

The Lawc protein is required for proper transcription by RNA polymerase II in *Drosophila*

Tracy Brandt · Victor G. Corces

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Abstract Genetic analysis of the *Drosophila leg-arista-wing complex (lawc)* gene suggests a role for the Lawc protein in chromatin-related processes based on its classification as a *trxG* gene but the molecular mechanisms of its function remain elusive. We have found that Lawc is a small, cysteine-rich protein that is present in most of the interbands of polytene chromosomes. In agreement with this observation, Lawc co-localizes with RNA polymerase IIo (Pol IIo) and it is recruited to transcribed loci after elongation by Pol IIo has begun. Lawc interacts with the nuclear proteasome regulator dREG γ in a yeast two-hybrid assay and both proteins co-localize on polytene chromosomes. In addition, a mutation in *lawc* interacts genetically with a mutation in a component of the proteasome. *lawc* mutants show decreased expression of some genes, while the levels of Pol IIo^{Ser2} increase. We conclude that Lawc is required for proper transcription by RNA polymerase II in a process that involves the nuclear proteasome.

Keywords Transcription · Lawc · RNA polymerase II · dREG γ · Proteasome

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T. Brandt · V. G. Corces
Department of Biology, Johns Hopkins University,
Baltimore, MD 21218, USA

Present Address:

V. G. Corces (✉)
Department of Biology, Emory University,
1510 Clifton Road NE, Atlanta, GA 30322, USA
e-mail: vcorces@emory.edu

Introduction

Transcription by RNA polymerase II (Pol II) is a tightly regulated and complex process designed to allow precise control of gene expression in eukaryotes. The first step in transcription by Pol II is the formation of the preinitiation complex. Sequence-specific activators bind to DNA elements near the gene and recruit ATP-dependent chromatin remodeling complexes, histone-modifying enzymes, and/or components of the mediator scaffold complex to serve as coactivators. Coactivators ultimately operate to make promoter-proximal chromatin accessible to additional factors required for transcription, including unphosphorylated Pol II (Pol IIa), as part of the preinitiation complex (Lu et al. 1991). The steady-state levels of transcription regulators at the promoter can be controlled by recruitment or by degradation. Here we explore the possibility that a new protein named Lawc is involved in regulating the activity of the REG γ proteasome to control transcription elongation.

During transcription, the recruitment of Pol IIa is followed by promoter melting, transcription initiation, and promoter clearance. The last component of the preinitiation complex to be recruited, TFIIF, can both unwind the DNA template and phosphorylate the carboxy-terminal domain (CTD) of the largest Pol IIa subunit on serine 5 of the heptapeptide repeat (Pol IIo^{Ser5}) (Lu et al. 1992; Schaeffer et al. 1993; Schwartz et al. 2003). CTD phosphorylation may disrupt contacts between Pol II and proteins involved in preinitiation; the Pol II complex can then move away from the promoter and transcription can progress. In addition, Pol IIo^{Ser5} recruits, and sometimes stimulates, factors needed for initiation and processing of the 5' end of the transcript, such as RNA-capping enzyme (Ho and Shuman 1999; Schroeder et al. 2000). DSIF and NELF proteins mediate promoter proximal pausing just downstream of the

transcription start site (Wu et al. 2005; Yamaguchi et al. 1999). The significance of this stage is not fully understood, but it may serve as a checkpoint for efficient mRNA capping. Next Pol IIO^{Ser5} is phosphorylated on CTD serine 2 (Pol IIO^{Ser2}) by p-TEFb and the pause is released so that Pol IIO can continue along the template in the elongation phase (Marshall et al. 1996).

The 26S proteasome also plays a role in transcription. This proteasome is both nuclear and cytoplasmic, and consists of a 20S catalytic core and two 19S lid/base subunits. A 19S subunit can recognize ubiquitinated substrates and funnel them into the core to be degraded (reviewed in Kinyamu et al. 2005). The 19S subunit and the 20S core have been detected at active genes and 19S seems to be involved in elongation (Ferdous et al. 2001; Gillette et al. 2004; Gonzalez et al. 2002). Moreover, the proteasome is required for transcriptional activation by many members of the Nuclear Hormone Receptor (NHR) family of transcription factors. It seems that the order of recruitment of coactivators to genes targeted by NHRs is important and that proteasome-mediated degradation of some NHRs and corepressors is required for proper transition between various complexes and successful transcription (Dennis et al. 2005; Reid et al. 2003).

