

Transcription regulation during stable elongation by a reversible halt of RNA polymerase II

Tim Patrick Kaminski, Jan Peter Siebrasse, and Ulrich Kubitscheck

Institute of Physical and Theoretical Chemistry, Rheinische Friedrich-Wilhelms-University Bonn, 53115 Bonn, Germany

ABSTRACT Regulation of RNA polymerase II (RNAPII) during transcription is essential for controlling gene expression. Here we report that the transcriptional activity of RNAPII at the Balbiani ring 2.1 gene could be halted during stable elongation in salivary gland cells of *Chironomus tentans* larvae for extended time periods in a regulated manner. The transcription halt was triggered by heat shock and affected all RNAPII independently of their position in the gene. During the halt, incomplete transcripts and RNAPII remained at the transcription site, the phosphorylation state of RNAPII was unaltered, and the transcription bubbles remained open. The transcription of halted transcripts was resumed upon relief of the heat shock. The observed mechanism allows cells to interrupt transcription for extended time periods and rapidly reactivate it without the need to reinitiate transcription of the complete gene. Our results suggest a so-far-unknown level of transcriptional control in eukaryotic cells.

Monitoring Editor

William P. Tansey
Vanderbilt University

Received: Feb 20, 2014

Revised: May 13, 2014

Accepted: May 14, 2014

INTRODUCTION

DNA transcription is the first step of gene expression, and modulation of transcriptional activity is a primary regulatory stage of this process. For a long time the regulated assembly of the preinitiation complex (PIC) was seen as the only regulatory step of transcription. Meanwhile, a complex postinitiation regulatory network of gene expression at the level of transcription has been known in eukaryotes—for example, by promoter-proximal pausing of RNA polymerase II (RNAP II; Rougvie and Lis, 1988) or premature transcription termination (Neil *et al.*, 2009). Promoter-proximal pausing, which was first observed at heat shock genes of *Drosophila* (*Sophophora melanogaster*) and the *MYC* gene of HL60 cells (Bentley and Groudine, 1986; Gilmour and Lis, 1986), was for a long time perceived as an isolated phenomenon, but now it is considered as an important general regulatory mechanism for numerous

genes (Margaritis and Holstege, 2008). Several regulatory interactions after formation of the PIC at the promoter are known. A key regulatory element is the C-terminal domain (CTD) of RNAPII, comprising a repetitive heptapeptide ($Y_1S_2P_3T_4S_5P_6S_7$). Posttranslational modifications of the CTD, especially phosphorylation of the serine residues, act as a transcriptional status indicator and form a scaffold coordinating the binding of various regulatory factors (Buratowski, 2009).

After PIC formation, the DNA double strand is melted and the synthesis of the first nucleotides begins. This may result in abortive initiation, when only a short portion, two or three nucleotides, of the RNA are synthesized. Alternatively, RNAPII dissociates from the PIC and enters early elongation. The dissociation is believed to be supported by phosphorylation of Ser-5 of the CTD (Nechaev and Adelman, 2011). After the transition to early elongation, the transcript remains stably associated with the transcription complex. During early elongation, there exists a tendency for backtracking and arrest of the early elongation complex. Promoter-proximal pausing takes place, when RNAPII needs additional factors for transition into productive elongation. The release from the pause can be very rapid, allowing highly dynamic regulation of transcription (reviewed in Saunders *et al.*, 2006). The transition into productive elongation is triggered by the phosphorylation of CTD Ser-2, DRB sensitivity-inducing factor, and negative elongation factor by positive transcription elongation factor b (P-TEFb; Nechaev and Adelman, 2011).

After entering productive elongation, RNAPII is believed to proceed without a major stop throughout the remainder of the gene (Saunders *et al.*, 2006). Several studies showed that productive elongation is a discontinuous process on short time scales (Darzacq *et al.*, 2007; Zhou *et al.*, 2013). Obstacles, topological constraints,

This article was published online ahead of print in MBoC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-02-0755>) on May 21, 2014.

Address correspondence to: Ulrich Kubitscheck (u.kubitscheck@uni-bonn.de).

Abbreviations used: aa, amino acids; BR, Balbiani ring; Cdk, cyclin-dependent kinase; CTD, c-terminal domain; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; FISH, fluorescence in situ hybridization; GRD, glycine-rich domain; HIV, human immunodeficiency virus; hrp, heterogeneous nuclear ribonucleoprotein; mRNP, messenger ribonucleoprotein particle; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PIC, preinitiation complex; P-TEFb, positive transcription elongation factor b; RNAPII, RNA-polymerase II; ROI, region of interest; Rpb, RNA polymerase B subunit; RRM, RNA recognition motif.

© 2014 Kaminski *et al.* This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (<http://creativecommons.org/licenses/by-nc-sa/3.0>).

“ASCB®,” “The American Society for Cell Biology®,” and “Molecular Biology of the Cell®” are registered trademarks of The American Society of Cell Biology.

DNA lesions, or certain sequences can cause short-term pausing of elongation complexes (Gómez-Herreros *et al.*, 2012). Additional regulatory processes may affect the elongation rate or induce pausing of RNAPII, which can affect splice-site recognition or termination (Kornblihtt *et al.*, 2004). Pausing of RNAPII results in continuation of transcription or backtracking of RNAPII (Gu and Reines, 1995). By backtracking, RNAPII loses contact between its active site and the extendable RNA 3'-OH end (Cheung and Cramer, 2011). Backtracked polymerases may become arrested. Once arrested, RNAPII requires accessory factors to resume elongation (Selth *et al.*, 2010). Numerous such accessory factors ensure that arrest of RNAPII does not become permanent. Permanently arrested RNAPII would block transcription of the gene, which could be lethal, depending on the affected gene. Several mechanisms exist to either reactivate stalled RNAPII or remove irreversibly arrested RNAPII to ensure clearance of the gene (Svejstrup, 2007). Not reactivatable, arrested RNAPII is targeted by monoubiquitinylation (Gómez-Herreros *et al.*, 2012), which can result in recovering processivity by recruiting accessory factors. As a last resort, Lys-48-linked polyubiquitinylation leads to proteosomal degradation of Rpb1, the largest subunit of RNAPII, and disassembly of the elongation complex to ensure clearance of the gene (Malik *et al.*, 2008; Wilson *et al.*, 2013). Preventing Rpb1 ubiquitinylation in yeast results in a lethal phenotype (Somesh *et al.*, 2007).

A mechanism that literally pushes RNAPII through pause sites is collision of a stalled RNAPII by a trailing RNAPII (Saeki and Svejstrup, 2009). Transcription of eukaryotes often occurs in bursts, leaving only distances of several hundred base pairs between individual elongation complexes (Lamb and Daneholt, 1979; Chubb *et al.*, 2006; Raj *et al.*, 2006). Such an arrangement allows elongation complexes just to stop forward movement for several seconds before they clash with a following RNAPII. This full elastic rear-end collision induces a force on the leading RNAPII, which helps to overcome the obstacle (Saeki and Svejstrup, 2009).

In eukaryotes, premature termination at protein-coding genes is described only for the transcription of *NRD1* in yeast and HIV transcription. Both transcription processes depend on the regulated readthrough of termination signals (Kessler and Mathews, 1992; Arigo *et al.*, 2006). In summary, several mechanisms ensure the processivity of RNAPII. Proteosomal degradation seems to be a last resort, which suggests that losing the largest RNAPII subunit and the transcript is evolutionary unfavorable.

