RESEARCH COMMUNICATION

Kinetics of promoter Pol II on *Hsp70* reveal stable pausing and key insights into its regulation

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The kinetics with which promoter-proximal paused RNA polymerase II (Pol II) undergoes premature termination versus productive elongation is central to understanding underlying mechanisms of metazoan transcription regulation. To assess the fate of Pol II quantitatively, we tracked photoactivatable GFP-tagged Pol II at uninduced *Hsp70* on polytene chromosomes and showed that Pol II is stably paused with a half-life of 5 min. Biochemical analysis of short nascent RNA from *Hsp70* reveals that this half-life is determined by two comparable rates of productive elongation and premature termination of paused Pol II. Importantly, heat shock dramatically increases elongating Pol II without decreasing termination, indicating that regulation acts at the step of paused Pol II entry to productive elongation.

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Many metazoan genes have a high occupancy of transcriptionally engaged RNA polymerase II (Pol II) paused near their promoters (Bentley and Groudine 1986; Rougvie and Lis 1988; Muse et al. 2007; Zeitlinger et al. 2007; Core et al. 2008). Increasingly, studies indicate that the transition of this promoter-proximal Pol II into productive elongation is one of the major regulatory checkpoints of gene expression. Previous studies hypothesized that the accumulated Pol II at the promoter is either stably paused or iteratively terminating prematurely during early elongation (Bentley and Groudine 1986; Rougvie and Lis 1988). Interestingly, recent reports indicate that both pausing (DSIF and NELF) (Wu et al. 2003; Rahl et al. 2010) and termination (Dcp1a, Xrn2, and TTF2) (Brannan et al. 2012) factors are enriched at metazoan promoters, and their depletions can alter the distribution of Pol II. Therefore, the relative contribution of pausing or premature termination to promoter Pol II accumulation and its regulation is a central question that has yet to be quantitatively addressed in vivo.

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The *Drosophila Hsp70* heat-shock gene possesses a promoter-proximal Pol II that has been extensively characterized (Fuda et al. 2009). Under basal conditions, Pol II on *Hsp70* pauses at sites 20–40 base pairs (bp) downstream from the transcription start site (Rasmussen and Lis 1993; Kwak et al. 2013), producing an accumulation of Pol II at the 5′ end of the gene and a basal distribution of Pol II along the gene body (Lis 1998). Upon heat-shock induction, the escape of paused Pol II to productive elongation as well as the initiation rate increase up to 100-fold, leading to the massive production of *Hsp70* mRNA.

High-resolution live-cell imaging of the polytene chromosomes in *Drosophila* salivary glands provides one method to analyze the kinetics of Pol II induction and elongation during the heat-shock activation of *Hsp70* (Darzacq et al. 2009). The heat-shock-activated endogenous *Hsp70* loci (87A/C) can be easily located in living polytene nuclei because they produce a distinct doublet of intense GFP-Pol II (or RFP)-containing puffs after the activation. Fluorescence recovery after photobleaching (FRAP) can then be used to measure dynamics and elongation rates (Yao et al. 2007). However, the dynamics of Pol II under uninduced conditions has been difficult to assay due to the challenges of locating the endogenous *Hsp70* loci without heat-shock activation.

Notably, many features of the paused Pol II at the uninduced *Hsp70* are similar to the large number (~70%) of active *Drosophila* genes containing paused Pol II (Core et al. 2012). In addition, many of the proteins identified to be involved in *Hsp70* gene regulation have corresponding activities at other genes in various organisms (Fuda et al. 2009). These findings indicate that the mechanisms governing *Hsp70* gene regulation are general. Therefore, in order to gain insights into the kinetic status of the promoter-proximal paused Pol II (paused Pol II that is stable vs. prematurely terminating), we measured the stability of paused Pol II at *Drosophila Hsp70* through a combination of complementing optical and biochemical strategies that resolves previous challenges. We found that promoter-paused Pol II is relatively stable, and its entry to productive elongation, not its termination, is regulated by heat-shock activation.

