The *Drosophila* P68 RNA helicase regulates transcriptional deactivation by promoting RNA release from chromatin

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Terminating a gene’s activity requires that pre-existing transcripts be matured or destroyed and that the local chromatin structure be returned to an inactive configuration. Here we show that the *Drosophila* homolog of the mammalian P68 RNA helicase plays a novel role in RNA export and gene deactivation. *p68* mutations phenotypically resemble mutations in *small bristles* (*sbr*), the *Drosophila* homolog of the human mRNA export factor NXF1. Full-length *hsp70* mRNA accumulates in the nucleus near its sites of transcription following heat shock of *p68* homozygotes, and *hsp70* gene shutdown is delayed. Unstressed mutant larvae show similar defects in transcript accumulation and gene repression at diverse loci, and we find that *p68* mutations are allelic to *Lighten-up*, a known suppressor of position effect variegation. Our observations reveal a strong connection between transcript clearance and gene repression. P68 may be needed to rapidly remove transcripts from a gene before its activity can be shut down and its chromatin reset to an inactive state.

Keywords: *p68*; RNA helicase; transcription; RNA export

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Programmed gene shut off plays an essential and complementary role to gene induction during the development of metazoan organisms, yet the mechanisms governing transcriptional deactivation are less well understood. Active and quiescent genes differ in at least two major respects. First, the organization and modification state of chromatin becomes specialized for gene activity [for review, see Henikoff and Ahmad 2005]. Second, active loci contain immature transcripts coated with protein complexes that mediate transcript initiation, elongation, termination, and processing [for review, see Aguilera 2005]. All the processes required to generate and export mature transcripts appear to be closely connected and coregulated [for review, see Reed 2003; Vinciguerra and Stutz 2004]. Hence, multiple protein complexes and their associated nascent transcripts must be appropriately disposed of when a gene is shut off.

Differences in active versus inactive gene chromatin have been well characterized using the stress-inducible *Drosophila hsp70* heat-shock genes. Upon heat shock, the transcription factor Heat-Shock Factor (HSF) forms trimers and binds to Heat-Shock Response Elements (HSEs) within response gene promoters [Wu et al. 1987; Westwood and Wu 1993]. Recruitment of HSF to DNA results in phosphorylation of histone H3 on Ser10 at those sites [Nowak and Corces 2000]. Moreover, the TAC1 complex acetylates histone H3, and this activity is essential for robust expression of heat-shock response genes [Smith et al. 2004]. Both histone phosphorylation and acetylation coincide with the recruitment of elongation factors, such as Spt6, to the Pol II complex [Andrulis et al. 2000]. Elongation proteins, along with TFIIIS, promote Pol II release from the promoter and productive polymerization of stress-induced transcripts during the duration of the heat shock [Adelman et al. 2005]. In addition, chromatin-bound poly(ADP-ribose) polymerase (PARP) at heat-shock response loci is modified with long poly(ADP-ribose) chains upon induction, and this process is essential for chromatin loosening and transcript production [Tulin and Spradling 2003]. When the *hsp70* genes shut down, these changes in histone modification and poly(ADP-ribose) accumulation are reversed.

Mechanisms used to export RNAs away from transcription sites and to proofread their structure [for review, see Fasken and Corbett 2005] are likely to facilitate transcript removal concomitant with gene shutdown. In yeast, finished transcripts accumulate at their sites of synthesis when export is defective [Jensen et al. 2001]. Interestingly, the yeast Rrp6p exoribonuclease, a component of the RNA processing and proofreading complex known as the exosome, is essential for transcript accumulation [Hilleren et al. 2001], suggesting that the exosome plays a role in signaling transcript release from the vicinity of the gene. The multiple subunits of the *Drosophila* exosome strongly resemble their yeast counterparts, and associate with Spt6 on actively transcribed genes, including *hsp70* genes [Andrulis et al. 2002]. How-

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ever, the function of the exosome in transcript export in *Drosophila* has not yet been tested.

Studies of RNA export in *Drosophila* suggest that the pathway has been highly conserved. Close homologs of the “export adaptor” proteins Y14 (Tsunagi) [Mohr et al. 2001], Aly/REF, and UAP56/BAT1 [Gatfield et al. 2001; Gatfield and Izaurralde 2002] and the “export receptor” components P15 and NXF1 [Herold et al. 2001; Wilkie et al. 2001] are also found within the *Drosophila* genome. The yeast THO–TREX complex is thought to mediate the association of some transcripts with the export receptor [Jimeno et al. 2002], and homologs of some but not all of these proteins are present in flies. The UAP56 RNA helicase encoded by the *Hel25E* gene and the NXF1 homolog encoded by *small bristles* (*sbr*) are essential for the export of an estimated 75% of *Drosophila* mRNAs in cultured cells [Gatfield et al. 2001; Herold et al. 2003], but Aly/REF is not essential for export of bulk RNA (Gatfield and Izaurralde 2002). When *Hel25E* is knocked down using RNA interference (RNAi), poly(A)-containing RNA is retained in the nucleus and expression of heat-shock proteins is blocked. RNAi inactivation of several THO components also disrupts the export of heat-shock transcripts, but affects only ~20% of genes overall [Rehwinkel et al. 2004]. Strong alleles of both *Hel25E* and *sbr* are lethal; animals bearing weaker *sbr* alleles survive to adulthood, but have abnormally thin thoracic bristles, and exhibit female sterility [Wilkie et al. 2001; Golubkova et al. 2004].

