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Efficient Release from Promoter-Proximal Stall Sites Requires Transcript Cleavage Factor TFIIS

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Summary

Uninduced heat shock genes are poised for rapid activation, with RNA polymerase II (Pol II) transcriptionally engaged, but paused or stalled, within the promoter-proximal region. Upon heat shock, this Pol II is promptly released from the promoter region and additional Pol II and transcription factors are robustly recruited to the gene. Regulation of the heat shock response relies upon factors that modify the efficiency of elongation through the initially transcribed sequence. Here, we report that Pol II is susceptible to transcription arrest within the promoter-proximal region of *Drosophila hsp70* and that transcript cleavage factor TFIIS is essential for rapid induction of *hsp70* RNA. Moreover, using a tandem RNAi-ChIP assay, we discovered that TFIIS is not required to establish the stalled Pol II, but that TFIIS is critical for efficient release of Pol II from the *hsp70* promoter region and the subsequent recruitment of additional Pol II upon heat induction.

Introduction

Precise control of transcription is critical for the regulation of gene expression and thus the differentiation, development, and survival of the cell. While transcription activation often involves the coordinated recruitment of upstream activators, transcription factors, and the RNA polymerase to the promoter, recent evidence has underscored the importance of continued regulation of transcription throughout mRNA synthesis. In particular, increasing interest has focused on factors that influence transcription elongation through chromatin and the coupling of transcription to mRNA processing (Arndt and Kane, 2003; Hartzog, 2003; Shilatifard et al., 2003; Sims et al., 2004). Moreover, localization of protein factors in vivo has revealed that a number of genes have general transcription factors bound and an RNA polymerase present within the promoter region prior to activation, suggesting that these genes are regulated at a step after the recruitment and formation of a preinitiation complex (reviewed in Lis, 1998).

One recognized mechanism for regulating transcription postrecruitment of the RNA polymerase involves modulating the efficiency of elongation through the initially transcribed region. This mechanism is fundamental for control of gene expression in organisms as diverse as bacteriophage λ, *E. coli*, *Drosophila*, human (e.g., c-*myc* and c-*fos*), and HIV-1 (Krumm et al., 1992; Laspia et al., 1993; Lis, 1998; Plet et al., 1995; Roberts et al., 1998; Strobl and Eick, 1992). In each of these systems, transcription factors function together with aspects of the initially transcribed sequence to control the progression of the RNA polymerase through the promoter-proximal region. Identifying the characteristics of the nucleic acid sequence and the protein factors that regulate the degree and duration of pausing that occurs is thus essential for understanding this method of transcription control.

The heat shock response in *Drosophila* represents a robust, highly inducible system for studying regulation at the level of transcription elongation. The *Drosophila* heat shock genes are primed for activation under uninduced conditions, with Pol II engaged but "paused" within the first ~20–45 nt (Rasmussen and Lis, 1993; Rougvie and Lis, 1988). The properties of "paused" Pol II at various sites in the promoter-proximal region are not uniform (Rasmussen and Lis, 1995); therefore, here we adopt the term "stalled" as a more general descriptor (Fish and Kane, 2002). The stalled Pol II is phosphorylated on Ser5 of the Pol II CTD (but not Ser2), and the nascent mRNA is partially capped (O'Brien et al., 1994; Rasmussen and Lis, 1993). Heat induction allows for the rapid release of this Pol II into the body of the gene, followed by recruitment of additional Pol II (Boehm et al., 2003; Lis, 1998). Escape from the promoter-proximal region has been shown to be the rate-limiting step in transcription of the heat shock genes both prior to and during the heat shock response (Lis, 1998; O'Brien and Lis, 1991). Thus, regulating the kinetics of Pol II recruitment and elongation through the initially transcribed sequence directly determines the levels of RNA produced.

Analysis of the *Drosophila hsp70* gene has revealed that, in addition to Pol II, a number of transcription factors are associated with the promoter region in the uninduced state, including the DNA binding GAGA factor, TATA binding protein, and the elongation factor Spt5 (Andrulis et al., 2000; Lis, 1998). Heat induction leads to the rapid recruitment to *hsp70* of Heat Shock Factor, Mediator, the Pol II CTD kinases P-TEFb and TFIIH, transcription factors Spt5 and Spt6, FACT, and the RNA processing exosome (Andrulis et al., 2000, 2002; Boehm et al., 2003; Lis et al., 2000; Park et al., 2001; Saunders et al., 2003; Schwartz et al., 2003). Recently, the putative transcription elongation factor NELF has been suggested to play a role in establishing a stalled Pol II at *hsp70* (Wu et al., 2003). However, the interplay between the general transcription factors and the factors that specifically modulate stalling versus release of Pol II within the promoter-proximal region remains to be determined.

In a search for elongation factors that directly affect

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the heat shock response, we identified, and report here, a role for the transcript cleavage factor TFIIS. Like the bacterial Gre factors, TFIIS rescues RNA polymerase that has undergone reverse translocation, or "backtracking" along the DNA template (Borukhov et al., 1993; Fish and Kane, 2002; Wind and Reines, 2000). Backward movement misaligns the 3′ end of the nascent RNA with the RNA polymerase active site, thereby prohibiting continued RNA synthesis (Komissarova and Kashlev, 1997). Transcript cleavage factors restart the arrested RNA polymerase by inducing internal cleavage of the RNA by the polymerase active site, creating a new 3′ end that is properly aligned for catalysis (Izban and Luse, 1992; Reines, 1992). The activity of transcript cleavage factors has been reported to stimulate promoter escape and transcription elongation and to decrease pausing (Guo and Price, 1993; Hsu et al., 1995). Recently published structural and functional analyses of transcript cleavage factors GreB and TFIIS complexed with their respective RNA polymerases elucidate the mechanism of this activity (Kettenberger et al., 2003; Laptenko et al., 2003; Opalka et al., 2003; Sosunova et al., 2003): TFIIS inserts a long coiled-coil domain into the RNA polymerase secondary channel, helping to coordinate a Mg^{+2} ion required for the reverse-catalytic reaction. However, although the detailed mechanism of TFIIS activity is known, the in vivo roles for this activity remain poorly defined.