Results and Discussion

To examine the kinetic fate of paused Pol II, we used an optical approach to measure the stability of Pol II fused to the photoactivatable GFP (paGFP) (Patterson and Lippincott-Schwartz 2002) at the *Hsp70* locus on *Drosophila* salivary gland polytene chromosomes (Fig. 1A). These interphase-like giant chromosomes have been used as a platform for high-resolution imaging of the dynamics of Pol II and other transcription factors by optical pulse-chase experiments at targeted genomic loci (Lis 2007). However, under uninduced basal conditions, identifying the endogenous *Hsp70* gene loci is technically challenging, since the paused Pol II signal from *Hsp70* is too weak to

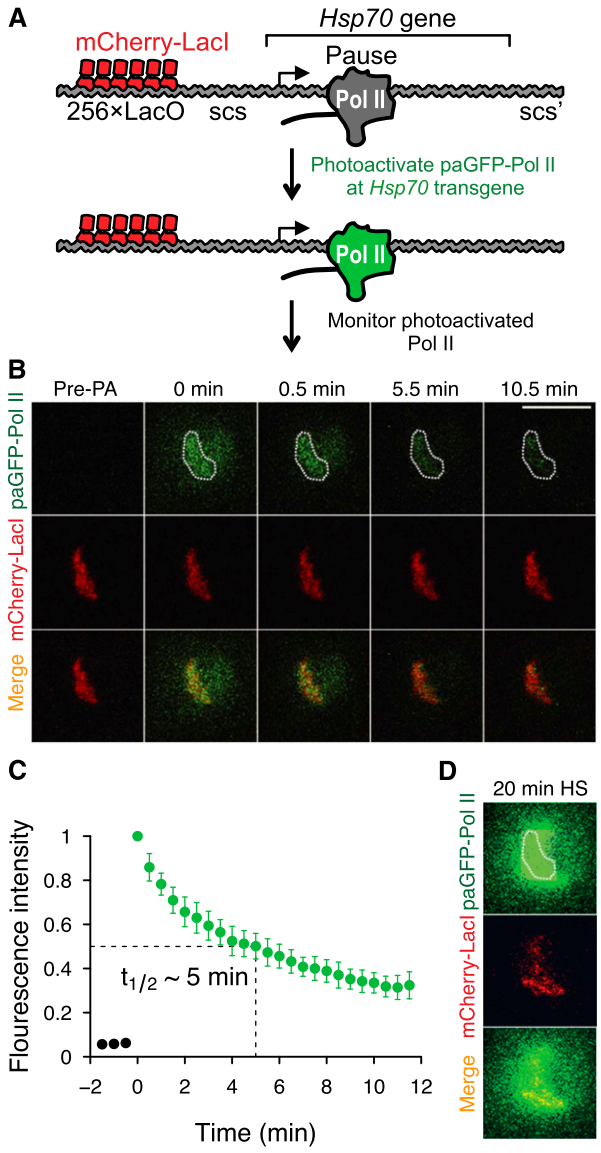

Figure 1. Imaging the stability of paused Pol II at the uninduced *Hsp70* transgene in living cells. (*A*) Schematic of the live-cell imaging experiment showing mCherry-LacI bound to the operator sites (red) and photoactivated Pol II (green). Scs and scs′ are the insulator elements flanking the *Hsp70* gene. (*B*) Uninduced mCherry-marked *Hsp70* transgene before and following the time course after paGFP-Pol II photoactivation. The region of interest is outlined by white dots. (*C*) Normalized fluorescence intensities of paGFP-Pol II fluorescence decay after photoactivation (FDAP) under the uninduced condition (n = 9). (*D*) After heat shock, images of mCherry-LacI, paGFP-Pol II (rephotoactivated after heat shock), and merge at the *Hsp70* transgene. Error bars indicate SD. Bar, 10 μm.

allow it to be easily distinguished from other Pol II signals (Supplemental Fig. 1a).