A common feature shared by many of the ribonucleoprotein [RNP] complexes at transcription sites is the inclusion of DEAD-box RNA helicases. DEAD-box RNA helicases unwind RNA:RNA and RNA:DNA duplexes [Pause and Sonenberg 1992; Huang and Liu 2002], disrupt RNA–protein interactions [Jankowsky et al. 2001], and have been implicated in rRNA processing, transcription, pre-RNA splicing, and mRNA transport [Rocak and Linder 2004]. For example, the helicase Sub2p, the yeast homolog of Hel25E, functions in splicing, plays a pivotal role in RNA export by facilitating transcript binding to the export receptor, and also interacts with the THO complex [Strasser et al. 2002]. P68 is another conserved RNA helicase that has been implicated in multiple functions within the nucleus. Mammalian P68 acts during splicing [Lin et al. 2005] but also functions in transcriptional repression [Wilson et al. 2004].

There are indications that disruption of gene processing can influence gene activity and genomic stability [Manley 2002]. Some processing factors in yeast have been shown to interact with basal transcription machinery [Kwek et al. 2002; Estruch and Cole 2003] or chromatin-modifying components [Rodriguez-Navarro et al. 2004]. When RNA export is perturbed, recombination is enhanced, and this has been ascribed to the disruptive effects of RNA:DNA hybrids that form at the affected loci [Huertas and Aguilera 2003]. In metazoan organisms, it remains unknown if RNA processing and export processes can influence gene activity.

Here, we report that in *Drosophila*, the P68 RNA helicase participates in transporting transcripts away from their sites of synthesis and in shutting off genes. Flies mutant for *p68* resemble *sbr* mutants, and retain complete mRNAs at their transcription sites. Unexpectedly, these defects in export are associated with problems in shutting off active genes. Our findings suggest that a fundamental connection exists between transcript clearance and gene repression.

**Results**

*Drosophila* P68 is found at sites of active transcription

Gene deactivation may require that nascent transcripts be cleared from transcription sites and exported or degraded before the chromatin within the region can be reset to a fully inactive state. To identify RNA processing and export factors that might be involved in transcriptional repression, endogenous *Drosophila* gene products were tagged with EGFP using a previously described P-element-based protein trap screening technique [Morin et al. 2001]. One line from our collection, *CB02119*, was of particular interest because the EGFP fusion protein localized to chromosomal sites of active transcription including the nucleolus and polytene chromosome puffs [Fig. 1A; data not shown]. The sequence of the flanking genomic DNA indicated that *CB02119* was inserted within an intron of *Rm62* [Fig. 1B]. The *Drosophila* ortholog of the *p68* RNA helicase, a protein that in mammals functions in RNA processing [Bates et al. 2001; FlyBase]. Multiple differentially spliced transcripts of this gene encode proteins of ~63 kDa as well as one isoform of 79 kDa [Fig. 1B; FlyBase]. Sequencing of RT–PCR-amplified transcripts from *CB02119* confirmed that this strain expresses an in-frame fusion between EGFP and the *p68*-RA isoform [Fig. 1B].

To characterize P68 expression, we generated a polyclonal antibody directed against an N-terminal region common to all isoforms. By Western blot, this antibody specifically recognizes an abundant band of ~63 kDa as expected in wild-type ovary extracts [Fig. 1F]. Costaining of RT–PCR-amplified transcripts from *CB02119* confirmed that this strain expresses an in-frame fusion between EGFP and the *p68*-RA isoform [Fig. 1F].

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*p68* mutant phenotypes are similar to RNA export factor mutations

To test the hypothesis that *p68* mediates some aspect of RNA metabolism at transcription sites, we sought to
identify mutants that disrupt \textit{p68} function. The \textit{Drosophila} genome project (Spradling et al. 1999) had previously generated several \textit{P}-element insertions within the 5' region of \textit{p68} that caused lethality, including \textit{l(3)01084} (Fig. 1B). The suggestion from Spradling et al. (1999) that the \textit{l(3)01084} lethal complementation group is allelic to a previously identified mutant, \textit{Lighten-up} (\textit{Lip}) (Csink et al. 1994), was verified by characterizing heteroallelic combinations of these alleles (Table 1). Moreover, these mutants expressed reduced amounts of endogenous \textit{P68} on Western blots (Fig. 1F).

Viable \textit{p68} mutant flies frequently had small, thin, or missing dorso-central and scutellar bristles (Fig. 1D). Both \textit{CB02119} homozygous and \textit{CB02119}/\textit{l(3)01084} transheterozygous females were also sterile. The nurse cell chromosomes of \textit{CB02119}/\textit{l(3)01084} mutant follicles failed to disperse during the fifth endocycle (Fig. 1E) as in wild type (Dej and Spradling 1999), and these follicles degenerated by stage 8 (data not shown). These phenotypic effects strongly resemble those caused by mutations in \textit{sbr}, which encodes a \textit{Drosophila} ortholog of the mammalian RNA export receptor subunit NXF1 (Gatfield et al. 2001; Herold et al. 2003). The bristle defects in \textit{sbr} and \textit{p68} adults are indistinguishable, and like

**Table 1. Complementation grid of \textit{p68} alleles**

|            | \textit{CB02119} | \textit{l(3)01084} | \textit{Lip}^\text{D} | \textit{Lip}^\text{E} | \textit{Lip}^\text{F} | \textit{Lip}^\text{H} | \textit{Lip}^\text{K} |
|------------|-----------------|-------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| \textit{CB02119} | Sterile         | Sterile/bristle   | Viable               | Viable               | Lethal               | Lethal               | Lethal               |
| \textit{l(3)01084} | Sterile/bristle | Lethal            | Lethal               | Viable               | Lethal               | Lethal               | Lethal               |
the viable p68 mutants, adults bearing conditional sbr alleles are female sterile at the restrictive temperature and undergo follicle degeneration (Wilkie et al. 2001; Golubkova et al. 2004). These similarities between the viable p68 and sbr phenotypes suggested that P68 might function in RNA export.