We report herein that TFIIS influences the kinetics of transcription of *Drosophila hsp70*, by modulating the rate-limiting process of escape from the promoter-proximal region. Using a combination of in vitro transcription elongation assays and in vivo functional and ChIP analyses, we demonstrate that Pol II is susceptible to backtracking and transcription arrest within the promoter-proximal region of *hsp70* and that TFIIS rescues the arrested Pol II so that it can be rapidly released into the body of the gene upon heat shock. These results define a specific in vivo target of TFIIS and reveal a mechanism through which TFIIS-induced cleavage activity is required for gene activation.

Results

Transcription through the Promoter-Proximal Sequence of *hsp70*

In vivo, Pol II stalled on the uninduced *hsp70* gene is located primarily within the interval between +21 and +35 nt with respect to the transcription start site (shown in Figure 1A) (Lis, 1998). To investigate the kinetics of Pol II movement through this region, we established an in vitro transcription assay that allowed us to monitor the efficiency and rate of transcript elongation (Experimental Procedures). Preinitiation complexes were formed on immobilized DNA templates containing *hsp70* sequences (from −365 to +500) in *Drosophila Kc* nuclear extract derived from non-heat-shocked cells. NTPs and heparin were added to initiate a single round of transcription. Aliquots were removed at the time points given (Figure 1B) and the reaction quenched immediately with EDTA. Unincorporated nucleotides and unbound material were washed away before analyzing the RNA species stably associated with the transcribing Pol II at each time point. We note that because our template was biotinylated on the downstream end, Pol II did not run off the end of our template and the full-length RNA was not released.

The data in Figure 1B reveal that the initially transcribed sequence of *hsp70* contains a number of specific sites at which Pol II stalls, with much of the Pol II remaining in the promoter-proximal region (denoted by a bracket) throughout the time course. Moreover, the short, promoter-proximal RNA species actually represent a large fraction of the total transcript formed since internal labeling of the nascent RNA results in the signal intensity from each band being proportional to the length of the RNA (and the number of U residues incorporated). The transient pauses observed in the promoter proximal region of *hsp70* in our in vitro system displayed half-lives that are consistent with the in vivo kinetics determined for escape from the *hsp70* promoter region in uninduced cells ($t_{1/2}$ for escape in vivo ≈ 10 min [Lis, 1998]; compared to $t_{1/2}$ ≈ 9 min in vitro).

In order to compare the locations at which Pol II stalls in vitro to the distribution of stalled Pol II determined previously in vivo (Rasmussen and Lis, 1993, 1995), the RNA species associated with elongation complexes halted after 30 s of transcription were analyzed on a denaturing gel that also contained short RNAs of known length, derived from *hsp70* by stepwise movement of Pol II through the initially transcribed sequence (see Supplemental Figure S1B at http://www.molecule.org/cgi/content/full/17/1/103/DC1/). This procedure allowed us to map the positions of the predominant stall sites as +26, +34, and +43 nt from the transcription start site (Supplemental Figure S1B). Importantly, Pol II stalled at these particular sites in isolated nuclei could not be efficiently stimulated to resume elongation upon heat shock during run-on analysis, even in the presence of sarkosyl at levels that would remove any negatively acting factors (Lis, 1998). In prior work, the inability of these particular Pol II species to be restarted lead to two possible explanations: (i) these RNAs were premature termination products; or (ii) Pol II stalled at these particular locations became inactive. The fact that short RNAs of these lengths remain associated with Pol II in vitro through several stringent washes indicates that they are unlikely to be termination products and suggests that Pol II stalled at these positions loses elongation competence.

Sarkosyl, When Added Immediately after Initiation, Diminishes the Appearances of Pauses

We sought to determine whether factors in the nuclear extract might alter the efficiency of transcription through the promoter-proximal region or the duration of pauses observed. As has been shown previously (Li et al., 1996), transcription of the *hsp70* promoter in nuclear extract yields only a single round of initiation (Supplemental Figure S2A), and while the addition of sarkosyl (to 0.5%) stimulates transcription significantly, it does so only when it is added shortly after the onset of transcription (Supplemental Figure S2B). These results have been interpreted to suggest that there is a limited time frame in which removal of negatively acting factors by sarkosyl can induce Pol II to resume transcription (Li et al., 1996). To evaluate the effects of sarkosyl in more detail, we

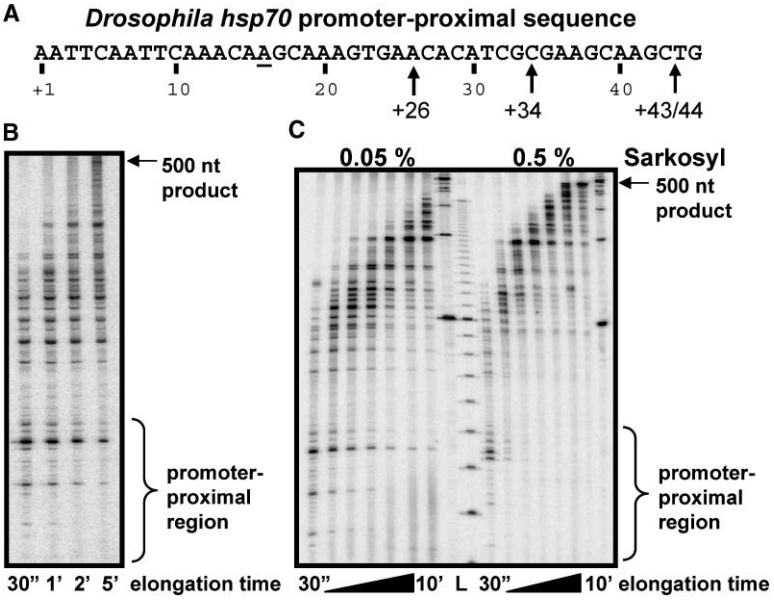

Figure 1. Pol II Pauses within the Promoter-Proximal Region of *hsp70*

(A) Sequence of the initially transcribed region of *Drosophila hsp70*. Arrows depict the locations at which the stalled Pol II does not efficiently resume elongation in vivo (Rasmussen and Lis, 1995).

(B) Biotinylated *hsp70* template was incubated in nuclear extract to allow preinitiation complex formation. A single round of transcription was initiated by addition of NTPs, including radiolabeled α-^{32}P-UTP, plus heparin (10 μg/ml). Samples were removed and elongation quenched at the times indicated. RNA that was stably associated with Pol II was analyzed on an 8% denaturing gel. The full-length, 500 nt product is shown at right by an arrow, and the promoter-proximal sequence shown in (A) is denoted by a bracket.