To circumvent this problem, we generated a transgenic *Hsp70* gene that can be easily identified on the polytene chromosomes for targeted analysis. The full-length *Hsp70* gene is marked with 256 repeats of *Escherichia coli* Lac operator sites (LacO) and can be rapidly identified by coexpressing a fluorescently tagged Lac repressor (mCherry-LacI) in salivary gland nuclei (Fig. 1A). We tested that this LacO-tagged transgenic *Hsp70* is functionally equivalent to the endogenous *Hsp70* gene (Zobeck et al. 2010) by examining the recruitment and levels of Pol II intensity at puffs in response to heat shock (Supplemental Fig. 1b,c). We also confirmed that the paGFP-labeled Pol II subunit (Rpb9) reliably tracks Pol II at both the endogenous *Hsp70* loci and the LacO-tagged *Hsp70* transgene (Supplemental Figs. 2, 3).

The paGFP-Pol II is fluorescently inert at the *Hsp70* transgene before photoactivation (Fig. 1B). To examine the dynamics of paused Pol II at the *Hsp70* loci, we used laser-scanning confocal microscopy to specifically photoactivate paGFP-Pol II at the LacO-marked *Hsp70* transgene under uninduced conditions (Fig. 1A). Figure 1, B and C, shows the time series of the fluorescence decay of paGFP-Pol II at the *Hsp70* transgene. Importantly, the fact that the paGFP-Pol II signal is heat-shock-inducible validates that the locus examined at the single mCherry-LacI band was the *Hsp70* transgene (Fig. 1D). Photobleaching is minimal during imaging (Supplemental Fig. 4), indicating that the decay of signal is due to the release of paGFP-Pol II from the transgene. The resulting decay approximates first-order kinetics with the half-life of ~5 min (Fig. 1C). Because the main form of Pol II at the uninduced *Hsp70* locus is paused Pol II (Core et al. 2012), this clearance half-life of 5 min indicates that paused Pol II is relatively stable but has a finite lifetime (Table 1).

The lifetime of the paused Pol II can be a consequence of the escape into productive elongation, premature termination, or both. To measure the contribution of each to the stability of paused Pol II, we developed an independent biochemical kinetic strategy in *Drosophila* S2 cells (Fig. 2A). First, we evaluated the rate of escape into productive elongation. If a certain fraction of paused Pol II escape into productive elongation every minute (k_{el}), these escaped Pol II will be distributed in the gene body region defined by the speed of elongation. Therefore, the rate of escaping Pol II can then be derived from the speed of Pol II elongation and the relative ratio between gene body and paused Pol II density (Fig. 2B,; Supplemental Material). For this measurement, we used pre-existing nuclear run-on sequencing (GRO-seq or PRO-seq) data sets in S2 cells (Fig. 2C; Core et al. 2012; Kwak et al. 2013), and the elongation speed of 1.5 kb/min for *Hsp70* from previous studies (Yao et al. 2007; Ardehali and Lis 2009). The run-on sequencing results show that at *Hsp70*, an average of 86% of engaged Pol IIs are restricted to the promoter-proximal region, and 13% are distributed in the 2.4-kb gene body region (Fig. 2C). From these estimates, we calculated that k_{el} = 0.094 min^{-1}; that is, 33% of paused Pol II escape into elongation every 5 min (Table 1).

Having measured the amount of paused Pol II that escapes into productive elongation, we then devised a biochemical measurement of the Pol II that terminates prematurely. Paused Pol II that terminates will dissociate from chromatin, and the engaged short nascent RNA will be released (Fig. 2A). Therefore, the amount of free short nascent RNA relative to the chromatin-associated (paused) short nascent RNA will reflect the rate of premature termination (Fig. 2B). We measured the amount of short nascent *Hsp70* RNA from chromatin-associated (paused) and free (terminated) Pol II using biochemical fractionation (Wuarin and Schibler 1994). Short nascent RNA was quantitatively measured by ligation-mediated quantitative RT–PCR (qRT–PCR) targeting most of the

Table 1. *Rate constants for the kinetics of promoter-proximal Pol II at uninduced* Hsp70

Method	Rate	Description	Value (min^{-1})	Half-life (min)	Fate after 5 min
Optical pulse chase	k_{ctrl}	Overall stability	0.135 (0.123–0.147)	5.4 (4.7–5.6)	51% paused
	$k_{t(FP)}$	Elongation blocked by FP	0.072 (0.058–0.086)		70% paused
Biochemical steady state	k_{cl}	Overall stability (= k_{el} + k_t)	0.149 (0.095–0.165)	4.7 (4.2–7.3)	47% paused
	k_{el}	Elongation by GRO-seq	0.094 (0.045–0.103)[a]		33% elongated
	k_t	Termination in steady state	0.055 (0.035–0.075)		19% terminated

[a]The range estimate of the elongation rate was provided by Yao et al. (2007).