Several modes of transcription regulation have been described so far. However, a general synchronous and reversible halt of all RNAPII of a gene at productive elongation has not yet been discovered.

The salivary gland cells of *Chironomus tentans* larvae contain polytene chromosomes consisting of 8200 or 16,400 perfectly aligned single chromatids exhibiting a characteristic banding pattern (Beermann, 1952; Lamb and Daneholt, 1979). Chromosome IV contains the so-called Balbiani ring (BR) genes, which code for especially large salivary proteins. On transcription of the BR genes, these chromosomal regions expand and form characteristic puffs, which can extend up to ~20 μm into the nucleoplasm and are well discernible by light microscopy. A single RNAPII needs ~20 min to synthesize the complete BR2.1 mRNA of the 30- to 35-kb-long BR2.1 gene (Lamb and Daneholt, 1979; Paulsson *et al.*, 1992), which contains five exons. Exon 4 comprises a high number of short repetitive



FIGURE 1: Schematic representation of BR2.1 gene structure. The repetitive sequence motifs, which were targeted by the used oligonucleotides, are indicated by yellow lines. They are distributed along almost the complete gene sequence. The lengths of the exons are 445, 6, 43, 26,000–31,000, and 609 base pairs for E1–E5, respectively, and are indicated by red bars; the intron lengths are 1009, 108, 1380, and 55 base pairs for introns 1–4, respectively. The data are taken from Paulsson *et al.* (1992).

sequences (Figure 1). BR2 genes are transcribed in a burst-like manner by transcriptional units. A single transcriptional unit contains in average of 123 RNAPII complexes with a distance of ~184 base pairs in between (Lamb and Daneholt, 1979). It is well established that during heat shock, BR gene expression is rapidly and efficiently down-regulated (Lezzi *et al.*, 1981). We exploited this effect to analyze transcription and its regulation using the well-defined native BR2 genes. We discovered a specific transcription regulation mechanism during productive elongation: a general, reversible halt of the RNAPII transcribing the BR2 gene. We propose this effect as a general transcriptional regulation mechanism.

RESULTS

We analyzed RNAPII-dependent transcription in salivary glands of heat-shocked and control larvae using RNA fluorescence in situ hybridization (FISH), immunostaining, potassium permanganate probing of transcription bubbles, and a recently developed *in vivo* transcription activity assay. This *in vivo* fluorescence assay represented a straightforward method to detect ongoing, active transcription sites in *C. tentans* salivary gland cells without using transcription inhibitors or isolation of nuclei (Siebrasse *et al.*, 2012; Kaminski *et al.*, 2013).

Using *hrp36* as probe to monitor RNAPII activity *in vivo*

The heterogeneous nuclear ribonucleoprotein (hnRNP) *hrp36* is a small mRNA-binding protein (297 amino acids [aa]; $M_r \approx 32.4$ kDa) and the *C. tentans* homologue of mammalian hnRNP A1 and *Drosophila* Hrb87F/Hrp40. It consists of three domains: two RNA-binding domains called RNA recognition motif (RRM) 1 and 2 and a C-terminal glycine-rich domain (GRD), which is required for steric regulation of *hrp36*–RNA interactions. It has been shown that multiple *hrp36* molecules are stably incorporated into nascent pre-mRNPs and then escort the mRNPs from the transcription site to the translation site in the cytosol (Visa *et al.*, 1996). This robust association has been used to track single mRNPs during nuclear trafficking and export after microinjection of fluorescent *hrp36* fusion proteins in salivary gland cells (Siebrasse *et al.*, 2008, 2012). However, since the incorporation of *hrp36* into pre-mRNPs is a nonrandom process and strictly dependent on ongoing mRNA transcription, it can be used as probe for active transcription sites. After microinjection of the recombinant, fluorescence-labeled *hrp36* the protein was quickly enriched at active transcription sites, which then appeared as intensely stained bands on the polytene chromosomes of the gland cells (Figure 2A and Supplemental Figure S1A). The characteristic polytene transcription pattern was lost when the salivary glands were pretreated with the RNAPII-specific inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB; Supplemental Figure S1B) or α -amanitin (Supplemental Figure S1C). DRB targets the elongation factor P-TEFb. It blocks the kinase activity of the Cdk9 subunit and hence Ser-2 phosphorylation of the CTD of the largest subunit of RNAPII at the heptapeptide repeats. α -Amanitin inhibits

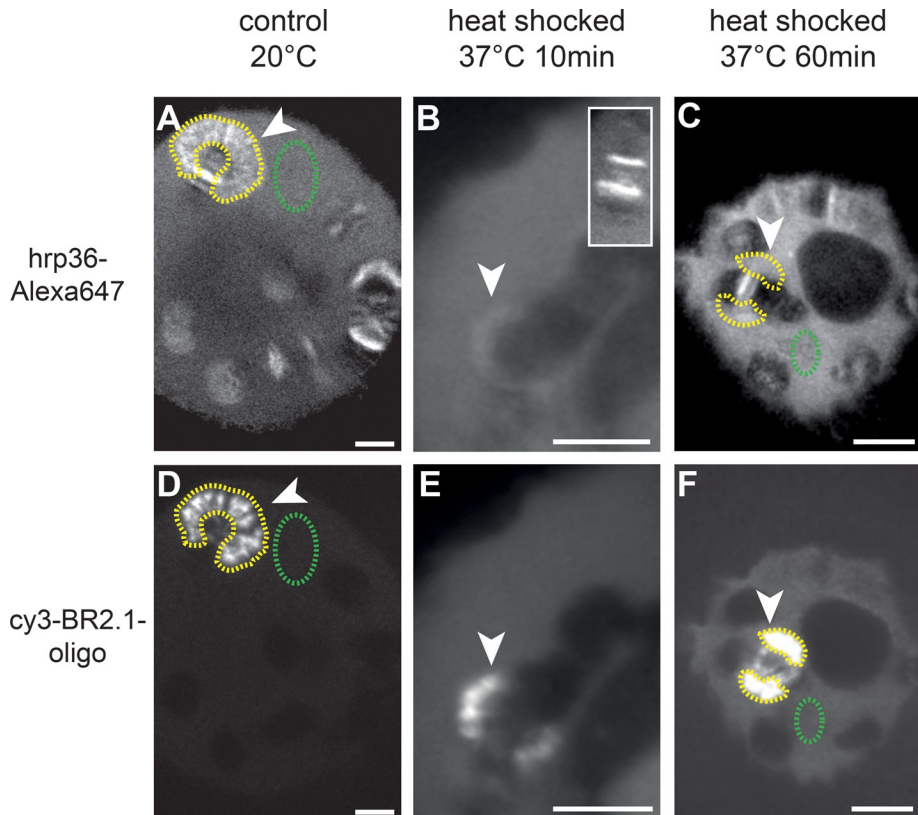


FIGURE 2: A stable transcription halt of BR2.1 was induced by heat shock, but the BR2.1 transcript remained at the BR transcription site. The images show confocal two-color fluorescence sections of salivary gland cell nuclei, in which the active transcription sites were labeled by hrp36–Alexa Fluor 647 (A–C), and the BR2.1 mRNA were labeled by the Cy3-BR2.1-oligo (D–F). The vertically arranged images show the same nuclei in the two different channels, respectively. Control salivary gland cells show active transcription at the BR2 puff (A) and the presence of high levels of BR2.1 mRNA (D). A heat shock for 10 min halted active transcription at most sites (B). The inset shows active transcription sites in this nucleus in a different Z-plane. BR2.1 mRNA was still present at the BR2 puff (E). Even after 1 h of heat shock, no transcriptional activity at the BR2 puff was detectable (C), whereas it still contained a high level of BR2-mRNA (F). The dashed lines label exemplary ROIs, which were used for quantification in Figure 3. The BR2.1 sites are marked by arrowheads; bars, 10 μm .