Loss of p68 results in accumulation of RNA at transcription sites

Since the inducible expression of hsp70 has been widely used to characterize defects in RNA export, we tested whether altering p68 activity affects the release of hsp70 RNA from its transcription sites following heat shock. Wild-type and p68 mutant third instar larvae were heat-shocked for 20 min, and hsp70 mRNA was localized in salivary glands from these animals using fluorescent in situ hybridization (FISH). In control animals, hsp70 mRNA was already abundant in the cell cytoplasm immediately after heat shock (AHS), and was also present in the nucleoplasm and at foci that corresponded to its sites of transcription at 87°C and 87A (Fig. 2A). In contrast, p68 mutant cells displayed almost no hsp70 RNA in the cytoplasm or nucleoplasm at this time (Fig. 2B). Instead most of the RNA was still found at its sites of synthesis. p68 mutants continued to exhibit transport defects 30 min after returning to normal temperature (data not shown). The slow movement of hsp70 RNA into the cytoplasm and its buildup at hsp70 gene loci indicates that P68 affects RNA clearance.

To rule out the possibility that P68 has a preferential role in removing heat shock gene transcripts, or transcripts from genes lacking introns, we pulse-labeled un-stressed wild-type and mutant salivary glands with Br-UTP and then treated them with α-amanitin to stop transcription (Fig. 2C,D). Tissue was then incubated for a further period to allow nascent RNAs time to be transported away from their transcription sites. Control cells displayed diffuse nucleoplasmic and cytoplasmic staining of Br-UTP-labeled transcripts after α-amanitin treatment, reflecting the movement of RNA away from chromatin and out of the nucleus (Fig. 2C). In contrast, labeled RNA in CB02119/LipF cells remained associated with the chromosomes in a tight banded pattern, indicating that many loci were affected (Fig. 2D). Little if any labeled RNA was detected in the cytoplasm of mutant cells. These data are consistent with the results obtained for hsp70 genes and demonstrate that reduced P68 activity disrupts RNA transport away from multiple transcription sites.

Loss of p68 results in redistribution of export factors within nuclei

If P68 functions just upstream or within the RNA export pathway, disruption of its action might alter the nuclear distribution of known RNA export components. In normal cells, RNA export proteins shuttle between transcription sites and nuclear pores. If P68 function occurs preferentially at genes before their newly made transcripts begin to move toward nuclear pores, as our experiments suggest, then other export factors might also accumulate around sites of active RNA production in p68 mutants.

We tested these expectations by examining the cellular location of two previously characterized export proteins, SBR (NXF1) (Herold et al. 2001) and Aly(REF1) (Rodrigues et al. 2001). Control and p68CB02119/LipF mutant salivary glands were stained with anti-SBR antibodies (E,F) or anti-ALY (REF1) antibodies (G,H). SBR and ALY are found along the nuclear periphery and in the nucleoplasm of control cells. In p68 mutant cells, their distribution is changed, and both export factors accumulate in the nuclear interior.

Figure 2. p68 mutants display RNA export defects. [A,B] Control (A) and CB02119/LipF (B) transheterozygotes labeled for hsp70 RNA immediately after a 20-min heat shock. The edge of representative nuclei is outlined in white [boxes]. p68 mutant salivary glands have less cytoplasmic hsp70 RNA and qualitatively higher levels of hsp70 RNA at transcription sites. [C,D] Control (C) and CB02119/LipF (D) salivary glands labeled with Br-UTP. Control cells exhibit cytoplasmic staining and punctuate nuclear Br-UTP staining, whereas p68 mutant salivary glands display robust Br-UTP labeling on polytene chromosomal bands. Little signal was observed in the cytoplasm of mutant cells. [E–H] Control (E,G) and CB02119/LipF (F,H) salivary gland cells stained with anti-SBR [NXF1] antibodies (E,F) or anti-ALY (REF1) antibodies (G,H). SBR and ALY are found along the nuclear periphery and in the nucleoplasm of control cells. In p68 mutant cells, their distribution is changed, and both export factors accumulate in the nuclear interior.
p68 mutants delay heat shock gene deactivation

Retarding RNA transport out of the nucleus would be expected to delay translation of the affected mRNAs. Therefore to further verify that P68 functions in RNA export, we studied the time course of HSP70 protein production following heat shock. Larvae were heat-shocked for 30 min and allowed to recover for up to 6 h, during which time samples were removed and analyzed for HSP70 protein and RNA production. In control larvae, HSP70 was readily detected during the 30-min heat shock (Fig. 3A). HSP70 protein levels reached a maximum 2 h later and then began to slowly fall, in accordance with previously described kinetics of the heat shock response [DiDomenico et al. 1982a]. CB02119/LipP larvae showed a very different pattern of HSP70 protein production. Detectable amounts of protein did not appear by the end of the 30-min heat shock, consistent with the strong reduction of hsp70 mRNA in the cytoplasm of the mutant cells. Interestingly, however, similar amounts of HSP70 protein did eventually accumulate, beginning 2 h after the start of the recovery period. Therefore, many of the hsp70 transcripts that accumulate near their transcription sites must be functional, eventually reach the cytoplasm, and be translated despite the delay. As a result, at 4 and 6 h AHS, the mutant animals contained higher levels of HSP70 than the controls.