(C) Preinitiation complexes were formed as described above and split into two identical reactions before NTPs were added to initiate transcription. Sarkosyl was supplied to the reactions 10 s later, to a final concentration of 0.05% sarkosyl (lanes at left), or 0.5% sarkosyl (lanes at right). Aliquots were taken and reactions quenched at 0.5, 1, 1.5, 2, 3, 5, and 10 min after transcription initiation. RNA species were isolated and analyzed as described above. L denotes a pair of lanes containing the 100 and 10 nt denatured DNA ladders used as relative size markers.

monitored the pattern of transcripts formed from *hsp70* in the presence of a low (0.05%) or high (0.5%) concentration of sarkosyl, added immediately after the NTPs. Figure 1C shows that the presence of 0.05% sarkosyl, a concentration frequently used to maintain a single round of transcription, yields a pattern of transcript formation that is similar to that shown in Figure 1B. In contrast, the addition of 0.5% sarkosyl, which has been shown to disrupt the interaction of elongation factors with Pol II, serves to markedly enhance the apparent rate of transcription of the *hsp70* gene. Moreover, this occurs largely through decreasing the appearance of pauses within the promoter-proximal region. Thus, these data are consistent with the hypothesis that negatively acting factors in the extract bind to Pol II and increase the frequency or duration of promoter-proximal pauses.

Pol II Is Highly Susceptible to Arrest in the Promoter-Proximal Region of *hsp70*

To determine whether rapid removal of negatively acting elongation factors from Pol II halted within the promoter-proximal region is sufficient to allow efficient restart of transcription, radiolabeled early elongation complexes (EEC) were stopped after 30 s of transcription with EDTA. The EEC were washed extensively with sarkosyl and high salt to remove bound elongation factors and termination products, equilibrated in transcription buffer, and supplied with unlabeled NTPs (1 mM). Figure 2A shows that the EEC halted within the initially transcribed region of *hsp70* are effectively unable to resume elongation. Only the Pol II halted beyond the promoter-proximal region appear to retain a perceptible level of elongation competence under these conditions (Pol II at + 43 nt is restarted with ~50% efficiency). Thus, the EEC formed in vitro remain largely inactive after the removal of associated transcription factors by sarkosyl, much like stalled Pol II found at these positions in vivo (Rasmussen and Lis, 1995).

To test whether Pol II complexes stalled within the promoter-proximal region were inactive due to transcription arrest, we investigated whether they could be rescued by transcript cleavage factor TFIIS. Stalled EEC formed in a partially fractionated embryo extract lacking TFIIS (compared to crude nuclear extract in Supplemental Figure S3B) were isolated and washed before restarting transcription in the presence or absence of purified TFIIS. We note that some endogenous species (such as tRNA) present in the extract (denoted by the bracket) were inadvertently 3′ end labeled during this reaction and were not fully removed during the wash steps.

The data in Figure 2B show that the addition of NTPs (200 μM) leads to little or no transcription elongation in the absence of TFIIS (and less at 200 μM NTPs than at 1 mM as in Figure 2A). The presence of purified TFIIS alone induced efficient cleavage of RNA products associated with stalled Pol II (observed as shorter RNA species). The sensitivity of specific RNAs to TFIIS-dependent cleavage signifies that these RNA species are associated with backtracked, arrested Pol II complexes. Cleavage of these RNAs in the presence of TFIIS reactivates the stalled complexes, allowing the labeled RNA species to be elongated upon addition of NTPs. We conclude that TFIIS-induced cleavage rescues the promoter-proximal stalled, arrested Pol II.

TFIIS Reduces the Duration of Promoter-Proximal Stalling and Enhances Elongation Efficiency

Taken together, the above data suggest that intrinsic pause sites within the promoter-proximal region of *hsp70* are recognized in vitro, perhaps with the aid of regulatory elongation factors, and that Pol II at these locations rapidly become inactive. However, the experiments demonstrating transcription arrest (Figures 2A

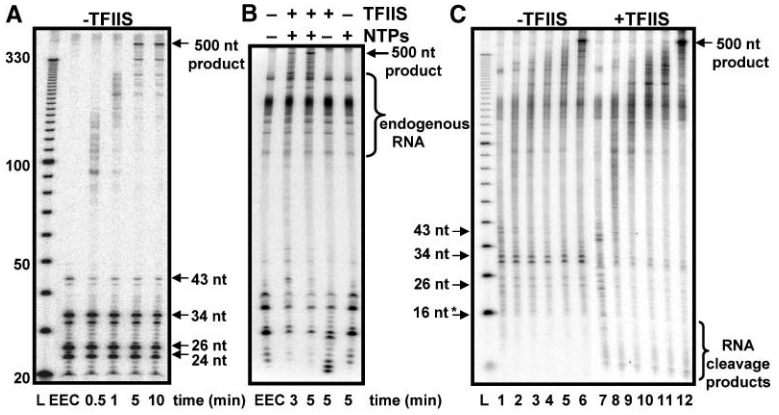

Figure 2. TFIIS Prevents Elongation Arrest and Reduces the Duration of Promoter-Proximal Stalling

(A) Labeled early elongation complexes (EEC), stalled after 30 s of transcription, were washed and reequilibrated in transcription buffer. Elongation was restarted by addition of unlabeled NTPs at 1 mM; after which point, aliquots were removed and the reaction stopped at the times indicated. RNA products were analyzed on an 8% denaturing gel. Denatured 10 bp ladder (L) was used as a relative size marker.

(B) Stalled radiolabeled EEC formed in a fractionated embryo extract lacking TFIIS were isolated as described above (EEC, first lane). These stalled Pol II were supplied with TFIIS (250 nM), NTPs (200 μM), or both (as indicated) for the times given before the reaction was quenched and the RNA products analyzed on a 10% denaturing gel.

