase of the TFIIH complex, fails to activate is worth noting, because it, like P-TEFb, can phosphorylate efficiently the CTD of Pol II. Perhaps these kinases have specificity for discrete steps in transcription. For example, P-TEFb may be capable of stimulating the elongation of the paused Pol II, whereas TFIIH

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kinase fulfills another role such as providing activity for an earlier step in transcription that does not necessarily lead to the full phosphorylation and maturation of elongationally competent Pol II. This specificity issue requires further, more focused investigation.

Direct recruitment of P-TEFb to an HIV promoter has been shown to activate HIV transcription fully and bypasses the need for Tat (Bieniasz et al. 1999). Although the activation of *hsp70* by directly recruiting P-TEFb that we observe in uninduced cells is strong, it is still less than that seen when the HSF activation domain is directly recruited. This fact suggests that HSF may be providing a function beyond triggering the events that lead to P-TEFb recruitment. The HSF activation domain is large enough to accommodate multiple interactions and functions (Wisniewski et al. 1996).

The colocalization of the hypophosphorylated Pol IIa with P-TEFb is intriguing, as the promoter-paused Pol II associated with all genes examined in *Drosophila* is hypophosphorylated (O'Brien et al. 1994). If the Pol IIa distribution is a general indicator of sites in which promoter-pausing is a part of the transcription mechanism, then P-TEFb may be stimulating the maturation of Pol II and its entry into productive elongation at a significant subset of active genes. We note that three of the four constitutively active genes that were reported previously to have promoter-paused Pol II (Rougvie and Lis 1990) are at chromosomal sites that show significant P-TEFb. These three genes, *polyubiquitin*, *β1-tubulin*, and *Gapdh-1* are at 63F2-4, 56D, and 43E respectively—sites that have levels of P-TEFb recorded as +++, +, and ++ (Table 1). The fourth, Gapdh-2, is at 13F, a region that shows light P-TEFb staining, but below a score of +. A higher resolution analysis will be required for a rigorous assignment the P-TEFb signals to these specific genes.

The failure to see a quantitative correlation of the intensity of staining of anti-Pol IIa and anti-P-TEFb at specific sites on polytene chromosomes (Fig. 5) is consistent with models in which the mechanism of generating paused Pol IIa is distinct from the mechanism that recruits P-TEFb. The extreme case of this is *hsp70*, in which, before heat shock, the promoter is fully occupied with Pol IIa, but has very little P-TEFb. Heat shock triggers the dramatic recruitment of P-TEFb (Fig. 2), and the accumulation of Pol IIo on heat shock puffs (Weeks et al. 1993). During heat shock, the paused Pol IIa still forms (O'Brien et al. 1994), but it escapes into productive elongation faster, once every 4 sec as compared with the uninduced level of once every 10 min. We hypothesize that P-TEFb participates in this escape at heat shock genes and the subset of other genes that have promoter-paused Pol II.

## Materials and methods

### *Antibodies*

*Affinity purification of* Drosophila *cyclin T antibodies* Antibodies produced against *Drosophila* cyclin T (Peng et al. 1998a) were affinity purified using a GST fusion protein containing the carboxy-terminal half of the protein. A total of 0.3 mg of the fusion protein containing amino acids 550–1097 from cyclin T (Peng et al. 1998a) was coupled to 0.2 ml of Affi-Gel 10 resin (BioRad Laboratories) according to the manufacturer's instructions. A total of 16 ml of crude antiserum was applied three successive times to a 1-ml column containing immobilized GST to remove anti-GST antibodies and the flowthrough was applied in a similar manner to the cyclin T resin. After washing with 16 ml of PBS, the IgGs were eluted with 3 ml of 50 mM glycine-HCl (pH 2.5) and collected into tubes containing Tris buffer (pH 9) to neutralize the solution.

*Other antibodies* The antibody to HSF was prepared in rat as described previously (Shopland et al. 1995). The antibody to the unphosphorylated CTD of RNA Pol II was the 8WG16 mouse ($IgG_{2a}$/k) antibody from Covance (Richmond, CA), and the antibody to the phosphorylated CTD was the monoclonal mouse H14 (IgM) antibody also from Covance. Fluorescently tagged secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

### *Plasmid construction*

The *hsp70* reporter plasmids were prepared by first inserting the 3′ untranslated sequences of the *ADH* gene from the plasmid pRmHa3 (Clos et al. 1993) into the end of the *hsp70* gene contained on plasmid HIC-L (Kraus et al. 1988). Specifically, an *ADH Sal*I/*Xba*I fragment, with *Xba*I site made blunt by Klenow treatment, was ligated to *Sal*I and *Eco*RV cut HIC-L vector. A portion of the bacterial malE sequence contained on a *Ecl*136II/*Bgl*II fragment of plasmid pMal-C2 (New England Biolabs) was then inserted between the *hsp70* and *ADH* sequences into this same *Sal*I site (after treatment of both vector and insert with Klenow). An *Eco*RV fragment containing two UASgal sites (Kraus et al. 1999) was inserted in the Klenow-filled *Xho*I site at –194 of the *hsp70* reporter plasmid.

The internal standard plasmid was prepared by insertion of the *Sac*I/*Bgl*II fragment from pMalC2 into the *Sac*I/*Bam*HI sites of pRmHa3.

