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.

Supplemental material is available for this article.

Received September 30, 2013; revised version accepted December 3, 2013.

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). Interestingly, recent reports 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). Notably, many features of the paused Pol II at the *Drosophila* Hsp70 gene 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 reveal pol II 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

[Keywords: RNA polymerase II, promoter-proximal pausing, termination, escape to productive elongation, photoactivation, nascent RNA]

© 2013 Buckley et al. This article is distributed exclusively by Cold Spring Harbor Laboratory Press for the first six months after the full-issue publication date (see http://genesdev.cshlp.org/site/misc/terms.xhtml). After six months, it is available under a Creative Commons License (Attribution-NonCommercial 3.0 Unported), as described at http://creativecommons.org/licenses/by-nc/3.0/.
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 IIIs 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

![Figure 1](image-url)
Our analysis further suggests that the loss of Pol II by mechanisms that are independent of P-TEFb activity, and we assume this is mainly due to 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 $k_{cl} = 0.149$ 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].

### 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         | $k_{el}$       | Overall stability            | 0.135 (0.123–0.147) | 5.4 (4.7–5.6)  | 51% paused      |
|                            | $k_{el,FP}$    | Elongation blocked by FP     | 0.072 (0.058–0.086) |                | 70% paused      |
| Biochemical steady state    | $k_d$          | Overall stability ($= k_d + k_t$) | 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].

**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). ($k_d$) Kinetic constant of paused Pol II elongation; ($k_t$) kinetic constant of paused Pol II termination; ($k_{cl}$) short nascent RNA decay constant; ($[RNA]_{ch}$) free Hsp70 short nascent RNA; ($[RNA]_{pr}$) 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). (C) Estimation of $k_{cl}$ 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 $k_t$ 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.
The fact that paused Pol II can increase the intriguing possibility that premature termination may play a key 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).
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 using a 405-nm laser. The fluorescence of both the mCherry-LacI and the locus was rephotoactivated.

Biochemical steady-state kinetic analysis

The rate constant of elongating Pol II from pausing \( k_{d} \) was derived from GRO-seq and PRO-seq data (Laybourn and Dahmus 2012; Laybourn et al. 2013) in Drosophila S2 cells at the Hsp70 gene. \( k_{d} = v_{r} / [\text{Pol II}]_{p} \), where \( v_{r} \) is the Pol II elongation speed (in kilobases per minute), \( \lambda \) is the gene body Pol II density (in reads per kilobase), and \( [\text{Pol II}]_{p} \) is the level of Pol II elongation speed (in kilobases per minute).

Acknowledgments

We thank Jie Yao (Yale University) for providing the range estimates of the elongation rate on Hsp70. This work was supported by a grant from the National Institute of Health (NIH GM087003 to M.S.B.) and the Howard Hughes Medical Institute (to H.K.).

References

Anindya R, Aygün O, Svejstrup JQ. 2007. Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1. Mol Cell 28: 386–389.

Ardehali MB, Lis JT. 2009. Tracking rates of transcription and splicing in vivo. Nat Struct Mol Biol 16: 1123–1124.

Bentley DL, Groudine M. 1986. A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature 321: 702–706.

Brannan K, Kim H, Erickson B, Glover-Cutter K, Kim S, Fong N, Kiemele L, Hansen K, Davis R, Lykke-Andersen J, et al. 2012. mRNA decapping factors and the exonuclease Xrn2 function in widespread premature termination of RNA polymerase II transcription. Mol Cell 46: 311–324.

Chao SH, Price DH. 2001. Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription in vivo. J Biol Chem 276: 31793–31799.

Core LJ, Waterfall JJ, Lis JT. 2008. Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. Science 322: 1845–1848.

Core LJ, Waterfall JJ, Gilchrist DA, Fargo DC, Kwak H, Adelman K, Lis JT. 2012. Defining the status of RNA polymerase at promoters. Cell Rep 2: 1025–1035.

Darzacq X, Yao J, Larson DR, Causse SZ, Bosanac L, de Turris V, Ruda VM, Lionnet T, Zenklusen D, Guglielmi B, et al. 2009. Imaging transcription in living cells. Annu Rev Biophys 38: 173–196.

Fuda NJ, Ardehali MB, Lis JT. 2009. Defining mechanisms that regulate RNA polymerase II transcription in vivo. Nature 461: 186–192.

Gollnick P, Bahitze P. 2002. Transcription attenuation. Biochim Biophys Acta 1577: 240–250.