transcription by direct interaction with the polymerase, thereby blocking its flexible bridge helix. The presence of α -amanitin interferes with the translocation of the enzyme complex on the DNA and considerably decreases its activity (Bushnell *et al.*, 2002). At higher α -amanitin concentrations, RNA polymerase III is also blocked, but since hrp36 does not bind to rRNA, the observed effect is obviously caused by RNAPII inhibition. The characteristic accumulation at the transcription sites was also completely suppressed when fluorescent hrp36 was coinjected with the DNA-intercalating drug actinomycin D (Supplemental Figure S1D). The inhibition controls proved that the hrp36 staining pattern was completely RNAPII dependent. However, mRNP–hrp36 engagement required more than simple mRNA binding, which becomes evident from an hrp36 deletion mutant that contained the RRM 1 and 2 domains but was lacking the C-terminal GRD. After coinjection of red-labeled wild type and green-labeled Δ GRD mutant, the wild-type but not the mutant hrp36 accumulated at the transcription sites (Supplemental Figure S1E). We concluded that hrp36 was cotranscriptionally and stably integrated into mRNPs as a structural component, and thus the respective labeling demonstrated ongoing transcription. Active transcription sites labeled by the fluorescent hrp36 displayed the

characteristic polytene banding pattern (Figure 2A; Beermann, 1952; Siebrasse *et al.*, 2012; Kaminski *et al.*, 2013). Of course, the characteristic BR2 puff was distinctly labeled by fluorescent hrp36 (Figure 2A). In the following experiments we used the presence or absence of hrp36 labeling as a proof of ongoing or halted transcription, respectively.

Monitoring transcription and transcripts before and during heat shock

It had been observed that a heat shock rapidly down-regulated the expression of most genes in *C. tentans*, as, for example, BR gene expression (Lezzi *et al.*, 1981). Accordingly, we observed labeling of the BR2 puff by hrp36 under normal conditions (Figure 2A) but did not observe significant labeling of the BR2 puff when we microinjected hrp36 after a heat shock of 10 (Figure 2B) or 60 min (Figure 2C). Obviously, the transcriptional activity at the BR2 puff was strongly diminished after 10 min, and after 1-h heat shock, transcription activity at the BR2.1 site completely ceased, as could be deduced from the absence of any hrp36 signal. Still, transcription continued at selected other sites (Figure 2, B and C).

The characteristic BR2 transcription site could be well recognized by visual inspection. We marked it additionally by nuclear injection of fluorescently labeled oligonucleotides complementary to sections of the BR2.1 mRNA (Figure 2D). This resulted in a strong intranuclear ring of fluorescence. Because the BR is approximately donut shaped, the appearance of the imaged structure depended on the actual position of the BR2 within the nucleus and how it was captured

by the optical section of the confocal microscope. To our surprise, we detected high amounts of BR2.1 mRNA at the BR2 transcription site not only after 10 min, but also still after 60 min of heat shock (Figure 2, D–F). Finally, we tested the presence of BR2 mRNA after 2 h of heat shock and found that the BR mRNA was still observable at the BR (Supplemental Figure S4).

These qualitative observations were then quantified by image processing. Regions of interest (ROIs) corresponding to either the BR2 puff or chromatin-free control regions were defined (e.g., see dashed lines in Figure 2). The ratio of the fluorescence intensity within these ROIs and the control ROI was calculated pixelwise, and the resulting values from several glands were pooled in respective histograms (Figure 3). Accumulation of fluorescent probes, either hrp36 or oligonucleotides, resulted in ratios >1 , as seen in the control experiments (Figure 3A; three glands, 1 nucleus/gland, average 1.52) due to the high amount of transcriptional activity at the BR2 puff. Evaluation of the hrp36 concentration at BR2 puffs of larvae that were heat shocked for 60 min before hrp36 injection demonstrated clearly that the transcriptional activity had stopped: the average normalized intensity was <1 (0.92; 11 larvae, 1 nucleus/gland; Figure 3B). Supplemental Figure S2 shows the results for the

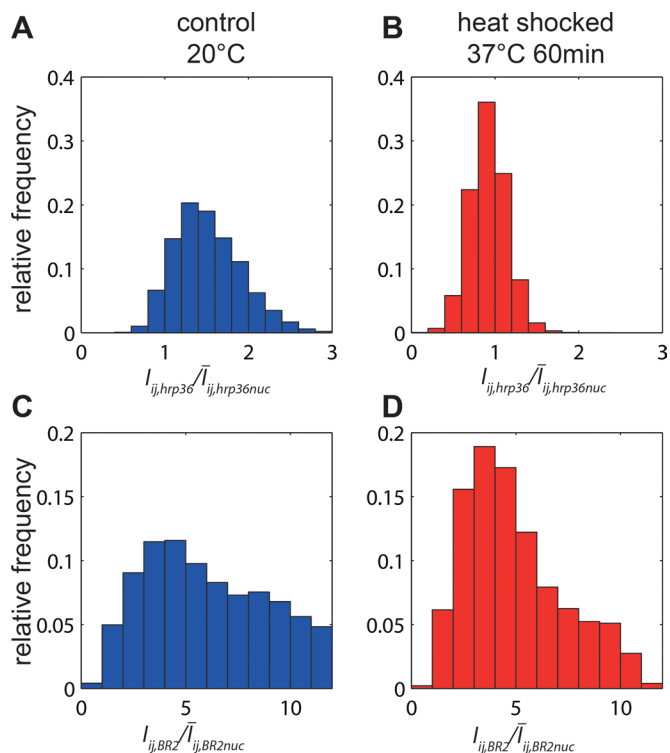


FIGURE 3: Quantification of the transcriptional activity and transcript concentration at the BR2 sites before and after heat shock. Hrp36 fluorescence intensity at (A) BR2 sites of control gland cell nuclei and (B) BR2 sites of 60-min heat-shocked gland cells. BR2.1 oligonucleotide fluorescence intensity at (C) BR2 sites of control gland cell nuclei and (D) BR2 sites of 60-min heat-shocked gland cells. The fluorescence intensity values in the region of the BR2 (BR2 region of interest [BR2-ROI]) were determined and normalized to the respective average concentration in the nucleoplasm. In control cells with normal transcriptional activity, hrp36 was clearly enriched at the BR2 puff (mean intensity, 1.44). The amount of hrp36 at the transcription sites in heat-shocked animals was close to the background (mean value, 0.93). The BR2.1 mRNA concentration at the BR2 puff was only slightly affected by the heat shock. The data in A and C are based on 11 independently examined glands, and those in B and D are based on 3 independently analyzed glands. Examples for BR2-ROI are shown in Figure 2 in yellow and those for background ROIs in green.

individual nuclei, proving the high degree of reproducibility of this experiment. We also quantified the presence of BR2 mRNA at the BR2 puff (Figure 3, C and D). In contrast to the transcription activity probe hrp36, the amount of BR2 mRNA at the BR2 puff was not altered by the heat shock.