Transcription of genes in response to hormones by activated class II NHRs has been extensively studied (reviewed in King-Jones and Thummel 2005). These NHRs bind to DNA and serve as transcriptional repressors in the absence of their ligand via corepressor recruitment. However, they bind DNA and become activators in the presence of the hormone ligand, usually in the form of a heterodimer. One well-studied class II NHR is the *Drosophila* Ecdysone Receptor (EcR). *Drosophila* chromosomes have many puff sites induced by the 20 hydroxyecdysone steroid (20E). Genes activated by 20E are involved in the development of the fly as it transitions from larva to adult. This activation is mediated by the EcR when bound to 20E and Ultraspirecle by recruiting coactivators such as Tai (Bai et al. 2000; Yao et al. 1993, 1992). The REG γ proteasome is a conserved nuclear complex consisting of the 20S core and 11S subunits. The 11S subunit is a REG γ homoheptamer and it substitutes for the 19S lid/base to degrade proteins in an ATP and ubiquitin-independent manner (Dubiel et al. 1992; Masson et al. 2001; Wilk et al. 2000). One of the few known targets of the REG γ proteasome is the SRC-3 coactivator, which is the mammalian homolog of the *Drosophila* Tai protein (Bai et al. 2000; Li et al. 2006).

Mutants in the *Drosophila melanogaster* *leg-arista-wing complex* (*lawc*) gene have been previously isolated and characterized. Flies carrying a mutation in *lawc* have partial arista-to-leg transformations and the *Drosophila lawc* gene was classified as a member of the *trxG* (Zorin et al. 1999). Given this link between *lawc* and chromatin,

we decided to analyze the role of the *Lawc* protein in chromatin structure and transcription. Here we find that *Lawc* is a novel, small, cysteine-rich nuclear protein that is present at most of the interbands of polytene chromosomes. *Lawc* co-localizes with Pol IIO at numerous chromosomal sites where it appears to be recruited after elongation by Pol IIO has begun. *lawc* mutants show an increase in the phosphorylated Pol IIO^{Ser2} form of RNA polymerase II. Nevertheless, some genes show decreased expression in *lawc* mutants, suggesting that the increase in Pol IIO^{Ser2} is not always productive. *Lawc* interacts with the nuclear proteasome regulator dREG γ in a yeast-two-hybrid screen and *lawc* interacts genetically with a mutation in a component of the proteasome core. We conclude that *Lawc* is required for proper transcription by RNA polymerase II in a process that involves the nuclear proteasome.

Materials and methods

Fly strains and construction of transgenic flies

Fly stocks were maintained in standard medium at 25°. Transgenic flies were obtained by P element-mediated transformation. A 6.5 kb fragment of DNA containing the coding region of *CG32711* plus 3,987 bp of 5' and 447 bp of 3' sequence was obtained by PCR of genomic DNA and cloned into the pCaSpeR vector. The phenotype of *y w ctⁿ lawc^{P1}* females was then compared to that of *y w ctⁿ lawc^{P1}* females homozygous for the transgene. *pros26 pb p^P/TM3 Sb Ser* and *pb p^P/TM3 Sb Ser* fly stocks were obtained from the Bloomington Stock Center. *w^a ctⁿ lawc^{P1}/FM7i* flies were crossed to test for phenotypic enhancement/suppression and *w^a ctⁿ/FM7i* was used in control crosses. The Chi squared test was used to determine the statistical significance of phenotypic enhancement or suppression.