(C) EEC formed in a fractionated embryo extract lacking TFIIS were radiolabeled during elongation to +16 nt in the presence of α-^{32}P-UTP. The sample was washed and split into two reactions that lacked (left side) or contained (right side) purified TFIIS (250 nM). Unlabeled NTPs were added (200 μM), and aliquots were removed for analysis at the following time points: 15, 30, 45, 60, 90 s, and 5 min; corresponding to lanes 1–6 (minus TFIIS) and lanes 7–12 (plus TFIIS). The RNA cleavage products that are released as a result of TFIIS activity are denoted at the bottom right.

and 2B) involve EEC that were artificially stalled and stringently washed prior to analysis, which does not accurately reflect the dynamics of *hsp70* transcription. Thus, to investigate whether Pol II actively transcribing through the promoter-proximal region is susceptible to arrest and to determine the role of TFIIS in this process, we performed a transcription assay in a fractionated *Drosophila* embryo extract that lacked TFIIS (Supplemental Figure S3B).

EEC were radiolabeled during elongation to position +16 nt (Figure 1A, position underlined; Figure 2C, position denoted at right with asterisk) and washed thoroughly with transcription buffer plus heparin (200 μg/ml) to remove unincorporated NTPs and unbound extract proteins and to prevent reinitiation. The resulting EEC were split into two equivalent reactions, one of which was supplemented with purified TFIIS (Figure 2C, minus TFIIS, lanes 1–6, plus TFIIS, lanes 7–12). Unlabeled NTPs were added to restart transcription, and aliquots were removed at the time points indicated. In the absence of TFIIS (lanes 1–6), Pol II accumulated in the promoter-proximal region and was not able to escape from sites of stalling during the time course (lanes 1–6). In contrast, inactive Pol II complexes were barely detectable in the presence of TFIIS (lanes 7–12). Instead, TFIIS stimulated rapid and efficient elongation of the labeled +16 nt RNA through the promoter-proximal region, leading to the formation of increased levels of full-length transcript. TFIIS activity also generated cleavage products that were released from Pol II (i.e., they were not observed when complexes were washed), shown at the bottom right. These data indicate that the initially transcribed sequence of *hsp70* contains intrinsic sites at which Pol II pauses or stalls during active transcription, and that TFIIS is critical for efficient elongation through this region.

TFIIS Is Required for an Optimal Heat Shock Response In Vivo

To verify the functional relevance of TFIIS in the heat shock response in vivo, we depleted TFIIS levels in *Drosophila* S2 cells using RNAi. S2 cells that were untreated or treated with dsRNA targeting TFIIS or β-galactosidase (LacZ) as a negative control were heat shocked to induce production of *hsp70* RNA before harvesting cells and isolating total RNA (Experimental Procedures). Figure 3A shows the resulting levels of TFIIS protein in the RNAi-treated cells (HSF levels are shown below as a loading control). The depletion of TFIIS was not complete, perhaps due to the abundance or low turnover of the TFIIS protein; nonetheless, TFIIS-depleted cells were estimated to contain only ~10% of normal levels of TFIIS.

Analysis of *hsp70* RNA levels by quantitative RT-PCR reveals that TFIIS-depleted cells are indeed deficient in the heat shock response. In particular, there is a dramatic delay in *hsp70* production in TFIIS-depleted cells, with *hsp70* levels barely increasing above background after 2.5 min of heat shock. The significant kinetic block in *hsp70* RNA production in TFIIS-depleted cells observed after a short heat shock (7- to 8-fold defect at 2.5 min, 4-fold defect at 5 min), begins to be overcome at later time points, leading to an overall heat shock response of approximately 50%–60% normal *hsp70* levels. The RT-rt-PCR results were confirmed by primer extension analysis of induced *hsp70* RNA levels (Supplementary Figure S5). These data demonstrate that TFIIS is required in vivo for maximal expression of *hsp70* and suggest that TFIIS may serve to regulate the kinetics of the heat shock response by maintaining Pol II in a readily inducible conformation.

TFIIS Is Recruited to Induced Heat Shock Loci

The results presented above suggest that TFIIS is involved in mediating the magnitude and efficiency of the heat shock response; additionally, TFIIS has been proposed to function broadly in transcription elongation by Pol II. To view the distribution of TFIIS both over the entire genome and at heat shock loci, we stained *Drosophila* polytene chromosomes with an antibody that is highly specific for TFIIS (Figure 4). Over 150 specific loci are stained by anti-TFIIS, including several in-

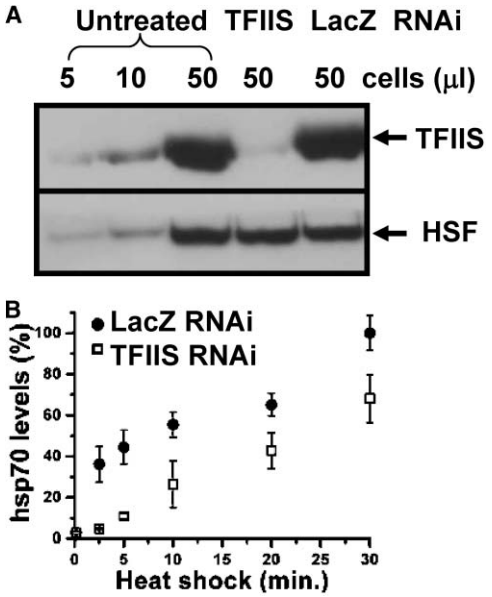

Figure 3. Depletion of TFIIS by RNAi Decreases the Level of Induced *hsp70* RNA

(A) Western analysis of protein levels in S2 cells that were untreated or treated with dsRNA corresponding to TFIIS or LacZ (as a negative control). The equivalent volume of cell culture loaded in each lane is given. The membrane was probed with an antibody against TFIIS (upper panel) and HSF (lower panel) as a loading control.

(B) Time course of *hsp70* RNA accumulation during heat shock. Cells were heat shocked for the times shown before centrifugation and isolation of total RNA. The level of *hsp70* RNA in each sample was determined by reverse transcription followed by quantitative real-time PCR, and was normalized for the amount of RNA in each sample using *rp49* RNA (a ribosomal protein gene) as an internal standard. Each time point was assayed in duplicate from two independent samples.

terbands and chromosomal puffs, which contain the Pol II-transcribed developmental genes (Figure 4A), the native and transgenic heat shock genes (Figure 4B), and the nucleolus organizer, which contains the Pol I-transcribed rRNA genes (Figure 4B, labeled NO). The consistent, prominent labeling of the nucleolus organizer suggests that TFIIS plays a role in Pol I elongation. A functional interaction between TFIIS and Pol I has been reported previously; however, conflicting reports have indicated that Pol I transcription is stimulated by a distinct transcript cleavage factor (Labhart, 1997; Schnapp et al., 1996).