Hsp70 pausing region within 25–40 bp downstream from the transcription start site (Supplemental Fig. 5). The nascent RNA of the free fraction was, on average, 45% of the chromatin-bound fraction level in uninduced *Hsp70* (Fig. 2D, UI), suggesting that a significant amount of Pol II may be terminating from the uninduced *Hsp70* pause sites.

For a quantitative estimation of the paused Pol II termination rate, we analyzed the steady-state kinetics of short *Hsp70* nascent RNA termination and decay (Fig. 2A; Supplemental Material). At steady state, the decay rate of the free nascent RNA should be equal to its production rate from the terminating Pol II. Therefore, the termination rate can be derived from the free RNA decay rate and the ratio between free and chromatin-bound short nascent RNA (Fig. 2B). To estimate the decay rate, we blocked the RNA production at the initiation step by using Triptolide, a potent chemical inhibitor of the TFIIH helicase XPB (Titov et al. 2011), and measured the time course of free nascent RNA decay. The time course showed a decay half-life of ~6 min and the decay constant k_d = 0.123 min^{-1} (Fig. 2E). This decay constant, when combined with the ratio between free and chromatin-bound short nascent RNA (described above), allows the calculation of the termination constant k_t = 0.055 min^{-1}, meaning that 19% of paused Pol II terminate prematurely every 5 min (Table 1). The rate of clearing of paused Pol II is the sum of termination and escape to elongation; adding these rates gives a Pol II that has a clearance kinetic constant (k_{cl}) of 0.149 min^{-1}, which corresponds to the half-life of 4.7 min (Table 1). Therefore, the two independent methods—biochemical steady-state kinetics and optical pulse-chase measurements—are in close agreement with each other.

P-TEFb (positive transcription elongation factor b) kinase activity is required for the escape of paused Pol II into productive elongation at most genes, including *Drosophila Hsp70* (Lis et al. 2000; Chao and Price 2001; Ni et al. 2008; Rahl et al. 2010). To obtain an independent estimate of the early termination rate of paused Pol II in vivo, salivary glands were treated with the P-TEFb kinase inhibitor Flavopiridol (Chao and Price 2001; Ni et al. 2008) to block transcription elongation (Fig. 3A). Assaying the fluorescence decay after photoactivation (FDAP) of paGFP-Pol II at the uninduced *Hsp70* transgene then reveals the loss of Pol II by mechanisms that are independent of P-TEFb activity, and we assume this is mainly by termination (Fig. 3B). We further analyzed the fluorescence using a quantitative decay model in both control and Flavopiridol treatment (Supplemental Fig. 6). The decay curves show the stabilization of Pol II (Fig. 3C; Supplemental Fig. 7) and near doubling of the half-life with Flavopiridol treatment (Fig. 3D; Table 1), indicating that the elongation and termination can also be distinctly measured using optical methods in vivo. Together, the optical and biochemical analyses provide a strong indication that paused Pol II is relatively stable in uninduced cells but does undergo slow transitions to both productive elongation or premature termination in living cells. This is additionally supported by a very recent independent and different approach showing that many other active *Drosophila* genes also have stably paused Pol II (Henriques et al. 2013).