Real-time RT-PCR

RNA was extracted from five male third instar larvae per genotype (*w^a ctⁿ lawc^{P1}* and the *w^a ctⁿ* control) using the RNA Bee isolation solution and protocol (AMS Biotechnology). RNA from third instar salivary glands of the above genotypes (40 pairs per genotype) was isolated using the QIAshredder columns and RNeasy Micro kit (Qiagen). RNA quantity was calculated spectrophotometrically. Two-step real-time RT-PCR was performed using QuantiTect kits (Qiagen) and a Bio-Rad iCYCLER iQ as instructed by manuals. QuantiTect primer assays were used: Dm_Act5C_1_SG, Dm_CG32711_1_SG, Dm_Trf2_1_SG, Dm_CG11190_1_SG, Dm_Act88F_1_SG, Dm_Hsp70Aa_1_SG, Dm_ACXD_1_SG, and Dm_CG9497_2_SG (Qiagen). Primer efficiency was verified on PCR products cloned into

pCR2.1. For comparing control and experimental transcript levels, Cycle threshold (C_t) levels of the transcript under investigation were subtracted from the *Act5C* C_t (ΔC_t). The Student's *T* test was used to determine statistical significance of ΔC_t values. One control larva was set as the reference, and then $\Delta \Delta C_t$ values for each larva were calculated and averaged to generate figure graphs.

Protein purification, antibody production and western analysis

The *Lawc* open reading frame was amplified by PCR and cloned into the pET100D-TOPO vector in-frame to the N-terminal His₆ tag. Cultures of His-*Lawc* in the BL21 bacterial strain were induced with 1 mM IPTG, grown for 2.5 h, lysed under denaturing conditions, and purified by Ni chromatography. The protein was then concentrated in a Centricon YM-10 (Millipore) and dialyzed into PBS via a 10,000 MWCO Slide-A-Lyzer dialysis cassette (Pierce). Purified His-*Lawc* was used to immunize rabbits by standard procedures (PRF&L). The specificity of the antisera versus that of the preimmune was verified by Western analysis of larval and His-*Lawc*-expressing bacterial protein extracts and by immunofluorescence microscopy using polytene chromosomes. For western analysis, third instar larvae (5 per genotype) were homogenized in protein sample buffer (PSB): 0.25 M Tris-HCl pH 6.8, 0.063 M Tris, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, 50 mM DTT, 1 × cOmplete, mini protease inhibitor (Roche), and 1 mM PMSF. For western analysis of Pol IIo, the PSB also contained 1:100 phosphatase inhibitor solution (Sigma) and 5 mM β-glycerophosphate. Extracts were run on Novex Tris-Glycine gels (4–20% for *Lawc* westerns and 4–12% for Pol IIo westerns) (Invitrogen). Gels were then transferred to 0.45 μm PVDF membranes in CAPS buffer (10 mM CAPS free acid, 3 mM DTT, 15% methanol, pH 10.5). Membranes were stained with Ponceau to confirm adequate transfer, destained in CAPS, blocked in PBS containing 5% milk and 0.25% Tween for 30 min, and exposed to the primary antibody overnight at 4°. Subsequently, the membrane was washed in PBS, 0.25% Tween, incubated with secondary antibody for 1 h at room temperature, and then washed again. Lastly, SuperSignal West Pico chemiluminescent substrate (Pierce) was added and detected on film. The following antibody dilutions were used: 1:1,000 rabbit α-*Lawc*, 1:5,000 rabbit α-Lamin, 1:500 H5 (Covance), 1:500 H14 (Covance), and 1:10,000 for all secondary antibodies (HRP α-rabbit and HRP α-mouse IgM).

Immunohistochemistry and fluorescent in situ hybridization

Wild-type and w^a ct^n third instar larvae were dissected and polytene chromosomes were fixed and squashed as

previously described (Ivaldi et al. 2007) with the following exceptions: the fix contained 50% acetic acid, 2% formaldehyde, and 0.1% Triton X-100, slides were processed immediately (not stored), in some instances, the antibody dilution buffer contained dry milk rather than BSA, a blocking step prior to antibody incubation was performed two times for 30 min, wash steps were performed with PBS, 0.1% Tween, and the DAPI concentration was 0.06 μg/mL. The following antibody dilutions were used: 1:20 or 1:30 rabbit α-*Lawc*, 1:150 rabbit α-dREGγ (Masson et al. 2001), 1:75 H5 (Covance), 1:75 H14 (Covance), 1:5 mouse-EcR (Developmental Studies Hybridoma Bank) (Talbot et al. 1993), and 1:250 for all secondary antibodies (Texas Red α-rabbit, FITC α-rabbit, FITC α-mouse IgM, and Texas Red α-mouse IgG). The fluorescent in situ hybridization (FISH) portion of the combined FISH and immunofluorescence on polytene chromosomes was performed as previously described (Ivaldi et al. 2007) with the following exceptions: freshly squashed slides were left out 1 h at room temperature before freezing and probes were denatured at 65°. The subsequent immunohistochemistry was performed as described above except that the primary antibody was incubated 2.5 h at 37°.