To investigate the structure and fate of the transcripts that accumulate at transcription sites in p68 mutants, we analyzed hsp70 mRNA by Northern blot analysis (Fig. 3B). As above, CB02119/LipP larvae were heat-shocked for 30 min and allowed to recover for up to 6 h, during which timed samples were removed and analyzed for RNA production. As expected, control larvae accumulated high levels of polyadenylated hsp70 mRNA only for a short period following the heat shock. Two hours later, hsp70 transcripts had dropped to near background levels, and no transcripts could be detected at 4 h, reflecting the known rapid shutoff of hsp70 transcription and turnover of hsp70 mRNA in the hours following a 30-min heat shock [DiDomenico et al. 1982b]. The hsp70 mRNA in the CB02119/LipP mutant larvae immediately after heat shock was indistinguishable in size and amount from the control RNA (Fig. 3B), despite the fact that it remains predominantly at its sites of transcription and is not translated. The large amount of transcript and absence of shorter forms strongly argue that hsp70 transcription, cleavage, and polyadenylation are normal in p68 mutants. We cannot rule out the existence of small changes in poly[A] length, however. Unlike wild type, in p68 mutants some of these mature transcripts persisted for up to 4 h AHS, in contrast to controls. Thus, the delayed export and abnormal persistence of mature hsp70 mRNA probably explains the kinetics of HSP70 expression in the mutant animals.

To further rule out the possibility that reduction of p68 function disrupts some aspect of normal transcription, and to look for defects in RNA splicing, we carried out additional Northern blot experiments using a 10-min heat shock and analyzed both hsp70 and hsp83 RNA at frequent intervals. Drosophila hsp83 is a constitutive gene with one intron whose transcription is strongly induced by heat shock. As we found in our previous experiments, hsp70 RNA was induced in p68^CB02119/LipP^ mutants.
mutant samples at similar levels to wild-type animals (after normalizing to the rp49 controls), but persisted longer during recovery (Fig. 3C). hsp83 mRNA followed a very similar pattern. Comparable amounts of normalized and polyadenylated hsp83 transcripts were made initially in the p68CB02119/LipF mutants and controls, while higher amounts persisted 60 and 90 min AHS.

The fact that we could observe a band at the expected size of unspliced hsp83 (Fig. 3C, asterisk) allowed the effects of p68 mutation on splicing to be addressed. The intensity of unspliced hsp83 RNA was no greater in the mutant than in controls during the initial 15 min, indicating that splicing was not significantly compromised. Unspliced hsp83 transcript in control animals was detected 15 and 30 min AHS, while higher amounts persisted 60 and 90 min AHS. The fact that we could observe a band at the expected size of unspliced hsp83 (Fig. 3C, asterisk) allowed the effects of p68 mutation on splicing to be addressed. The intensity of unspliced hsp83 RNA was no greater in the mutant than in controls during the initial 15 min, indicating that splicing was not significantly compromised. Unspliced hsp83 transcript in control animals was detected 15 and 30 min AHS, while higher amounts persisted 60 and 90 min AHS. However, the small increase and the persistence of a pool of intron-containing hsp83 transcript probably just reflects continued hsp83 transcription in the mutant compared with wild-type animals [see below]. Our results argue that transcription, splicing, cleavage, and polyadenylation of the hsp70 and hsp83 genes still occurs normally when p68 levels are reduced, but suggest that in addition to retarding RNA export, defects in p68 increase the time required to shut off the transcription of these genes.

p68 mutation impairs gene shutdown after heat shock

The observation that p68 mutants express heat shock transcripts for a prolonged period is interesting in light of the aforementioned finding that p68 is allelic to the Lip locus. Loss-of-function Lip mutations are suppressors of position effect variegation (PEV) (Csink et al. 1994), indicating a possible function in gene silencing and heterochromatin formation. This suggested that the persistence of hsp70 and hsp83 transcripts following heat shock might result from a weakened capacity to repress gene transcription, rather than from an alteration in the kinetics of heat-shock recovery induced by the delay in hsp70 export or some other change in RNA stability. If so, the normal turnoff of cellular gene transcription that accompanies heat shock might also be affected.

To help distinguish between these possibilities, we directly compared chromatin structure in control and mutant samples. Active genes can be distinguished by the presence of histone modifications and specific transcription factors. In unstressed wild-type and p68 mutant cells, histone H3 phosphorylation at Ser10 (H3phosS10) generally marked transcriptional activity at many loci scattered throughout the genome, including P11-positive puff sites (Fig. 4A). Upon heat shock, transcription of hsp70 and other response genes depends on direct HSF binding within their promoters, and this binding is accompanied by the accumulation of H3phosS10. To probe whether loss of p68 prolongs hsp70 transcription, we examined polytene chromosomes to determine whether H3phosS10 and HSF persist on the hsp70 gene loci longer than normal in p68CB02119/LipF mutant animals (Fig. 4B, boxed regions). Immediately following a 20-min heat

Figure 4. Reduced P68 activity results in persistent transcription factor binding to chromosomes. [A] Control and CB02119/LipF polytene chromosomes from unstressed larvae stained with anti-H3phosS10 and P11 antibodies. [B] Control and CB02119/LipF polytene chromosome squashes from larvae heat-shocked for 20 min and dissected immediately (0 min AHS) or 60 min AHS. These samples were stained with anti-H3phosS10 (green) and anti-HSF (red) antibodies. HSF binding to heat-shock response puffs appears to be comparable in control and mutant samples immediately after heat shock. However, the level and number of sites labeled with the anti-H3phosS10 antibody are greater in p68 mutants. While HSF is lost from heat-shock puffs in control samples [control inset] 60 min AHS, HSF binding persists in p68 mutant chromosomes (p68−/− inset). Insets show the hsp70 loci at 87A and 87C.
shock, the hsp70 genes were extensively modified with H3phosS10 and contained abundant HSF in both wild-type and p68 mutant cells. After 1 h of recovery, both the level of H3phosS10 modification and HSF binding were greatly reduced in wild-type cells. In contrast, high levels of both markers of hsp70 gene activity remained in the p68 mutant animals. Thus, reducing the level of p68 prolongs the accumulation of H3phosS10 and HSF at heat-shock loci, further supporting the view that p68 mutation blocks the shutoff of hsp70 transcription.