Monitoring RNAPII and transcription bubbles before and during heat shock

Because we observed that the BR2 transcripts were still located at the transcription site, although the heat shock had stopped transcription since 60 min, we asked whether possibly also RNAPII was still present at the transcription site. Thus we probed for the presence of RNAPII by immunofluorescence of heat-shocked and control glands. Figure 4 shows unambiguously that RNAPII was present not only at the BR2 puff, but also actually at numerous other common transcription sites. The control and heat-shocked samples showed principally the same staining pattern. RNAPII binds stably to the DNA, but the observation of transcriptionally inactive RNAPII

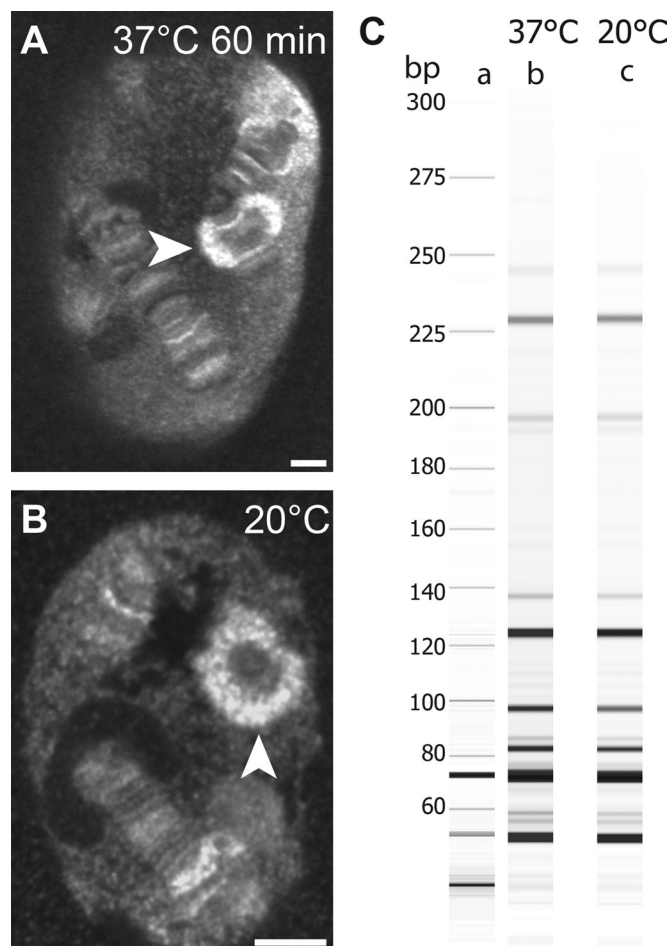


FIGURE 4: RNAPII-transcribing BR2 genes stopped transcriptional activity upon heat shock but remained at the BR2 puff and still formed transcription bubbles. Immunostaining of RNAPII in (A) heat-shocked and (B) control salivary glands. Arrowheads indicate the BR2 site. Bars, 10 μ m. (C) After \sim 1 h without transcriptional activity, RNAPII still formed open complexes at the BR2.1 gene. (a) DNA size marker. “Virtual gel view” of capillary gel electrophoresis results demonstrated that the RNAPII distribution at BR2.1 of (b) heat-shocked salivary glands matched the RNAPII distribution of (c) control salivary glands. Peak positions and their intensity were remarkably similar.

after \sim 60 min at the transcription site was again unexpected, since several mechanisms exist that rapidly remove persistently stalled RNAPII during productive elongation, as discussed in the *Introduction*.

Finally, we wanted to know whether RNAPII was still in a transcriptionally competent state, that is, whether it still formed transcription bubbles. Thus the presence of transcription bubbles at the BR2.1 gene was tested by potassium permanganate footprinting and subsequent linker-mediated PCR (LM-PCR). The BR2.1 gene encodes a saliva protein. To examine the sensitivity and specificity of the potassium permanganate footprinting, midgut tissue, which does not transcribe the BR2.1 gene, was analyzed as negative control. The results of this control are shown in Supplemental Figure S3. We found that after a 60-min heat shock, transcription bubbles were still present in the BR2 gene and displayed the same appearance and distribution as those of the control (Figure 4C). The genomic primers used for the assay were complementary to a repetitive sequence motif of exon 4, whose location is well known

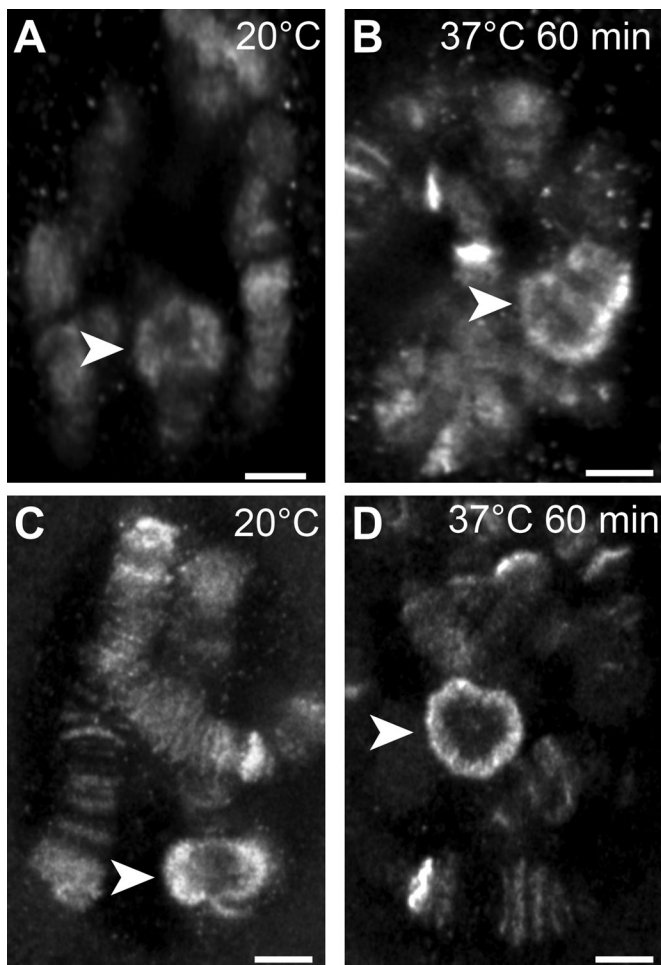


FIGURE 5: The transcription halt was not accompanied by notable changes of the Rpb1-CTD phosphorylation pattern. (A) Immunostaining of RNAPII phospho S2 CTD of a control salivary gland. (B) Immunostaining of RNAPII phospho S2 CTD of heat-shocked larvae (1-h heat shock). (C) Immunostaining of RNAPII phospho S5 CTD of control salivary gland. (D) Immunostaining of RNAPII phospho S5 CTD of larvae that had been heat shocked for 1 h. Bars, 10 μ m.

(Figure 1). We concluded that the detected open transcription bubbles were located at least \sim 1600 base pairs downstream of the promoter and \sim 2000 base pairs upstream of the termination site. The observed transcriptional halt and retention of RNAPII at the BR2.1 gene after heat shock was linked to neither a regulatory mechanism at the transition from early to productive elongation nor impaired termination.