Perhaps most surprising is the strong staining of many condensed chromosomal bands (Figure 4, see DNA/TFIIS merges). These are sites that are not actively transcribed by RNA Pol I, II, or III. These transcriptionally inactive regions of TFIIS accumulation may be indicative of an as yet uncharacterized function of TFIIS, or may represent storage or proposed transcriptosome assembly loci akin to the TFIIS-containing Cajal Bodies in *Xenopus* oocytes (Smith et al., 2003).

Upon stimulation of the heat shock response, TFIIS accumulates at heat shock loci (Figures 4B, 4C, and 4E). However, in contrast to many other transcription factors (Andrulis et al., 2000; Lis et al., 2000; Ni et al., 2004; Saunders et al., 2003; Schwartz et al., 2003), TFIIS can still be observed at many other loci on the chromosomes, and in particular, the strong colocalization with condensed DNA bands persists (DNA/TFIIS in Figure 4B). This result is consistent with recent data on the localization of TFIIS in yeast (Pokholok et al., 2002), where it was noted that TFIIS was not generally required for Pol II transcription but appeared to be specifically recruited to actively transcribed genes during times of cellular stress and when transcription was compromised (i.e., 6-AU treatment or temperature shift). In agreement with these results, *Drosophila* TFIIS is recruited to heat shock loci rapidly after heat induction (Figures 4C–4E) and TFIIS appears to travel into the body of the gene along with Pol II, since it can be seen to colocalize throughout the puff with active Pol II (Figure 4D).

TFIIS Colocalizes with the Promoter-Proximal Pol II Prior to Heat Shock

To analyze the localization of TFIIS at *hsp70* at higher resolution, we performed chromatin immunoprecipitation (ChIP) assays followed by real-time PCR. This method allows for quantitative analysis of both the spatial and temporal distribution of TFIIS on the *hsp70* gene. As has been previously reported, Pol II (detected using an antibody that recognizes the Pol II Rpb3 subunit) is associated specifically with the promoter region of *hsp70* prior to heat shock (Figure 5B, top panel, yellow bars and Boehm et al. [2003]). Upon heat induction, Pol II is rapidly detected in the body of the gene and a robust recruitment of additional Pol II is observed (Figure 5B, top panel, and Boehm et al. [2003]).

Strikingly, TFIIS is also present at the uninduced *hsp70* promoter (Figure 5B, lower panel, yellow bars). This result is consistent with the idea that TFIIS associates with the promoter-proximal stalled Pol II to rescue it from arrest, thereby maintaining the Pol II in a rapidly responsive, active state. During heat shock, TFIIS is further recruited to the promoter region of *hsp70* and TFIIS is seen to track along with the elongating Pol II into the body of the gene (Figure 5B, lower panel), in agreement with its role as an accessory factor for transcription elongation.

Depletion of TFIIS Leads to Reduced Recruitment of Pol II upon Heat Shock

All of the above data are consistent with the hypothesis that the promoter-proximal stalled Pol II has a tendency to fall into transcription arrest and that TFIIS serves to rescue the arrested Pol II so that it can be induced to elongate upon heat shock. Thus, one would predict that, in the absence of TFIIS, Pol II that becomes inactive in the promoter-proximal region would remain inactive, thereby presenting a steric obstacle to the rapid recruitment of additional Pol II molecules upon heat shock. The Pol II density at the *hsp70* promoter before heat shock would thus remain unchanged (i.e., one Pol II present within each *hsp70* promoter region), but the movement of Pol II into the body of the gene and the recruitment of additional Pol II upon heat shock should be diminished or delayed.

To test this idea, we developed a protocol to perform ChIP on S2 cells that had been depleted of TFIIS by RNAi (Experimental Procedures). TFIIS and LacZ RNAi-