Further evidence that p68 plays a role in gene deactivation comes from examining the shutdown of endogenous genes following heat shock [Fig. 4B]. Immediately after a 20-min heat shock, most previously active genes had shut down in control samples, as measured by the level of H3phosS10 labeling [Fig. 4, cf. B and A]. However, after this same time period, p68CB02119/LipF mutant animals continued to display a broad pattern of H3phosS10 labeling. Comparison of banding patterns suggested that many of these were normal loci that had failed to shut off following heat shock. After 1 h of recovery, H3phosS10 had reappeared at sites throughout the chromosomes in wild type, reflecting the recovery of general transcription [Fig. 4B]. The failure to ever lose H3phosS10 at the sites of normally active genes suggests that p68 is not only required to shut down hsp70 during recovery from heat shock, but is also needed to shut down non-heat-shock genes immediately after the onset of heat shock.

To confirm that the additional loci containing H3phosS10 in stressed p68 animals represent sites of active transcription, we examined the distribution of active RNA polymerase II. The large subunit of Pol II is modified by specific serine phosphorylations within its repetitive C-terminal domain (CTD) when active, a change recognized by the H5 antibody. Following a 20-min heat shock of wild-type animals, heat-shock loci were strongly labeled, but H5 reactivity had been lost from most chromosomal sites due to the cessation of general transcription [Fig. 4B]. The failure to ever lose H3phosS10 at the sites of normally active genes suggests that p68 is not only required to shut down hsp70 during recovery from heat shock, but is also needed to shut down non-heat-shock genes immediately after the onset of heat shock.

While chromosomal genes clearly retained hallmarks of active transcription following heat shock in p68 mutants, we confirmed their continued activity by analyzing Br-UTP incorporation. Salivary glands from control and p68 mutant larvae were dissected and heat-shocked in culture for 10 min. Br-UTP was then added to the culture, and the glands were kept at 37°C for an additional 20 min. The samples were immediately fixed and stained for incorporated Br-UTP [Fig. 5E–H]. As expected, control samples showed very little evidence of Br-UTP incorporation except at a few foci that likely correspond to heat-shock gene loci [Fig. 5E, G, arrows]. p68CB02119/LipH mutant animals, on the other hand, incorporated Br-UTP at similar loci, and at multiple additional sites on the chromosomes and in nucleoli [Fig. 5F, H]. The incorporation of Br-UTP within nucleoli suggests that rRNA genes remain active during stress conditions in p68 mutant cells. Thus, many loci not only retain the hallmarks of gene activity in stressed p68 mutants, but continue to synthesize RNA for a prolonged period. All of these assays argue strongly that p68 is required for gene deactivation in addition to its role in RNA export.
P68 localizes to genes targeted for repression

P68 might facilitate gene shutdown simply by continuing to remove completed transcripts, or it might act by a mechanism distinct from its steady-state role in RNA export. If facilitating export was its only role, the level of P68 would not be expected to change dramatically as the time of shutdown. However, if more P68 function was required for gene shut off, additional P68 might be recruited prior to gene deactivated. To look for evidence of such a change, we studied P68 levels on active heat-shock genes and on genes undergoing deactivated after a 30-min heat shock. Before heat shock, P68 and H3phosS10 are both present on several ecdysonic puffs and many but not all other secondary loci (Fig. 6, no HS). Frequent colocalization is not surprising given the likely role of P68 in exporting RNA from active loci.

These experiments documented a striking correlation in the level of P68 at the site of hsp70 genes undergoing transcriptional shutdown. As expected, following a 30-min heat shock, general H3phosS10 staining was lost and heat-shock loci became strongly labeled (Fig. 6, 0 min heat shock, general H3phosS10 staining was lost and heat-shock loci became strongly labeled [Fig. 6, 0 min AHS]). However, despite the fact that the hsp70 genes have already been active for nearly 30 min, P68 at this time was still distributed at multiple sites with only very low amounts detectable at the hsp70 loci. These dispersed sites may represent normal genes that have lost H3phosS10 modifications, but are still undergoing P68-mediated deactivation. After 10 min of recovery, P68 begins to accumulate at high levels at heat-shock loci (Fig. 6, 10 min AHS). Thirty minutes after heat shock, normal loci (including ecdysonic puffs) are beginning to recover based on H3phosS10 staining (Fig. 6, 30 min AHS). P68 is now present at even higher levels at the newly regressed heat-shock puffs. The dynamic localization patterns suggest that P68 does not simply follow the timing of gene activity, but accumulates at higher levels on loci undergoing gene shutdown. The simple interpretation of these observations is that the rate of RNA export away from transcription sites increases when a gene is being shut down.

Loss of P68 prolongs ecdysonic-responsive gene expression

The heat shock response may represent a special case in which the dynamic localization of P68 is required for transcriptional repression. To test whether P68 acts as a specific or general repressor, we looked for other potential targets of p68 regulation. Some p68 transheterozygous mutants remain as third instar larvae for up to 5 d, whereas control animals enter preupal development after 1–2 d. Delayed molting phenotypes have also been described in mutants that disrupt synthesis and response to the steroid hormone ecdysone [Thummel 1996]. Therefore, we tested whether disruption of p68 leads to an alteration in the normal time course of ecdysonic-induced gene expression.