The phosphorylation of the C-terminal domain of RNAPII was not altered during heat shock

The phosphorylation of the CTD of RNAPII is essential for its regulation and acts as a transcription status indicator for several regulatory factors (Phatnani and Greenleaf, 2006). We speculated that the observed transcription halt during heat shock might have been a consequence of a modified phosphorylation state of RNAPII. This possibility was examined by immunofluorescence using antibodies specific for phosphorylated RNAPII Ser-2 and -5. However, no reduction of RNAPII Ser-2 and -5 phosphorylation was detected on comparing control and 60-min heat-shocked salivary gland cell nuclei by immunostaining (Figure 5). The Ser-2 phosphorylation

revealed that during heat shock at the BR2 puff, remaining RNAPII already entered productive elongation (Figure 5B). Thus the loss of transcription activity was not linked to a major change in RNAPII CTD phosphorylation.

Halted RNAPII at the BR2 transcription site can be reactivated

Halted RNAPII that remained at the BR2.1 transcription site was still bound to the DNA during heat shock, as indicated by the presence of transcription bubbles. To examine whether the halted RNAPII was reactivated after release of the heat shock, we used two different transcription inhibitors.

In the first experiment, we used flavopiridol, which is a very potent inhibitor of the transition from initial to productive elongation (Bensaude, 2011). Like DRB, it inhibits the Cdk9 subunit of P-TEFb but can be used at much lower concentrations. Application of this inhibitor inhibits the start of new stable transcription processes but allows the completion of ongoing ones. Early transcription processes are not detected by the complementary oligonucleotides used here. Salivary glands dissected from heat-shocked animals for 60 min were examined for the presence of BR2.1 mRNA immediately after the heat shock (control) and then again after incubation for 60 min at 20°C in hemolymph containing 300 nM flavopiridol. As before, the fluorescent BR2.1-specific 2'-O-methyl-RNA oligonucleotides were microinjected into dissected gland cell nuclei and the respective mRNA marked. Imaging of the salivary gland cells revealed again the characteristic BR2.1 mRNA enrichment at the BR transcription site (Figure 6A). The samples that had additionally been incubated at room temperature with flavopiridol, however, did not show any specific fluorescence staining of the BR2 puff (Figure 6B), which can easily be visually recognized. We concluded that the halted and bound RNAPII complexes had been reactivated and completed the transcription processes that had temporarily been halted due to the heat shock. As a control, we incubated salivary glands under the same experimental settings after heat shock in hemolymph containing 300 nM flavopiridol and 5 μ g/ml actinomycin D. Actinomycin D inhibits the overall transcription activity by intercalation in GC-rich DNA sequences. We expected the halted RNAPII complexes to remain bound to the BR2 puff. Indeed, in this case we observed no reduction of BR2 mRNA at the BR2 transcription site compared with the initial situation directly after the 60-min heat shock (Figure 6, C and D). Our results are in good agreement with work by Lezzi *et al.* (1981), who demonstrated that recovery of BR gene transcription occurred only minutes after heat shock release.

RNAPII at BR sites were not inactivated due to a lack of cellular resources

It can be speculated that all available cellular transcription machinery was used by the heat-shocked genes upon a raise of temperature. Possibly, all energy and resources were used by these genes, leaving not enough for continued transcription at the BR genes. If that were the case, the inactivation of genes besides heat shock genes would be a passive side effect rather than an active regulation. To examine this option, we performed further experiments based on the following considerations. We demonstrated earlier that ongoing transcription processes can be finished during flavopiridol incubation at 20°C but that no new transcription processes enter stable elongation (Figure 6). At 37°C, application of flavopiridol would have the same effect on the heat shock transcription processes. After sufficient time at 37°C in the presence of flavopiridol, the heat shock gene transcription would be finished and the

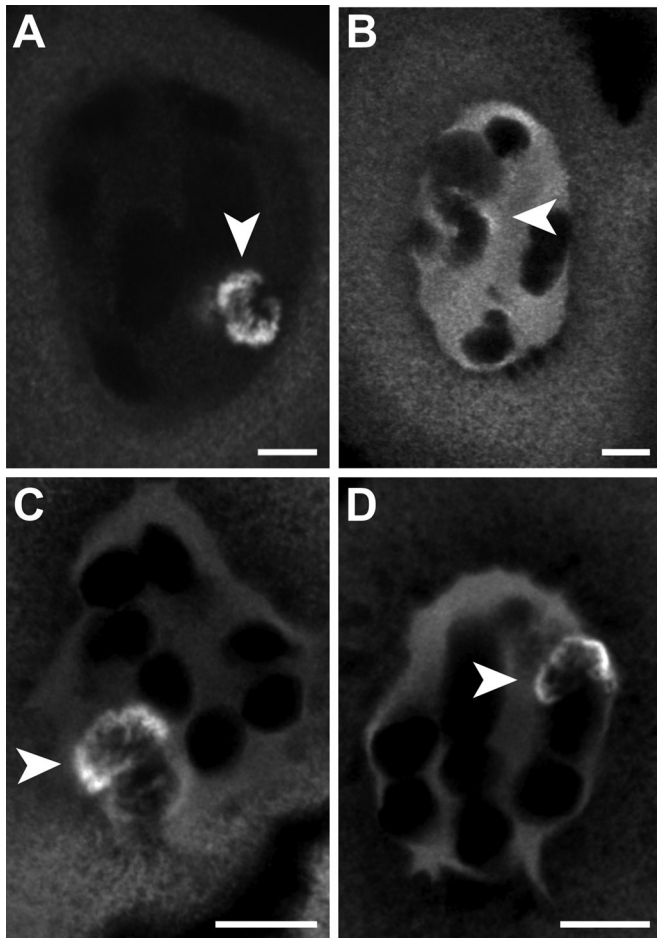


FIGURE 6: Halted RNAPII resumed activity upon heat shock release. Confocal sections of salivary gland cell nuclei after labeling by BR2.1-specific oligonucleotides. (A) Directly after heat shock, BR2.1 mRNA accumulated at the BR2 puff, as shown by mRNA FISH (arrowhead; refer also to Figure 2). (B) The heat-shocked sister gland was returned to 20°C and incubated in hemolymph containing 300 nM flavopiridol, which inhibited the start of new transcription processes. After 60 min, BR2.1 mRNA was no longer present at the transcription site. Halted transcription processes were resumed and completed, and the site was emptied. (C) Nucleus of a gland that was fixed directly after heat shock showed the presence of BR2.1 mRNA, as did (D) a nucleus of the sister gland, which was incubated after the heat shock for 60 min at 20°C in hemolymph containing 300 nM flavopiridol and 5 µg/ml actinomycin D. The latter inhibited any progress of transcription, and the transcripts remained at the BR. Arrowheads indicate the position of the BR2.1. Bars, 10 µm.

putatively limited resources would be available again for BR2.1 gene transcription, resulting in their completion. Hence after sufficient time there should be no BR2.1 mRNA detectable at the BR. Thus, if the observed transcription halt were merely caused by a lack of resources, we would expect not to detect any BR2.1 mRNA localized at the BR2 puff upon incubation with flavopiridol at 37°C for a duration required by the heat shock gene transcription and BR2.1 transcription.