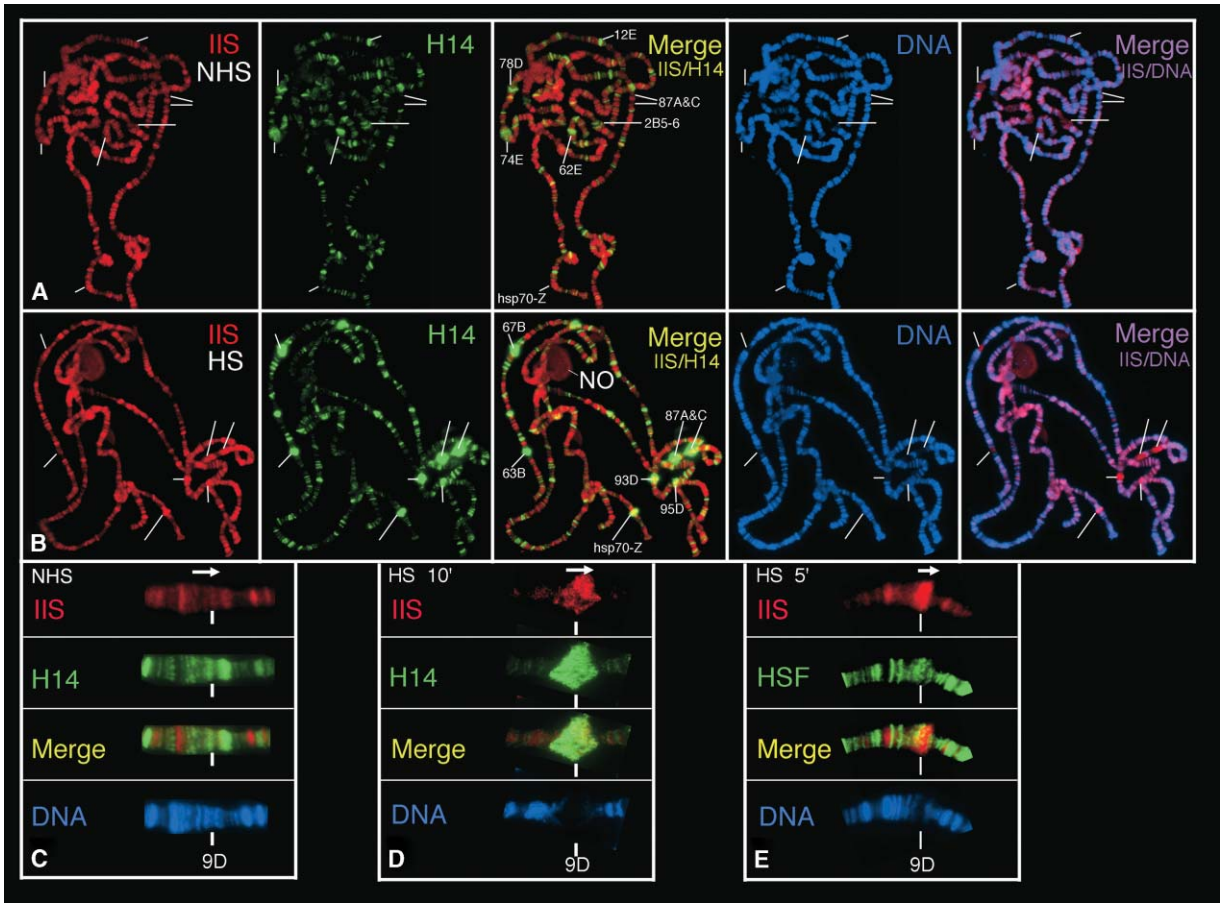

Figure 4. Immunofluorescence Showing the Distribution of TFIIS on Polytene Chromosomes from Uninduced and Heat Shock-Induced *Drosophila* Larvae

Salivary glands were dissected from third instar larvae ([A–B] Z243 fly strain; [C–E] Bg9 strain), and nuclei were spread on slides and processed as previously described (Schwartz et al., 2004). The nucleolar organizer and major heat shock loci are indicated with lines and are labeled in the central (merge) panel.

(A) Chromosomal spreads from non-heat-shocked (NHS) salivary glands, showing the distribution of TFIIS (red), Ser5 phosphorylated Pol II (green), and DNA (blue). The Ser5-phosphorylated C-terminal domain of the largest subunit of Pol II was detected with mouse monoclonal antibody (H14, Covance). DNA was stained with Hoechst.

(B) TFIIS (red) colocalizes with Ser5 phosphorylated Pol II (as in [A]) on nuclei of heat-shocked (HS) salivary glands. The representative larva shown was heat shocked for 20 min.

(C–E) Increased magnification views of TFIIS at an *hsp70*-lacZ transgene, located at chromosomal position 9D. The panel on the left shows the costaining of TFIIS and Ser5 phosphorylated Pol II before heat shock (C), whereas the center panel represents the same costaining after a 10 min heat shock (D). The panel on the right (E) shows the localization of TFIIS relative to the promoter-associated heat shock factor (HSF, shown in green, antibody used at a 1:50 dilution) after a 5 min heat shock.

treated cells were crosslinked directly, or after a short, 2.5 min, heat shock. As shown in Figure 5C, depletion of TFIIS has no effect on the level of Pol II detected in the *hsp70* promoter region before heat shock (top panel, yellow bars). This result, in agreement with the data shown in Figure 2, indicates that TFIIS is not required for Pol II to stall within the promoter-proximal region. However, depletion of TFIIS leads to a significant reduction in the heat shock-induced recruitment of Pol II to the promoter (Figure 5C, upper panel, red bars). In fact, the Pol II density remains equivalent to that observed before heat induction. Moreover, the reduction in recruitment of Pol II is accompanied by a decrease in the Pol II signal throughout the body of the gene. This result indicates that the stalled Pol II is not efficiently released into the gene in the TFIIS-depleted cells, and that this "stuck" Pol II blocks recruitment of additional Pol II.

As a control for the level of depletion, we also assayed for the presence of TFIIS at *hsp70* in LacZ and TFIIS-treated cells. The 10-fold depletion of TFIIS observed by Western analysis (Figure 3A) leads to a similar reduction in TFIIS detectable on the *hsp70* gene under both NHS and HS conditions (Figure 5C, lower panel, compare the 2.5 min HS values of ~0.2% input at position +58 in LacZ-treated cells with ~0.02% in the TFIIS-depleted cells). Importantly, depletion of TFIIS has no effect on the levels of HSF recruited to *hsp70* upon heat shock, indicating that TFIIS-depleted cells did not display a general, nonspecific loss of factor recruitment (Supplemental Figure S6). These results demonstrate

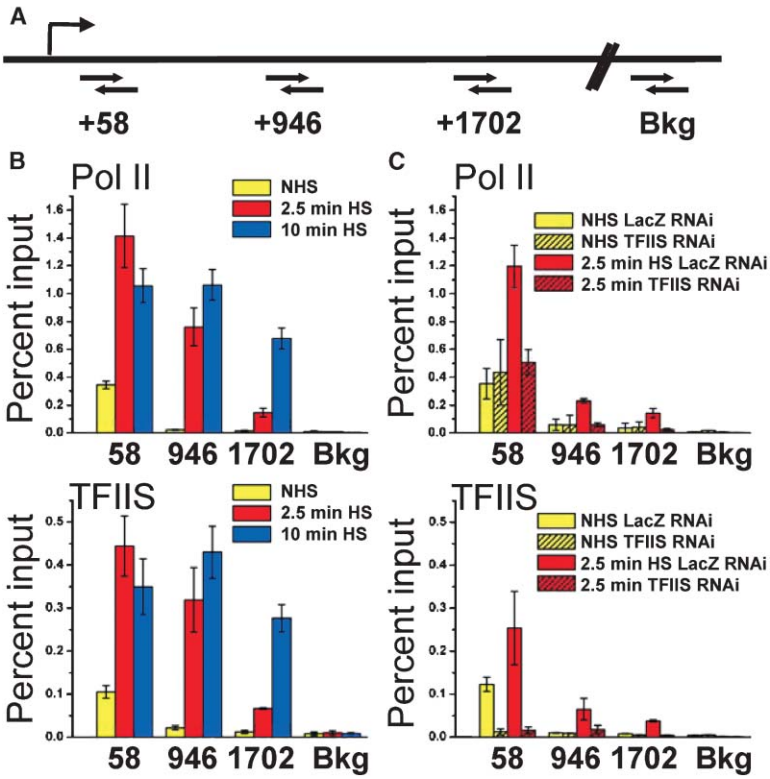

Figure 5. TFIIS Colocalizes with Pol II at *hsp70* and Is Required for Efficient Recruitment of Pol II upon Heat Shock