Binding of ecdysonic to its receptor induces the expression of a set of early response genes that include E74 and E75. Previous results have shown that specific E74 and E75 isoforms exhibit dynamic expression patterns during late larval and prepupal development [Karim and Thummel 1991, 1992]. To test whether P68 regulates the expression of ecdysonic-responsive genes, we examined the expression of E74 and E75 during prepupal development.

Total RNA was isolated from control and CB02119/Lip-animals at specific time points relative to spiracle excision. We performed RT–PCR on these samples using primers specific for different isoforms of the E74 and E75 genes [Fig. 7]. In control samples, E74B levels are high in wandering larvae, leading up to puparium formation. Once these animals form puparia, the expression of E74B gives way to high levels of E74A expression within 2–4 h. E74A expression then decreases over the next few hours. A similar shut off of E75B also takes place 2–4 h after puparium formation [APF]. In p68 mutants, neither E74B nor E75B shut off on schedule [Fig. 7]. RNA levels of both genes started to decline at least 2 h later than normal, and unlike controls, complete shutdown is never observed. The decrease in E74A expression normally observed by 6 h after puparium formation in control animals was also delayed and incomplete. E74A RNA did not decline in the p68 mutant animals until 10 h APF, and remained at higher levels than in the control animals.

This experiment confirms that p68-mediated transcriptional regulation is not limited to stress response genes.

Discussion

P68 is required for transcriptional deactivation

We found that p68 mutant animals turn off genes slowly and incompletely. These include the developmentally regulated early ecdysone response genes E74 and E75, rDNA genes within the nucleolus, and many other active genes that normally cease transcription following heat shock, and the heat shock response genes themselves during recovery from stress. This prolonged gene activity in p68 mutant animals appears to be normal in regard to chromatin organization, RNA production, and RNA processing. For example, long after hsp70 genes have shut off in wild-type animals, hsp70 genes in p68 mutants retain high levels of H3phosS10 modification, abundant HSF transcription factor, and active RNA polymerase II, and they continue to incorporate Br-UTP into RNA.

Our experiments rule out several potential explanations for the continuing gene activity in p68 mutant animals. First, mammalian P68 has been proposed to facilitate splicing by unwinding base-pairing between the U1 RNA and the 5’-splice junction [Lin et al. 2005]. Thus, effects on gene shutdown might be secondary to mis-pairing of direct shutdown regulators. However, we found no evidence that Drosophila P68 affects splicing. The level of unspliced hsp83 transcripts did not increase beyond a small rise attributable to ongoing transcription, nor could unspliced E74 or E75 gene transcripts be detected in p68 mutants. Genes lacking introns such as hsp70 were affected in the same manner as intron-containing genes. Second, mammalian P68 has been implicated as a direct transcriptional regulator, and may participate in activating p53 target genes [Bates et al. 2005].
However, *Drosophila* P68 seems unlikely to act as a transcriptional activator, because the affected genes were diverse and unrelated in function (including the nucleolus), and were up-regulated rather than down-regulated.

A final possibility is that secondary affects of *p68* on nuclear physiology feed back and arrest gene shutdown. For example, the delay in HSP70 production caused by *p68* mutation might slow the activation of the negative

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**Figure 6.** The localization of P68 changes during heat shock. Salivary gland polytene chromosome squashes from wild-type wandering third instar larvae. Samples were unstressed (no HS) or subjected to a 30-min heat shock at 37°C followed by no recovery period (0 min AHS), 10 min of recovery at room temperature (10 min AHS), and 30 min of recovery (30 min AHS). Squashes were stained with anti-H3phosS10 and anti-P68 antibodies. Relevant cytological loci are indicated in each panel. In non-heat-shocked animals, there is robust colocalization between H3phosS10 and P68 on a few developmental puffs. Upon heat shock, H3phosS10 accumulates at heat-shock puffs (87A, C) and decreases at other sites (74E, F, 75B). However, P68 remains bound to developmental puffs. After a 10-min recovery period, P68 begins to colocalize with H3phosS10 on heat-shock puffs. This accumulation of P68 on heat-shock puffs continues to increase up to 30 min after heat shock. Thirty minutes into recovery, H3phosS10 staining begins to accumulate on other bands, while P68 remains highly enriched on heat-shock puffs.
feedback loop by which chaperone proteins including HSP70, HSP90, and HSP40 are proposed to normally shut down heat-shock-induced gene expression after removal from stress [Ali et al. 1998; Duina et al. 1998; Shi et al. 1998; Bharadwaj et al. 1999]. However, it seems highly unlikely that the diverse genes whose shutdown depends on p68 function, including rDNA genes, ecdysone response genes, and the cellular genes repressed by heat shock, are all subject to such feedback regulation. Instead, the available evidence argues strongly that P68 functions directly in shutting down gene activity, and consistent with such direct action we observed that P68’s abundance increases on loci prior to and during the shutdown process.

P68 also functions in RNA clearance from transcription sites

How, then, does a direct role for P68 in gene shutoff relate to the additional function in RNA export we observed for this molecule? Loss-of-function p68 mutations result in bristle and ovary defects similar to those seen in sbr mutants, a known component of the RNA export machinery [Herold et al. 2001, 2003; Wilkic et al. 2001]. p68 mutant animals accumulate RNA at transcription sites scattered at many locations around the genome. Furthermore, newly transcribed and processed hsp70 transcripts accumulate at the site of transcription for prolonged periods, causing a pronounced delay in HSP70 translation. These findings suggest that P68 functions at a very early step in RNA export, or at a novel step prior to export that is required to clear completed transcripts from their sites of transcription.