Henriques T, Gilchrist DA, Nechaev S, Bern M, Muse GW, Burkholder A, Fargo DC, Adelman K. 2013. Stable pausing by RNA polymerase II provides an opportunity to target and integrate regulatory signals. Mol Cell 52: 517–526.

Kao SY, Calman AE, Luciw PA, Peterlin BM. 1987. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature 330: 489–493.

Kwak H, Fuda NJ, Core LJ, Lis JT. 2013. Precise maps of RNA polymerase reveal how promoters direct initiation and pausing. Science 339: 950–953.

Laybourn PJ, Dahmus ME. 1990. Phosphorylation of RNA polymerase IIA occurs subsequent to interaction with the promoter and before the initiation of transcription. J Biol Chem 265: 13165–13173.

Lis JT. 1998. Promoter-associated pausing in promoter architecture and posttranscriptional regulation. Cold Spring Harb Symp Quant Biol 63: 347–356.

Lis JT. 2007. Imaging Drosophila gene activation and polymerase pausing in vivo. Nature 450: 198–202.

Lis JT, Mason P, Peng J, Price DH, Werner J. 2000. P-TEFB kinase recruitment and function at heat shock loci. Genes Dev 14: 792–803.

Muse GW, Gilchrist DA, Nechaev S, Shah R, Parker JS, Grissom SF, Zeitzling J, Adelman K. 2007. RNA polymerase II pausing is poised for activation across the genome. Nat Genet 39: 1507–1511.

Ni Z, Saunders A, Fuda NJ, Yao J, Suarez J-R, Webb WW, Lis JT. 2008. P-TEFB is critical for the maturation of RNA polymerase II into productive elongation in vivo. Mol Cell Biol 28: 1161–1170.

Patterson GH, Lippincott-Schwartz J. 2002. A photoactivatable GFP for selective photolabeling of proteins and cells. Science 297: 1873–1877.

Rahl PB, Lin CY, Seila AC, Flynn RA, McCuine S, Burge CB, Sharp PA, Young RA. 2010. c-Myc regulates transcriptional pause release. Cell 141: 432–445.

Rasmussen EB, Lis JT. 1993. In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes. Proc Natl Acad Sci USA 90: 7923–7927.

Rougvie AEA, Lis JT. 1988. The RNA polymerase II molecule at the 5’ end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged. Cell 54: 795–804.

Sacki H, Svejstrup JQ. 2009. Stability, flexibility, and dynamic interactions of colliding RNA polymerase II elongation complexes. Mol Cell 35: 191–205.

Somesh BP, Reid J, Liu W-F, Søgaard TMM, Erdjument-Bromage H, Tempst P, Svejstrup JQ. 2005. Multiple mechanisms confining RNA polymerase II ubiquitylation to polymerases undergoing transcriptional arrest. Cell 121: 913–923.

Titov DV, Gilman B, He Q-L, Bhat S, Low W-K, Dang Y, Smeaton M, Demain AL, Miller FS, Kugel JF, et al. 2011. XFP, a subunit of TRF1I, is a target of the natural product triptolide. Nat Chem Biol 7: 182–188.
Stable Pol II pausing regulates *Hsp70*

Wu C-H, Yamaguchi Y, Benjamin LR, Horvat-Gordon M, Washinsky J, Enerly E, Larsson J, Lambertsson A, Handa H, Gilmour D. 2003. NELF and DSIF cause promoter proximal pausing on the *hsp70* promoter in *Drosophila*. *Genes Dev* 17: 1402–1414.

Wuarin J, Schibler U. 1994. Physical isolation of nascent RNA chains transcribed by RNA polymerase II: Evidence for cotranscriptional splicing. *Mol Cell Biol* 14: 7219–7225.

Yao J, Ardehali MB, Fecko CJ, Webb WW, Lis JT. 2007. Intracellular distribution and local dynamics of RNA polymerase II during transcription activation. *Mol Cell* 28: 978–990.

Zeitlinger J, Stark A, Kellis M, Hong J-W, Nechaev S, Adelman K, Levine M, Young RA. 2007. RNA polymerase stalling at developmental control genes in the *Drosophila melanogaster* embryo. *Nat Genet* 39: 1512–1516.

Zobeck KL, Buckley MS, Zipfel WR, Lis JT. 2010. Recruitment timing and dynamics of transcription factors at the *Hsp70* loci in living cells. *Mol Cell* 40: 965–975.