The corresponding experiment was performed as follows. Salivary glands were explanted after heat shocking the larvae for 60 min. One gland of each animal was subsequently incubated for additional 60 min at 37°C in hemolymph containing 300 nM flavopiridol. The sister gland was fixed directly after dissection for

comparison. The additional incubation time of 60 min was long enough to ensure that BR2.1 and the shorter heat-shock gene transcription would be completed because the total transcription time of the long BR2.1 gene is only ~20 min. Subsequently the BR2.1 mRNA distribution was analyzed in all glands by means of FISH. We found that the BR2.1 mRNA distribution of control and flavopiridol-incubated glands did not show any difference: both glands revealed the previously observed presence of BR2.1 mRNA (Supplemental Figure S5). This demonstrated that a putative lack of resources did not cause the transcription halt. At the same time, it represented an independent experiment demonstrating the transcription halt without using the in vivo transcription assay based on *hrp36*.

DISCUSSION

Transcription is a key process for every cell, and gene expression outcome is carefully regulated at various distinct levels. Still, to our knowledge, a gene-wide, regulated, and reversible halt of RNAPII processivity during productive elongation had not previously been known.

Here we report the observation of such transcription regulation as consequence of heat shock. Experimentally we exploited the fact that the BR transcription sites of *C. tentans* salivary gland cell nuclei are light microscopically very well discernible, such that the presence or absence of BR transcripts and RNAPII could well be analyzed at a specific fully native transcription site.

It was well known that the transcription of most genes of *C. tentans* could be down-regulated by heat shock (Sass, 1995). However, that transcription is not generally stopped in the nucleus; instead, heat shock-responsive genes become activated or more strongly expressed. We demonstrated here that upon heat shock, specific transcripts, namely BR2.1 transcripts, and RNAPII remained associated to the DNA template. Furthermore, we demonstrated that the transcription bubbles remained open, presumably due to the presence of bound but halted RNAPII–transcript complexes. The detected open transcription bubbles were located clearly downstream of the promoter and upstream of the termination site, which indicated that the transcription halt was linked to neither a regulatory mechanism at the transition from early to productive elongation nor impaired termination. We concluded that RNAPII was gene-specifically arrested since transcription at several other prominent transcription sites—most likely harboring heat shock-induced genes—was even up-regulated. We demonstrated that the observed transcription halt was not a mere passive side effect of the up-regulation of heat shock genes. Finally and most important, we found that upon relief of the heat shock, transcription readily resumed. We conclude that the transcription of the examined BR2.1 gene was halted simultaneously for all chromatids of the polytene chromosome in a regulated and reversible manner as a consequence of the heat shock.

A central question is, by what mechanism was RNAPII-mediated transcription transiently halted? The unmodified RNAPII distribution over a major portion of the gene demonstrated that the halt of RNAPII activity occurred ubiquitously and independently of the position on the BR2.1 gene. Thus it might be that the stable arrest is induced by factors binding to the DNA or RNAPII that block the transcription activity. Alternatively, regulatory factors might dissociate from the RNAPII upon a special signal. It is also possible that RNAPII is primed from transcription initiation on to be down-regulated at BR2.1 upon heat shock. A priming of a similar kind occurs, for example, when the promoter structure is involved in downstream events like alternative splice-site selection (Cramer *et al.*, 1997). The long-term stability of the transcriptional halt suggested that it was

achieved by a mechanism that protected the halted RNAPII from ubiquitin-mediated degradation.

It is useful to view our findings in the given biological context. As outlined, BR2.1 mRNA is an especially long transcript, which encodes a huge, silk-like salivary protein with a molecular mass of ~1.2 MDa. Like other salivary proteins, it is expressed in very high numbers because it is a component of the larval housing tubes. An abort of transcription and subsequent reinitiation would waste an enormous amount of cellular resources. Therefore a transient freezing of RNAPII activity during the elongation process would be biologically rational because it avoids wasting the energy for decomposing the nascent mRNAs. In addition, it significantly decreases the temporal delay of regulation.

In summary, we demonstrated for the first time that the transcriptional activity of RNAPII can be switched on and off during stable elongation in a controlled and reversible manner. In this study we focused on the transcription of the *C. tentans* BR2.1 gene because of its characteristic and light-microscopically recognizable appearance. However, RNAPII and its associated factors are highly conserved across eukaryotes. Therefore, we speculate that the observed transcription regulation is not limited to the BR2.1 gene and does not exist only in *C. tentans* but instead is of very general importance. Our results demonstrate that eukaryotic cells have a much wider regulatory access to transcription than previously thought.

MATERIALS AND METHODS

Preparation and microinjection of *C. tentans* salivary glands

C. tentans midguts were cultivated as described previously (Kaminski *et al.*, 2013). For heat shocking, living larvae were transferred into 37°C prewarmed culture medium for 10, 60, or 120 min. For FISH and immunostaining, salivary glands were dissected in phosphate-buffered saline (PBS) at room temperature and directly fixed by PBS plus 4% paraformaldehyde (PFA). Dissection took maximally 2 min. For *in vivo* experiments, larvae were transferred to poly-L-lysine-coated cover slides, incubated in hemolymph, and covered by oil to prevent evaporation (M8410; Sigma-Aldrich, St. Louis, MO). Microinjection was performed with 70- to 90-hPa holding and 500- to 1000-hPa injection pressure for 0.1–0.3 s using the manual injection mode and a 40× water immersion objective lens.

In vivo labeling of active transcription sites

The *in vivo* labeling of active transcription sites was based on the incorporation of the fluorescently labeled *C. tentans* homologue of the mRNA-packaging protein hnRNPA1 (*hrp36*) and coinjected target mRNA-specific molecular beacons conjugated to Cy3 as described earlier (Siebrasse *et al.*, 2012; Kaminski *et al.*, 2013), with minor modifications. The *hrp36* cDNA was cloned in a modified *pet28a* vector in which a tetracycline tag (*tc*; *cys-cys-progly-cys-cys*) was placed in front of the hexahistidine (*his*) tag. The *tc-his*-tagged protein was expressed in BL2(DE3) and purified using metal affinity resin (Talon beads; Life Technologies GmbH, Darmstadt, Germany). Site-directed fluorescence labeling of the *hrp36* at the N-terminal *tc* tag was done with maleimide dyes (Alexa Fluor 647). Microinjection was performed as described (Kaminski *et al.*, 2013). Microinjected salivary gland cells were imaged 5 min after injection with a LSM-510-META to detect *hrp36* incorporation and monitor transcription activity. To label BR2.1 mRNA in living cells, the following beacon was used: Cy3-ACGACUUGGCUUGCUGUGIIUGCUUGGIIUGCUCGUG-BHQ2 (Cy3-BR2.1-oligo; Rinne *et al.*, 2013).

Light microscopy

Confocal imaging was performed using a Zeiss (Jena, Germany) LSM-510-META with incubator XL LSM using a 40× lens (C-Apochromat, numerical aperture 1.2) and microinjection device (Femtojet; Eppendorf, Hamburg, Germany). For analysis of heat-shocked larvae, the stage was heated to 37°C for 4 h before imaging the glands.