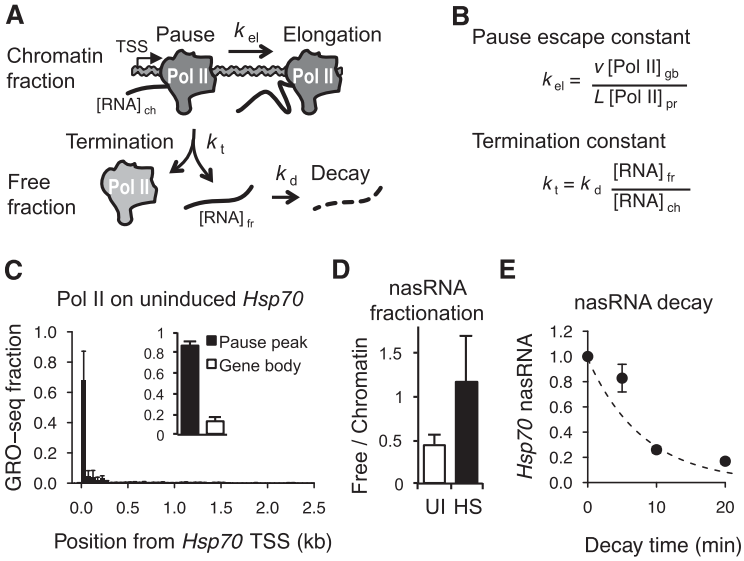

Figure 2. Biochemical analysis of steady-state paused Pol II kinetics. (*A*) Schematic showing the kinetic fates of paused Pol II and short nascent RNA (nasRNA). (k_{el}) Kinetic constant of paused Pol II elongation; (k_t) kinetic constant of paused Pol II termination; (k_d) short nascent RNA decay constant; ($[RNA]_{fr}$) free *Hsp70* short nascent RNA; ($[RNA]_{ch}$) chromatin-bound *Hsp70* short nascent RNA. (*B*) The equations used to calculate the pause Pol II elongation and termination rate constants (see the Supplemental Material). (v) Elongation rate; (L) gene length; ($[Pol\ II]_{pr}$) Pol II fraction in pause region; ($[Pol\ II]_{gb}$) Pol II fraction in gene body region. (*C*) Estimation of k_{el} from GRO-seq (and PRO-seq) read fractions in *Hsp70* (50-bp bins; n = 14 independent data sets). The *inset* shows read fractions in pause region (−50 to +250 from the transcription start site [TSS]) and gene body (+300 to 2.4 kb). (*D*) Measuring the ratio between free and chromatin-bound short nascent RNA by qRT–PCR in uninduced (UI; n = 9) and 15-min heat-shock (HS; n = 8) conditions. (*E*) Estimation of k_d from free *Hsp70* short nascent RNA decay after Triptolide (10 μM) addition (qRT–PCR). Each time point is normalized to the pretreatment level. (*C–E*) Error bars indicate the SEM.

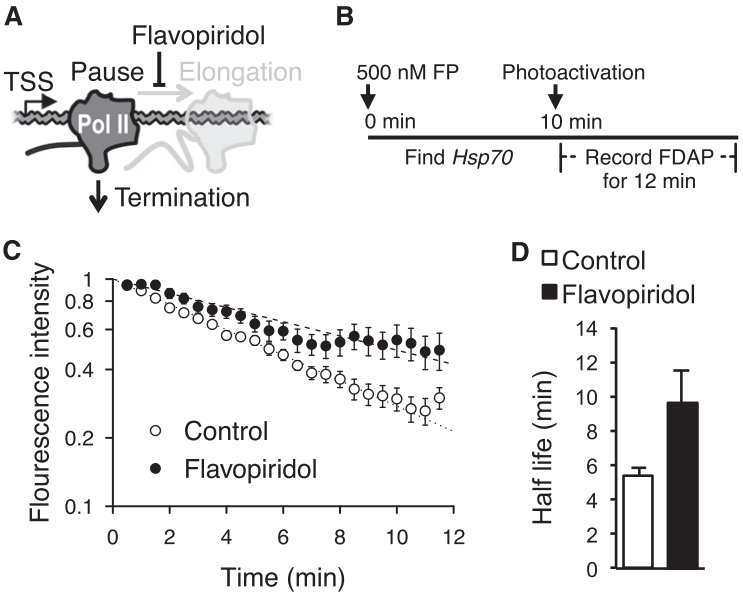

Figure 3. Optical measurement of the termination rate of paused Pol II at the *Hsp70* transgene in living cells. (*A*) Schematic diagram outlining overall logic of using Flavopiridol to measure paused Pol II termination kinetics. (*B*) Illustration of the experimental scheme. (*C*) Semi-log plot of normalized fluorescence intensities of paGFP-Pol II under uninduced condition in control (n = 9) and with Flavopiridol treatment (n = 7). Data are corrected for background Pol II signal (see the Materials and Methods). Error bars indicate SD. (*D*) Half-lives of paused Pol II decay with Flavopiridol treatment and control. (*C*,*D*) Error bars indicate SEM.