Localization of Pol II and TFIIS at *hsp70* before and after instantaneous heat shock. Graphs show the percent input of starting DNA that was immunoprecipitated in different regions of the *hsp70* gene before and following various periods of heat shock. The x axis refers to the region of the *hsp70* gene, as shown in (A), with positions given representing the center nucleotide of the amplified DNA. Background (Bkg) refers to a chromosomal region that is free of annotated genes, and is 32 kb downstream of the third *hsp70* copy at 87C. Error bars represent the SEM. (B) Pol II association with the *hsp70* gene, detected using an antibody against the Rpb3 subunit of Pol II (top panel), is compared with the localization of TFIIS (bottom panel). (C) Analysis of the effects of TFIIS-depletion on localization and recruitment of Pol II (upper panel) using an antibody that recognizes the Pol II Rpb3 subunit. The bottom panel represents a comparison of TFIIS levels detected in LacZ-treated versus TFIIS-depleted cells.

that, while TFIIS is not required to establish the stalled Pol II at *hsp70*, depletion of TFIIS interferes with efficient release of Pol II from the promoter region and the rapid recruitment of additional Pol II.

Discussion

Pol II and/or general transcription factors have been found to occupy a growing number of promoters of preactivated genes (Dellino et al., 2004; Lis, 1998). These varied promoters may utilize similar mechanisms for selectively recruiting certain components of the transcription machinery and for regulating transcription initiation and elongation through the promoter-proximal region. The efficiency of synthesis through the initially transcribed sequence is particularly sensitive to perturbation and is thus a prime target for gene regulation (Luse and Samkurashvili, 1998; Pal et al., 2001). Factors that impede the progress of the RNA polymerase within the first 10–40 nt, which often include both protein components and the nucleic acid sequence, have been shown to influence transcriptional pausing, arrest, and termination efficiency (Krumm et al., 1992; Laspia et al., 1993; Lis, 1998; Marshall and Price, 1992; Plet et al., 1995; Price, 2000; Roberts et al., 1998; Strobl and Eick, 1992). Identification and characterization of the factors that modulate the regulatory pausing and/or stalling of Pol II within the promoter-proximal region is essential to understanding the regulation of genes like *hsp70*, wherein this step is rate limiting for gene expression.

Transcription of *hsp70* in vitro revealed positions of pausing that corresponded faithfully with locations that had been identified in vivo as harboring Pol II complexes that were not efficiently elongated. Likewise, Pol II artificially halted at these positions in vitro rapidly lost the capacity to resume transcription, even after removal of negatively acting elongation factors through stringent washing with sarkosyl. These results are similar to in vitro data reported by Gilmour and colleagues (Li et al., 1996), demonstrating that Pol II can become inactive within the promoter-proximal region. Our work expands upon these observations by establishing that the inactive Pol II can be rescued by transcript cleavage factor TFIIS and thus represent arrested species. It is interesting to note that the predominant sites at which Pol II is found on the uninduced *hsp70* gene (Rasmussen and Lis, 1993, 1995) are positions to which Pol II stably backtracks in vitro.

The Role of TFIIS in *hsp70* Transcription

Our data suggest the following model for the role of TFIIS in *hsp70* gene expression (Figure 6). Under uninduced conditions, Pol II (red rocket) is recruited to the *hsp70* promoter and begins to transcribe through the promoter-proximal region. Intrinsic pause sites within the initially transcribed sequence induce transient stops in elongation, giving the regulatory negative-elongation factors (shown for simplicity as a single yellow oval) time to bind and impede further movement into the gene. However, Pol II stalled for an extended time at the pausing sites have a tendency to backtrack along the template, displacing the 3′ end of the RNA from the catalytic site (purple sphere) and prohibiting further elongation. In the absence of TFIIS (pathway shown at left), the arrested, inactive Pol II are unable to resume transcription rapidly upon heat induction, even after the nega-

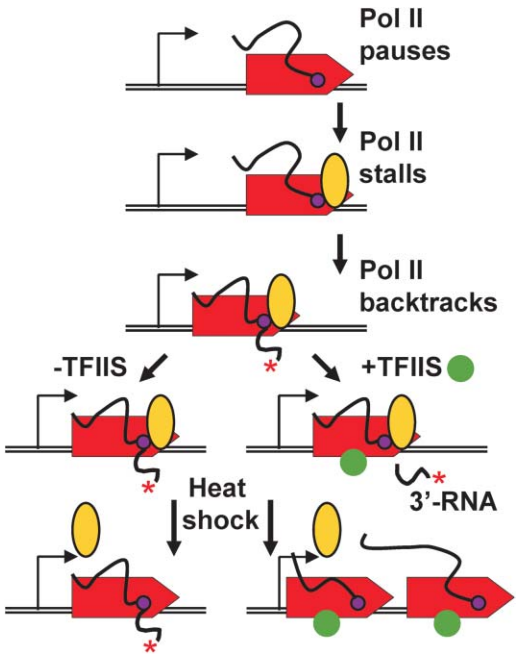

Figure 6. Model Depicting the Role of TFIIS in the Regulation of *hsp70* Transcription

Pol II (red rocket) pauses in the early phases of transcription elongation and is held in the promoter-proximal region by accessory elongation factors (illustrated in yellow). Backtracking of the Pol II along the DNA template leads to the displacement of the 3′ end of the RNA (black) from the Pol II active site (purple dot). TFIIS (green) stimulates cleavage of the backtracked RNA, aligning the new RNA 3′ end with the active site. Upon heat shock, active Pol II (but not the arrested Pol II) is released from the promoter-proximal region and TFIIS tracks with the elongating Pol II into the body of the gene. Movement of the initial Pol II from the promoter region allows recruitment of additional Pol II.

tively acting factors have been removed. However, in the presence of TFIIS (pathway shown at right, TFIIS depicted as green circle), TFIIS-dependent cleavage returns inactive Pol II to a transcriptionally active conformation so that, upon heat shock and the removal of negatively acting factors, Pol II can be rapidly released from the promoter region. The movement of the first Pol II away from the promoter region allows for the recruitment of subsequent Pol II molecules (shown also as red rockets). We note that this model is supported by a recent study of factors that interact genetically with Dst1 (the yeast gene encoding TFIIS), which suggested a general role for TFIIS in the transition from initiation to elongation (Malagon et al., 2004).