FISH

Whole-mount FISH of *C. tentans* salivary glands was done as previously described (Kaminski *et al.*, 2013), with minor modifications. The glands were dissected in PBS, fixed for 20 min in PBS with 4% PFA (Sigma-Aldrich), and washed five times with PBS. After treatment with proteinase K, the glands were fixed again for 20 min in PBS/PFA, washed in PBS, and finally hybridized overnight. For hybridization, BR2.1-specific (ACU UGG CUU GCU GUG UUU GCU UGG UUU GCU), 2'-O-methyl-RNA oligonucleotides 5' labeled with ATTO647N were used.

Image analysis

For quantification, 10 min after coinjection, the BR2 transcription site was imaged with the LSM-510-META. The fluorescence of *hrp36* and the BR2.1 molecular beacon was determined in the appropriate confocal images. An ROI was defined around the BR2 puff labeled by the BR2.1 molecular beacon (e.g., see the dashed yellow lines in Figure 3). A second ROI was defined next to the BR2 site in the nucleoplasm devoid of any chromosomes or nucleoli (dashed green lines in Figure 3). The pixel values $I_{ij, BR2}$ of the BR2 ROI were divided by the average intensity of the nucleoplasmic ROI, I_{BR2nuc} . Similarly, the relative pixel intensities of the *hrp36* channel were determined as $I_{ij, hrp36}/I_{hrp36nuc}$. The heat-shocked larvae were incubated for 60 min at 37°C before salivary gland dissection and microinjection. In total, 14 nuclei in the salivary glands of 11 heat-shocked and 3 control larvae were analyzed.

Whole-mount immunostaining

Preparation, fixation, and permeabilization were done as described above for FISH experiments. Primary antibodies were incubated overnight. Glands were washed 5 times for 45 min with PBS plus 0.2% Tween (PBT), and then samples were blocked for 4 h with PBT plus 2% bovine serum albumin. Secondary antibodies were incubated overnight. Samples were washed five times for 45 min with PBT before mounting on a coverslip with ProLong Gold (Invitrogen) and sealed with nail polish. The primary antibodies were the mouse anti-RNA polymerase II clone ARNA-3 CBL221 (Merck-Millipore, Darmstadt, Germany), a rabbit polyclonal anti-RNA polymerase II CTD repeat YSPTSPS (phospho S5) antibody (ab5131; Abcam, Cambridge, UK), and a rabbit polyclonal antibody against RNA polymerase II CTD repeat YSPTSPS (phospho S2; ab5095; Abcam). As secondary antibodies we used Alexa Fluor 647 goat anti-mouse immunoglobulin G1 (IgG1; a-21240; Life Technologies GmbH), Alexa Fluor 532 goat anti-mouse IgG1 (a-11002; Life Technologies GmbH), and Alexa Fluor 633 rabbit anti-goat IgG (a-21086; Life Technologies GmbH). DNA was stained by propidium iodide.

Potassium permanganate RNAPII footprinting

Eight salivary gland pairs were collected in 100 µl of ice-cold PBS. A 100-µl amount of 40 mM ice-cold potassium permanganate solution was added and incubated for 2 min on ice. A 200-µl amount of stop solution (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 40 mM Na₂EDTA, 1% SDS, 400 mM 2-mercatopethanol, pH 8) was added and samples shaken until all coloration vanished. DNA was purified by proteinase K digestion and subsequent phenol-chloroform

precipitation. The DNA pellet was resuspended in 50 μ l of 10 mM Tris-HCl + 0.1 mM EDTA. From 10 to 20 μ g of DNA was diluted to 15 μ l of 10 mM Tris-HCl + 0.1 mM EDTA and mixed with 75 μ l of water and 15 μ l of piperidine in a 1.5-ml reaction tube. After incubation for 30 min at 90°C, condensate was spun down and 200 μ l of water was added. Piperidine was extracted two times with 700 μ l of isobutanol and finally with 700 μ l of ether. DNA was precipitated by two volumes of ice-cold ethanol and centrifuged for 30 min with 16,000 \times g at 4°C. DNA pellets were resuspended in 10 μ l of Tris-HCl + 0.1 mM EDTA. The resuspended samples were used for LM-PCR (Carey *et al.*, 2009).

LM-PCR

A 20- μ mol amount of Linker1 and a 20- μ mol amount of Linker2 (see later description) were mixed, denatured for 5 min at 95°C, incubated at 70°C, slowly cooled to room temperature, and stored overnight at 4°C. For first-strand synthesis, 2 μ g of piperidine-digested DNA was mixed with 5 \times Herculanase II reaction buffer (Agilent Technologies Deutschland GmbH, Böblingen, Germany), 0.625 μ l of dNTP (10 μ M), 0.5 μ l of genomic primer (1 μ M), and 0.2 μ l of Herculanase II and filled up to 30 μ l with PCR-grade water, with subsequent PCR (5 min, 95°C; 30 min, 60°C; 10 min, 72°C).

To ligate the linker, 10 μ l of T4 DNA-Ligase Buffer (New England Biolabs), 10 μ l of 50% PEG4000 solution (New England Biolabs), 5 μ l of linker mix, and 2 U of T4 Ligase (New England Biolabs) were mixed with the PCR, added to 50- μ l total volume with water, and incubated overnight at 4°C. DNA was precipitated and the pellet resuspended in 10 μ l of 10 mM Tris-HCl + 0.1 mM EDTA.

To amplify the first strand, the following components were added in the following order: 36.85 μ l of water, 10 μ l of 5 \times Herculanase II reaction buffer, 1.25 μ l of dNTPs (10 mM), 0.2 μ l of linker primer (100 μ M; see later description), 0.2 μ l of genomic primer (100 μ M), 1 μ l of ligation reaction, and 0.5 μ l of Herculanase II. To fluorescently label PCR products, 5 μ l of ATTO-488 UTP (1 mM; Jena Bioscience, Jena, Germany) was added to the PCR.

Oligonucleotides

Linker1, GCGAGACGTATCCCCGGTTGCTGCTTGGG; Linker2, CCCAAGCAGCAACCGGGAATAC; BR2 genomic primer, ACTTGGCTTGCTGTGTTGCTTGGTTTGGCT; glycylamide ribonucleotide synthetase AAGGAATGCCGATGGCTGT; linker primer, CGGTGCTGCTTGGG.

DNA fragment analysis

The LM-PCR mixture was ethanol precipitated after thermocycling and resuspended in 5 μ l of 10 mM Tris-HCl + 0.1 mM EDTA. As gel matrix, 10–16% acrylamide/bis-acrylamide (19:1; Carl Roth) in a Protean II xi Cell was used. Gels were stained for 60 min with 3 \times GelRed solution (Biotum) and imaged with a gel documentation system. For high-resolution fragment analysis, the PCR was directly analyzed by a 3130xl Genetic Analyzer (Life Technologies GmbH) using a POP7 36-cm capillary and Internal Lane Standard 600 (Promega GmbH, Mannheim, Germany). The chromatograms were analyzed and visualized as virtual gel images by custom-built MATLAB (MathWorks, Natick, MA) routines.

ACKNOWLEDGMENTS

We gratefully thank Alexander Heckel and Jennifer Rinne for the generous gift of molecular beacons specific for the BR2.1 mRNA, Ruth Raff and Dietlinde Steinen for help with fragment length analysis, and Bertil Daneholt and Neus Visa for constructive comments on a previous version of the manuscript. Furthermore, we thank

Claudio Nietzel for excellent technical assistance. U.K. and T.K. gratefully acknowledge financial support by Deutsche Forschungsgemeinschaft Grants Ku 2474/7-1 and Ku 2474/8-1 and the German National Academic Foundation, respectively.