The fact that paused Pol II can terminate raises the intriguing possibility that premature termination may play a role in *Hsp70* gene regulation, as demonstrated in the examples of prokaryotic or viral promoters (Kao et al. 1987; Gollnick and Babitzke 2002). We assessed the contribution of premature termination to the regulation of *Hsp70* expression level upon heat-shock induction based on our kinetic findings. The rate of Pol II escape into elongation rapidly increases after the heat-shock induction. This increase is equal to the initial rate of Pol II recruitment to the activated *Hsp70*, which was measured previously using a high-temporal-resolution recruitment study of Pol II in living cells (Zobeck et al. 2010), and here we estimate this rate to be about six molecules per minute per promoter (Fig. 4A). If all prematurely terminating Pol II are converted to productively elongating Pol II, the expected increase would be 0.055 molecules per minute per promoter (Table 1), which explains only a small fraction of the elongating Pol II (Fig. 4B). In addition, we observed an increase rather than the decrease of terminated nascent RNA fractions from paused Pol II upon heat shock (Fig. 2D, HS), opposite to the expectation if down-regulation of premature termination contributes to the heat-shock induction. Collectively, these findings provide a comprehensive assessment of paused Pol II kinetics of elongation and termination on *Hsp70* (Fig. 4C). Although cycles of initiation and premature termination occur at the promoter-proximal Pol II on *Hsp70*, this process is slow, and Pol II at the pause is relatively stable; the changes in premature termination do not appear to contribute to *Hsp70* activation. Rather, the regulation of the *Hsp70* gene upon heat shock occurs mainly through stimulation of escape from the stable pause.

Our optical approach addresses the long-standing question of paused Pol II stability. One possible interpretation of the optical analysis is that the photoactivated paused Pol II in uninduced cells may be frequently terminated and recycled at the same locus. However, the paused Pol II is known to be phosphorylated at Ser5, and it is the unphosphorylated form of Pol II that is used in the reinitiation step (Laybourn and Dahmus 1990). Additionally, we observed previously in FRAP assays that, during the early phases of induction of the *Hsp70* gene, Pol II dissociates from the locus rather than recycling and only partially recycles after accumulating at the locus at high concentrations (Yao et al. 2007; Zobeck et al. 2010). In addition, we further provide orthogonal, biochemical evidence of the stability of paused Pol II using nascent RNA, which can be assessed independently from recycling.

The molecular mechanism of premature termination still remains to be investigated. 3′ RNA processing and termination factors may have a role in this termination by targeting the nascent RNA engaged with paused Pol II near the promoter (Brannan et al. 2012). However, nascent RNA extending outside of the Pol II complex may not have enough accessibility for RNA processing factors, since the positions of promoter-proximal pausing in *Hsp70* are, like many other paused genes in *Drosophila*, relatively close to the transcription start site (Rasmussen and Lis 1993; Kwak et al. 2013). Nonetheless, this mechanism may still exist if additional premature termination takes place after Pol II escapes pausing in nearby downstream regions or for other *Drosophila* genes shown to have more distal pausing (Kwak et al. 2013).