During transcription, various proteins that mediate transcriptional elongation, RNA processing, and RNA export coat nascent transcripts to form large ribonucleoprotein complexes [mRNPs] [Stutz and Izaurralde 2003]. Work in a variety of organisms suggests that the assembly of these mRNPs is under strict quality controls. Disruption of RNA processing or association of export factors with transcripts results in RNA accumulation at gene loci and the activation of the RNA surveillance pathway. Defective transcripts are targeted for degradation by the exosome, an evolutionarily conserved multiscubunit RNase complex. The exosome associates with elongation factors and localizes to actively transcribed genes in Drosophila [Andrulis et al. 2002], suggesting that transcripts are constantly monitored for defects. Interestingly, mutations in the rrp6p exosome gene not only result in the stabilization of RNAs but also lead to their improper release from chromatin [Hilleren et al. 2001]. These results suggest that the exosome tethers transcripts to chromatin and ensures that only properly processed RNAs are released for export. P68 may function by interacting with nascent transcripts in a manner that confers competence for a transcript to leave its site of synthesis and undergo transport to nuclear pores. This P68-sensitive step would act as a checkpoint for final release of a completed transcript into the nucleoplasm.

P68-dependent transcript clearance may be required to shut off genes

We favor the idea that P68’s requirement for gene shutoff can be explained by its proposed role as a mediator of transcript release. There are two general mechanisms by which nascent transcripts at the site of an active gene might impede transcriptional deactivation. First, some positively acting transcriptional cofactors associate with nascent transcripts rather than remaining at promoter or enhancer regions [Aguilera 2005]. Transcript-bound factors would remain at very high local concentrations relative to those that dissociate into the nucleoplasm prior to rebinding. Transcripts bearing such factors would be expected to promote continued gene activity, as part of an equilibrium of positively and negatively acting mechanisms that determine the instantaneous rate of transcriptional activity. During repression, up-regulated RNA release mediated by P68 would lower the local concentration of such factors and help shift the equilibrium toward a state that favors gene deactivation.

Figure 7. Ecdysone response genes exhibit prolonged expression in the absence of p68. [A] RT–PCR reactions using E74A, E74B, and rpo9 specific primers. Total RNA was isolated from control and C802119/Lipfr animals at the various points after puparium formation. Expression of E74B in controls peaks at ~3 h APF and then decreases from 2 to 10 h APF. A second peak of E74B expression is observed beginning at 12 h APF. E74A expression in control animals first peaks at 2 h APF and then decreases to low levels between 6 and 8 h APF, followed by a second peak at 10 h APF that declines rapidly. Mutant animals exhibit prolonged expression of both E74A and E74B relative to the control samples.

[B] RT–PCR using E75A– and E75B-specific primers. p68 mutant animals exhibit prolonged expression of both E75A and E75B relative to the control samples.
Gene activity is also controlled by the state of chromatin decondensation [for review, see Jenuwein and Allis 2001]. Decondensation is correlated with high levels of histone acetylation [Jenuwein and Allis 2001], phosphorylation [Nowak and Corces 2004], and ADP-ribose modification [Tulin et al. 2003], and these modifications must be reversed to complete gene shut-off. The presence of bulky mRNPs around a gene might simply block chromatin from reassembling into the highly compact state characteristic of inactive genes. Transcripts and their associated protein complexes might also be inhibitory to chromatin-remodeling processes due to specific protein–protein interactions. The targeting of extra P68 to genes at the onset of gene shutdown might stimulate export of the remaining transcripts that would counteract the repression process.

Multiple genes within a chromosome region are sometimes shut down coordinately through the process of heterochromatin formation. The ability of p68 to stimulate gene deactivation via transcript release may play a role in forming heterochromatin as well as in shutting off individual genes, because p68/Lip was previously identified as a suppressor of position effect variegation [Csink et al. 1994]. This suggests that transcript removal may be an important part of heterochromatin formation and spreading. The RNAi pathway has been shown to facilitate heterochromatization [Bernstein and Allis 1999], and spreading. The RNAi pathway has been shown to facilitate heterochromatization [Bernstein and Allis 1999], and the remaining transcripts that would counteract the repression process.

**Materials and methods**

**Fly strains and genetics**

Fly stocks were maintained at 20°C–23°C on standard cornmeal–agar–yeast food. The y w strain used for generating CB02119 was used as a control in all experiments. CB02119 was generated by mobilizing the GFP-trapping P-element described in Spradling et al. [1999]. and the remaining transcripts that would counteract the repression process.

The sequence corresponding to the first 147 amino acids of P68-RB was cloned into a modified pPRO-EXHTC [Invitrogen] vector [gift from J. Wilhelm, Carnegie Institution of Washington, Baltimore, MD] using a LR clonase reaction [Invitrogen] following the manufacturer’s protocol. This bacterial expression vector places a 6xHIS tag at the N terminus of the ORF. Expression of this construct in a large-scale culture was induced using 0.2 mM IPTG for 4 h. Cells were then lysed in 6 M guanidine and incubated with Ni-beads [Qiagen]. These beads were then washed into 6 M urea, and P68 recombinant protein was eluted off the beads using 300 mM imidazole in 6 M urea. Various elution fractions were run on 10% SDS-PAGE gels and stained with Coomassie blue to check for purity. Antibodies against recombinant P68 protein were generated in guinea pigs by Covance Research Products.