REFERENCES

- Arigo JT, Carroll KL, Ames JM, Corden JL (2006). Regulation of yeast NRD1 expression by premature transcription termination. *Mol Cell* 21, 641–651.
- Beermann W (1952). Chromosome constancy and specific modifications of the chromosome structure in development and organ differentiation of *Chironomus tentans* [in German]. *Chromosoma* 5, 139–198.
- Bensaude O (2011). Inhibiting eukaryotic transcription: which compound to choose? How to evaluate its activity? *Transcription* 2, 103–108.
- 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.
- Buratowski S (2009). Progression through the RNA polymerase II CTD cycle. *Mol Cell* 36, 541–546.
- Bushnell DA, Cramer P, Kornberg RD (2002). Structural basis of transcription: alpha-amanitin-RNA polymerase II cocystal at 2.8 Å resolution. *Proc Natl Acad Sci USA* 99, 1218–1222.
- Carey MF, Peterson C L, Smale ST (2009). *Transcriptional Regulation in Eukaryotes: Concepts, Strategies and Techniques*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Cheung ACM, Cramer P (2011). Structural basis of RNA polymerase II backtracking, arrest and reactivation. *Nature* 471, 249–253.
- Chubb JR, Trcek T, Shenoy SM, Singer RH (2006). Transcriptional pulsing of a developmental gene. *Curr Biol* 16, 1018–1025.
- Cramer P, Pesce CG, Baralle FE, Kornblihtt AR (1997). Functional association between promoter structure and transcript alternative splicing. *Proc Natl Acad Sci USA* 94, 11456–11460.
- Darzacq X, Shav-Tal Y, de Turris V, Brody Y, Shenoy SM, Phair RD, Singer RH (2007). In vivo dynamics of RNA polymerase II transcription. *Nat Struct Mol Biol* 14, 796–806.
- Gilmour DS, Lis JT (1986). RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in *Drosophila melanogaster* cells. *Mol Cell Biol* 6, 3984–3989.
- Gómez-Herreros F, de Miguel-Jiménez L, Millán-Zambrano G, Peñate X, Delgado-Ramos L, Muñoz-Centeno MC, Chávez S (2012). One step back before moving forward: regulation of transcription elongation by arrest and backtracking. *FEBS Lett* 586, 2820–2825.
- Gu W, Reines D (1995). Identification of a decay in transcription potential that results in elongation factor dependence of RNA polymerase II. *J Biol Chem* 270, 11238–11244.
- Kaminski TP, Spille J-H, Nietzel C, Siebrasse JP, Kubitschek U (2013). Nuclear trafficking and export of single, native mRNPs in *Chironomus tentans* salivary gland cells. *Methods Mol Biol* 1042, 73–85.
- Kessler M, Mathews MB (1992). Premature termination and processing of human immunodeficiency virus type 1-promoted transcripts. *J Virol* 66, 4488–4496.
- Kornblihtt AR, de la Mata M, Fededa JP, Muñoz MJ, Nogues G (2004). Multiple links between transcription and splicing. *RNA* 10, 1489–1498.
- Lamb MM, Daneholt B (1979). Characterization of active transcription units in Balbiani rings of *Chironomus tentans*. *Cell* 17, 835–848.
- Lezzi M, Meyer B, Mähr R (1981). Heat shock phenomena in *Chironomus tentans* I. In vivo effects of heat, overheat, and quenching on salivary chromosome puffing. *Chromosoma* 83, 327–339.
- Malik S, Bagla S, Chaurasia P, Duan Z, Bhaumik SR (2008). Elongating RNA polymerase II is disassembled through specific degradation of its largest but not other subunits in response to DNA damage in vivo. *J Biol Chem* 283, 6897–6905.
- Margaritis T, Holstege FCP (2008). Poised RNA polymerase II gives pause for thought. *Cell* 133, 581–584.
- Nechaev S, Adelman K (2011). Pol II waiting in the starting gates: regulating the transition from transcription initiation into productive elongation. *Biochim Biophys Acta* 1809, 34–45.
- Neil H, Malabat C, d'Aubenton Carafa Y, Xu Z, Steinmetz LM, Jacquier A (2009). Widespread bidirectional promoters are the major source of cryptic transcripts in yeast. *Nature* 457, 1038–1042.
- Paulsson G, Bernholm K, Wieslander L (1992). Conserved and variable repeat structures in the Balbiani ring gene family in *Chironomus tentans*. *J Mol Evol* 35, 205–216.

- Phatnani HP, Greenleaf AL (2006). Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev* 20, 2922–2936.
- Raj A, Peskin CS, Tranchina D, Vargas DY, Tyagi S (2006). Stochastic mRNA synthesis in mammalian cells. *PLoS Biol* 4, e309.
- Rinne JS, Kaminski TP, Kubitscheck U, Heckel A (2013). Light-inducible molecular beacons for spatio-temporally highly defined activation. *Chem Commun (Camb)* 49, 5375–5377.
- Rougvie AE, 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.
- Saeki H, Svejstrup JQ (2009). Stability, flexibility, and dynamic interactions of colliding RNA polymerase II elongation complexes. *Mol Cell* 35, 191–205.
- Sass H (1995). Transcription of heat shock gene loci versus non-heat shock loci in *Chironomus* polytene chromosomes: evidence for heat-induced formation of novel putative ribonucleoprotein particles (hsRNPs) in the major heat shock puffs. *Chromosoma* 103, 528–538.
- Saunders A, Core LJ, Lis JT (2006). Breaking barriers to transcription elongation. *Nat Rev Mol Cell Biol* 7, 557–567.
- Selth LA, Sigurdsson S, Svejstrup JQ (2010). Transcript elongation by RNA polymerase II. *Annu Rev Biochem* 79, 271–293.
- Siebrasse JP, Kaminski T, Kubitscheck U (2012). Nuclear export of single native mRNA molecules observed by light sheet fluorescence microscopy. *Proc Natl Acad Sci USA* 109, 9426–9431.
- Siebrasse JP, Veith R, Dobay A, Leonhardt H, Daneholt B, Kubitscheck U (2008). Discontinuous movement of mRNP particles in nucleoplasmic regions devoid of chromatin. *Proc Natl Acad Sci USA* 105, 20291–20296.
- Somesh BP, Sigurdsson S, Saeki H, Erdjument-Bromage H, Tempst P, Svejstrup JQ (2007). Communication between distant sites in RNA polymerase II through ubiquitylation factors and the polymerase CTD. *Cell* 129, 57–68.
- Svejstrup JQ (2007). Contending with transcriptional arrest during RNAPII transcript elongation. *Trends Biochem Sci* 32, 165–171.
- Visa N, Alzhanova-Ericsson AT, Sun X, Kiseleva E, Bjorkroth B, Wurtz T, Daneholt B (1996). A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. *Cell* 84, 253–264.
- Wilson MD, Harreman M, Svejstrup JQ (2013). Ubiquitylation and degradation of elongating RNA polymerase II: the last resort. *Biochim Biophys Acta* 1829, 151–157.
- Zhou J, Schweikhard V, Block SM (2013). Single-molecule studies of RNAPII elongation. *Biochim Biophys Acta* 1829, 29–38.