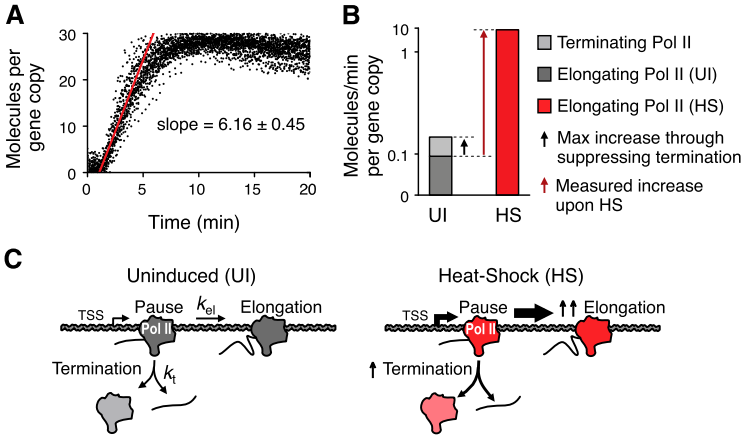

Figure 4. Kinetics of early elongating Pol II at *Hsp70* during heat-shock induction. (*A*) Estimation of initial Pol II escape rate using live-cell imaging at the endogenous *Hsp70* upon heat shock. The imaging data from a previous study (Zobeck et al. 2010) were analyzed (n = 33). (*B*) Contribution of premature termination to Pol II escape rate upon heat-shock induction. (UI) Uninduced condition; (HS) heat-shock induction. The *Y*-axis is in a nonlinear scale. (*C*) Model of early elongating Pol II kinetics under uninduced and heat-shock-activated conditions. The width of an arrow reflects the magnitude of the rate.

The prolonged pausing itself may also lead to spontaneous Pol II termination, similar to what was observed in Pol II pausing at DNA damage sites (Somesh et al. 2005; Anindya et al. 2007). In addition, it is possible that a trailing or a newly initiating Pol II molecule may collide with the paused Pol II and result in the termination of the leading Pol II molecule, as seen in in vitro studies (Saeki and Svejstrup 2009). This may explain our observation of increased short terminated transcripts under heat-shock induction, where initiation is much more frequent. Thus, premature termination appears to be a consequence of the promoter dynamics rather than a control mechanism for Pol II productive elongation.

Materials and methods

FDAP of polytene nuclei

Intact *Drosophila* salivary glands were dissected from third instar larvae and transferred to Grace's medium. For drug experiments, glands were transferred to 500 nM Flavopiridol (Sigma-Aldrich) diluted in medium. Laser-scanning confocal microscopy of salivary glands was carried out on a Zeiss 710 microscope. The mCherry-LacI-tagged *Hsp70* transgene was identified using a 561-nm laser. Samples were photoactivated using a circular region of interest limited to the dimensions of the mCherry-LacI spot using a 405-nm laser. The fluorescence of both the mCherry-LacI and Rpb9-paGFP was imaged using 561- and 488-nm lasers every 30 sec for 12 min. Photoactivation curves were normalized for the first image to equal 1. Half-lives were obtained by fitting the FDAP data to an exponential component using a grid-search regression in a mixed linear decay model. To confirm that the *Hsp70* gene was targeted, an objective preheated to 37°C (Bioptechs) was used to heat-shock samples for 20 min, and the locus was rephotoactivated.

Biochemical steady-state kinetic analysis

The rate constant of elongating Pol II from pausing (k_{el}) was derived from GRO-seq and PRO-seq data (Core et al. 2012; Kwak et al. 2013) in *Drosophila* S2 cells at the *Hsp70* gene: $k_{el} = v\lambda/[\text{Pol II}]_{pr}$, where v is the Pol II elongation speed (in kilobases per minute), λ is the gene body Pol II density (in reads per kilobase), and $[\text{Pol II}]_{pr}$ is the level of promoter-proximal Pol II (in reads). The kinetic constant of Pol II termination (k_t) was determined from nascent RNA fractionation in S2 cells: $k_t = k_d([\text{RNA}]_{fr}/[\text{RNA}]_{ch})$, where $[\text{RNA}]_{ch}$ is the nascent RNA in chromatin fraction, $[\text{RNA}]_{fr}$ is the nascent RNA in free fraction, and k_d is the free nascent RNA decay constant. Nascent RNA fractionations were carried out as described previously (Wuarin and Schibler 1994), and a short spike-in RNA sequence from an *Arabidopsis* gene (RCP1) was added for the normalization after ligation-mediated qRT–PCR analysis.

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