Conservation of TFIIS/Gre Function

Our results are reminiscent of the role of bacterial Gre factors in mediating transcription efficiency through a regulatory pause in the late gene operon of λ bacteriophage (Marr and Roberts, 2000). In the λ system, interactions between the RNA polymerase σ subunit and the promoter-proximal DNA sequence induce a transient pause in transcription, during which the λ Q protein binds and modifies the RNA polymerase, rendering it termination resistant. The Gre proteins modulate the kinetics of transcription through the pause site and are required for efficient function of the λ Q protein. Similarly, the activity of transcript cleavage factor TFIIS is necessary for efficient induction of *hsp70* through its activation of promoter-proximally stalled Pol II. Thus, our results indicate that, in addition to structural and mechanistic similarity between the Gre and TFIIS proteins, these factors may perform similar roles in vivo, serving to mediate the expression of genes that undergo pausing within the initially transcribed sequence.

Experimental Procedures

Protein Purification and Clones

Biotinylated DNA containing *Drosophila hsp70* sequences were generated by PCR with biotinylated primers. Nuclear extracts were prepared from *Drosophila Kc* cells using the protocol described in Price et al. (1987). Preparation of fractionated *Drosophila* embryo nuclear extract and antibody generation is described in detail in Supplemental Data. For polytene immunofluorescence, the TFIIS antibody was used at a 1:250 dilution, while H14 (Covance) and HSF antibodies were used at a 1:50 dilution. TFIIS cDNA was cloned into pET19 (Novagen) vector, and the N-terminal 6His-tag was used to purify TFIIS to homogeneity for use in enzymatic assays.

In Vitro Transcription Elongation Assays

Transcription using *Drosophila Kc* nuclear extracts (Figures 1 and 2A, Supplemental Figures S1 and S4) from non-heat-shocked cells (120 μg total protein) employed a DNA template (100 ng per reaction) that contained *hsp70* sequences from −365 to +500 and was biotinylated on both ends to allow for immobilization on streptavidin-coated magnetic beads (Dynal) and to prevent DNA degradation. Preinitiation complexes were formed during a 30 min incubation at room temperature in transcription buffer (20 mM HEPES [pH 7.6], 10% glycerol, 0.2 mM EDTA, 1 mM DTT, and 60 mM potassium glutamate). NTPs were added at a final concentration of 100 μM (except UTP, which was 5 μM unlabeled and 0.2 μM of radiolabeled α-^{32}P UTP), $MgCl_2$ at 5 mM, followed 10 s later by heparin or sarkosyl (at concentrations given in the figure legends) to maintain a single round of transcription. Reactions were quenched at the times indicated with 20 mM EDTA in wash solution (20 mM HEPES, pH 7.6, 1 M KCl, and 1% sarkosyl). Ternary complexes bound to beads were washed three times to remove unincorporated nucleotides and unbound material before being reequilibrated in transcription buffer, or processed as described below. Proteins were digested with Proteinase K before phenol/chloroform extraction of nucleic acids. RNA species were precipitated, resuspended in formamide buffer, and resolved on a denaturing gel. Full-length RNA remained bound to Pol II during the washes (data not shown). RNA products were visualized using a PhosphoImager and analyzed using the ImageQuant software (Molecular Dynamics). The signal intensity within the interval +15 to +45 nt was quantified at each time point (for three experiments) and fit to exponential decay to determine the half-life of escape of Pol II from the promoter proximal region, yielding a $t_{1/2} \approx 9$ min. Transcription assays in fractionated embryo extract (Figures 2B and 2C) were performed as described above, with the following modifications: DNA templates bore a single biotin on the upstream end (at −450) and the stringent sarkosyl and high-salt washes were omitted, with free Pol II and radiolabeled nucleotides removed by washes in transcription buffer plus heparin (200 μg/ml).

RNAi

dsRNA for RNAi experiments was produced as described in the Ambion MEGAscript manual. Primers used to amplify a 944 bp segment from *Drosophila* TFIIS cDNA contained the T7 promoter sequence plus the following sequences complementary to TFIIS: (F) 5′-GAGAGCAAGATGGCCAGCGACGGC-3′ and (R) 5′-GACCGCAC TCGTTGCACATGACG-3′. S2 cells at a cell density of 0.3×10^6 cells/ml were treated with 3.3 μg of dsRNA/ml for 72 hr. Before harvesting cells for RNA analysis, cultures were heat shocked for the times indicated at 36.5°C. TFIIS-depleted cells were compared to cells that had been treated with RNAi against β-galactosidase

(LacZ), as a control for nonspecific effects of treatment with double-stranded RNA. Quantitative RT-rt-PCR was used to measure the levels of *hsp70* and *rp49* RNA present in each sample, using the primers described in Ni et al. (2004). The *rp49* ribosomal protein gene was selected as an internal control because *rp49* mRNA is extremely stable (Ni et al., 2004).

ChIP

ChIP assays were performed essentially as described in Boehm et al. (2003) with several modifications. *S2* cells were grown to a density of 4–6 $\times$ 10^6 cells/ml in HyQ Sfx media (HyClone) before harvesting. Crosslinked cells were resuspended at 1 ml/10^8 cells in sonication buffer containing 0.5% SDS. DNA was sonicated to an average fragment size of 500 bp by 12 $\times$ 20 s bursts on ice. Samples were centrifuged to remove cellular debris before immunoprecipitation (5 $\times$ 10^6 cells/ IP). TFIIS and HSF antibodies (lab stocks) were used at 2 μl per immunoprecipitation, whereas 4 μl of antibody against the Pol II subunit Rpb3 (lab stock) was used. Immunoprecipitated DNA was analyzed by real-time PCR, using primers for *hsp70* sequences centered at +58 (from +4 to +112), +946 (from +872 to +1019), and +1702 (from +1649 to +1754) as described in Boehm et al. (2003). Each data set shown represents results from crosslinking of three to four independent cell cultures.

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