Gal4 fusions were constructed by in-frame insertion of PCR products or plasmid fragments for Cdk2, Cdk9, Cdk7, and cyclin T into the *Nhe*I and *Sal*I sites of plasmid pG (Wisniewski et al. 1996) either directly or after treatment with Klenow where appropriate to maintain the proper reading frame. Where applicable, specific point mutations were introduced by PCR, and verified by sequencing and/or restriction enzyme analysis.

The plasmid for expression of Cdk9–Flag was prepared by inserting a PCR fragment flanked by *Eco*RI and *Xho*I sites containing the Cdk9 ORF fused to a 3′ Flag tag directly into the *Eco*RI and *Sal*I sites of pRmHa3.

The plasmid for expression of CycT–6His was prepared by inserting a PCR fragment flanked by *Eco*RI and *Bcl*I sites containing the CycT ORF directly into the *Eco*RI and *Bam*HI sites of pRmHa3. The 3′ portion of the CycT ORF was then replaced by treatment of the pRm–CycT plasmid with *Xba*I (partial, followed by Klenow treatment) followed by complete digestion with *Xho*I to remove the native CycT 3′ end. This was then replaced with the *Eco*RI (filled in)/*Xho*I fragment from the plasmid pBac-CycT-6His (gift of Zhuoyu Ni, Cornell University).

### *Indirect immunofluorescence*

Salivary gland polytene chromosomes were mounted on slides and stained as described (Shopland et al. 1995) with the follow-

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ing modifications. Coverslips were silanized with Rain-X (Blue Coral-Slick 50, Ltd.). The glands were dissected in 100 µl of Dissection buffer [100 µl of Buffer A (Dequin et al. 1984)] and fixed by a 30″ treatment in Fixing solution [100 µl of Buffer A containing 3.4% EM grade Formaldehyde (Electron Microscopy Sciences, Catalog #15710)] as usual, but the final fixation for 3 min contained only 50% acetic acid. Slides of fixed squashed glands were stained with a 1:20 dilution of affinity-purified cyclin T antibody, and a 1:5 dilution of either RNA Pol II antibodies, 8WG16 and H14, or a 1:50 dilution of HSF alone or in various pairwise combinations. All antibody stainings contained 5% normal donkey serum as a nonspecific blocker (Jackson ImmunoResearch Laboratories, Inc), and incubations were for 1 hr in a moist chamber. The slides were washed, and secondary antibody stainings were performed with a 1:100 dilution of Texas red-conjugated donkey anti-rabbit, or a 1:10 dilution of FITC-conjugated anti-mouse, or a 1:100 dilution of Cy2-conjugated anti-mouse, or 1:100 dilution of rhodamine Red-X-conjugated anti-mouse IgM, or a 1:100 dilution of TRITC-conjugated anti-rat in various pairwise combinations. These secondary stainings also contained 5% normal donkey serum, and were for 1 hr. Slides were washed again, and mounted with 20 µl of glycerol containing 2.5% n-propylgallate and a coverslip. An aliquot of 5 µl of a 1:100 dilution of 0.5 µm of TetraSpeck Fluorescent Microspheres (Molecular Probes, T-7281) was added for aligning color merges. Those slides that were double labeled were examined, images recorded, and then, if necessary, coverslips were removed and slides were stained with 1 µg/ml Hoechst 33258 (Sigma). When the slides were labeled with only one antibody, the slides were stained with 1 µg/ml Hoechst 33258 (Sigma) prior to the mounting of the coverslip. We have found that the frequency of obtaining high quality slides can be improved by using slides that are base treated as follows. Slides are incubated for 2 hr in chamber on orbital shaker containing a solution of 70 grams of NaOH dissolved in 280 ml of water with 420 ml of 95% ethanol added. Slides are washed vigorously with water multiple times and put in a drying oven overnight.

### *Cell transfections and primer extension assays*

*Drosophila* Kc cells were maintained in HyQ-CCM3 medium (HyClone) and plated at a density of ~$1.5 \times 10^6$/ml, 2 ml per 35-mm dish. Lipid/DNA complexes were formed by incubation of ~1 µg each of reporter, standard, and effector plasmids with 9 µl of CellFECTIN (Life Technologies) in 200 µl of medium for 30–45 min at room temperature. Complexes were then diluted to 1 ml with medium, and applied to cells following removal of original medium. After 12–14 hr, transfection mixes were replaced with 2 ml of fresh medium. Following 10–12 hr of recovery, 1 ml of medium was replaced with 1 ml of fresh medium containing 1.4 mM CuSO4. After 12–14 hr of copper treatment, cells were harvested and assayed.

Total RNA was prepared from harvested cells using an RNeasy kit (Qiagen Inc.), and RNA preparations were analyzed for reporter activity by primer extension with reverse transcriptase as described previously (Giardina and Lis 1995). Samples were subsequently treated sequentially with RNase A, phenol extracted, precipitated, and analyzed by polyacrylamide gel electrophoresis.

For Western blot analysis, ~$2 \times 10^5$ cells were lysed by boiling in SDS dye, run on 10% SDS–polyacrylamide gels, and analyzed by immunoblotting with the antibody RK5C1 (Santa Cruz Biotechnology, Inc.), which recognizes the Gal4 DNA-binding domain.

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