**P68 regulates transcriptional deactivation**

**RNA in situ and Br-UTP labeling**

FISH using DIG-labeled hsp70 antisense probes was performed on salivary glands as described in Franke and Baker [1999]. The DIG-labeled RNA was detected using sheep anti-DIG antibodies [1:2000, Roche] and anti-sheep Alexa 568 antibodies [1:200, Molecular Probes]. A final concentration of 2 mM Br-UTP [Sigma] was used to pulse-label nascent RNA in salivary glands. Br-UTP-labeled RNA was detected using rat anti-BrdU antibodies [1:50, Serotec].

**Immunostaining and polytene chromosome squashes**

Whole-mount samples were fixed with 4% paraformaldehyde for 10 min and processed using standard procedures. Squashes of third instar larval salivary glands were performed as described in Paro [2000] with the following modifications: Samples were dissected in 1× PBS, 0.1% Triton X-100, and the glands were then fixed in 4% paraformaldehyde, 50% acetic acid, 1% Triton X-100 for 2 min and squashed in 45% acetic acid.

Primary antibodies were diluted in PBT (1× PBS, 0.3% Triton X-100, 0.5% BSA) and used at the following dilutions: rabbit anti-GFP [1:2000, Torrey Pines Biolabs], guinea pig anti-P68 [1:500], mouse anti-Hrb87 [1:50, kind gift from H. Saumweber, Institut für Biologie, Berlin, Germany], rabbit anti-H3phosS10 [Upstate], anti-HSF [1:50; kind gift from J. Lis, Cornell University, Ithaca, NY], anti-NXF1 [1:100, kind gift from E. Izaurralde, European Molecular Biology Laboratory, Heidelberg, Germany], anti-REF1 [1:100, kind gift from E. Izaurralde], and mouse anti-AJI [1:20, kind gift from J. Gall, Carnegie Institution of Washington, Baltimore, MD]. The following secondary antibodies were used: goat anti-rabbit, goat anti-mouse, or goat anti-guinea pig conjugated to either Alexa 488 or Alexa 568 [1:200, Molecular Probes].

**Western blot analysis**

Standard Western blotting techniques were used. Ponceau S staining of membranes was used to confirm equal loading between lanes immediately after transfer. Antibodies were used at the following concentrations: guinea pig anti-P68 [1:3000], mouse anti-Actin [1:2000], mouse anti-HSF [1:1000], goat anti-mouse HRP [1:2000, Jackson Labs], goat anti-rabbit HRP [1:2000, Jackson Labs], and goat anti-guinea pig HRP [1:2000, Jackson Labs].

**Northern blot**

Total RNA was isolated using Trizol reagent [Invitrogen] and fractionated on a 1% formaldehyde-agarose gel. The gel was soaked in 0.05 N NaOH for 10 min and washed in dH2O. RNA was transferred to a nitrocellulose membrane using 10× SSPE and then cross-linked with a UV-Stratalinker. Membranes were then prehybridized with DIG Easy Hybrid Granules [Roche] for 30 min at 65°C and then hybridized with DIG-labeled hsp70 and rp49 RNA antisense probes overnight at 65°C. The membrane was then washed in Post-Hybridization washes: two times for 5 min in 2× SSC, SDS [0.1%] at 15°C–25°C followed by two times for 15 min in 0.5× SSC, SDS [0.1%] at 65°C under constant agitation. The membrane was incubated for 30 min in blocking solution [1% blocking reagent [Roche] in 0.1 M maleic acid at pH 7.4, 0.15 M NaCl], followed by a 30-min incubation with sheep anti-DIG-AP [Roche] in blocking solution. The membrane was then washed extensively, soaked with CDP-Star reagent [Roche], and exposed to film.
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**RT–TAIL–PCR and RT–PCR**

The insertion site of CB02119 was originally determined using an inverse PCR protocol (Bellen et al. 2004). To confirm that CB02119 trapped p68, an RT reaction was coupled to a modified TAIL–PCR reaction (Singer and Burke 2003). Briefly, total RNA was isolated from adult flies using a 96-well RNAqueous kit (Ambion) following the manufacturer’s protocol. Five microliters of total RNA was used in a 50-µL RT reaction using Qiagen’s One Step RT–PCR kit following the manufacturer’s protocol. This same reaction was then subjected to three rounds of TAIL–PCR, using the following GFP-specific primers: GFP-For1, 5’-GCACTCTGAGACCACTGG-3’; GFP-For2, 5’-CAACGTTCACTATGAGCCG-3’; GFP-For3, 5’-AGACCCCAACGGAGAAGCCG-3’; GFP-Rev1, 5’-GTCCCTGCTGTCTGATGCTG-3’; GFP-Rev2, 5’-GACACCTGTAATCTTGTTGCG-3’; GFP-Rev3, 5’-AGCTCTCGCCTTGGCTCACC-3’. The resulting products were then sequenced.

For E74 and E75 RT–PCR reactions, control and CB02119/Lip samples were staged as previously described except that slow wandering larvae were used for the −3-h time point, and fast crawling larvae were used for the −12-h time point. Total RNA from these samples was isolated using Trizol reagent according to the manufacturer’s instructions. Equal amounts of RNA were used in a One Step RT–PCR reaction (Qiagen) using E74 and E75 isoform-specific primers as described in Huet et al. (1993). tp49 primers were included in the E74 reactions, and the resulting RT–PCR product served as a control. Products were run on a 2% agarose gel.

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The *Drosophila* P68 RNA helicase regulates transcriptional deactivation by promoting RNA release from chromatin

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