Yeast two-hybrid screen

The yeast two-hybrid screen was carried out with the *lawc* ORF as previously reported (Capelson and Corces 2005) with the following exceptions: western blot analysis confirmed the expression of *lawc* in the yeast and more than six positive clones were selected for sequencing.

Results

lawc corresponds to a previously uncharacterized gene

The *Drosophila melanogaster lawc* gene was identified based on the mutant phenotype caused by a P-element insertion between 7D10 and 7F1, generating *lawc^{P1}* (Zorin et al. 1999). This mutant phenotype consists of an arista-to-leg transformation and an enhancement of the *cut* wing phenotype of ct^n . We noted that this allele is semilethal, with about 35–65% of *lawc^{P1}* mutant larvae surviving to adulthood (data not shown), and it is therefore likely that the allele is hypomorphic. Excision of the P element reverts the mutant phenotype to wild type, indicating that the mutation is caused by the presence of the P element (Zorin et al. 1999). The P-element insertion site in *lawc^{P1}* was mapped 400 bp upstream of the previously uncharacterized gene *CG32711* (I. Zorin and T. Brandt, unpublished data). The UCSC genome browser (<http://genome.ucsc.edu/>) was used to determine that *CG32711*

is conserved among *Drosophila* species but we were unable to find a *CG32711* homologue outside of *Drosophila* (data not shown) (Kent et al. 2002). *CG32711* has an unusual structure. The open reading frame is very short, encoding only 73 amino acids, and the termination codon is located in the first of four exons (Crosby et al. 2007). It is therefore possible that the apparent absence of a *lawc* gene homologue from the genome of other higher eukaryotes could be due to incomplete annotation of small genes in these genomes.

In order to test whether *CG32711* corresponds to *lawc*, we introduced this gene into the *Drosophila* genome by P element-mediated transformation. Although *CG32711* has a very large 5' regulatory region, we were able to almost completely rescue the *lawc^{P1}* mutant phenotype with a transgenic construct containing the *CG32711* coding sequence plus 3,987 bp of 5' and 447 bp of 3' sequences (Fig. 1a). *lawc^{P1}* flies carrying the *CG32711* transgene show a 75% reduction in the frequency of strong arista-to-leg transformations ($\alpha < 0.025$) and a 44% reduction in the frequency of very cut wings ($\alpha < 0.05$). In addition, the transgene also rescues the larval/pupal lethality of the *lawc^{P1}* allele (data not shown). The failure to completely rescue the *lawc^{P1}* phenotype may be due to the absence of regulatory sequences required for proper expression of *CG32711* located outside of the region used in the transgene.

To further test the hypothesis that the P-element in *lawc^{P1}* affects the expression of the *CG32711* gene, transcript levels in *ctⁿ lawc^{P1}* larvae were measured by quantitative real-time RT-PCR (qRT-PCR) and compared to transcript levels in control *ctⁿ* larvae. RNA was extracted from five larvae per genotype and qRT-PCR experiments were run in triplicate. Transcript levels were normalized to *Actin 5C (Act5C)* mRNA. *lawc^{P1}* mutant larvae have 58% less *CG32711* transcript than the control ($\alpha < 0.004$), while transcription of the gene immediately upstream, *Trf2*, and the gene immediately downstream, *CG11190*, remain unaffected (Fig. 1b). These results were reproducible and similar results were obtained normalizing *CG32711* transcript levels to *Act88F* (data not shown). In order to determine whether the levels of the *CG32711* protein are also affected by mutations in the *lawc* gene, an antibody was created against the predicted protein product of *CG32711*. This antibody detects a band of approximately 10 kDa on western blots of larval protein extracts (Fig. 1c). This molecular weight is very close to the predicted size of the *CG32711* protein. Importantly, this band is not recognized by preimmune serum (data not shown). We observed a reduction in the *CG32711* protein in *ctⁿ lawc^{P1}* larvae relative to *ctⁿ* larvae used as a control, confirming that *lawc* is *CG32711* (Fig. 1c).

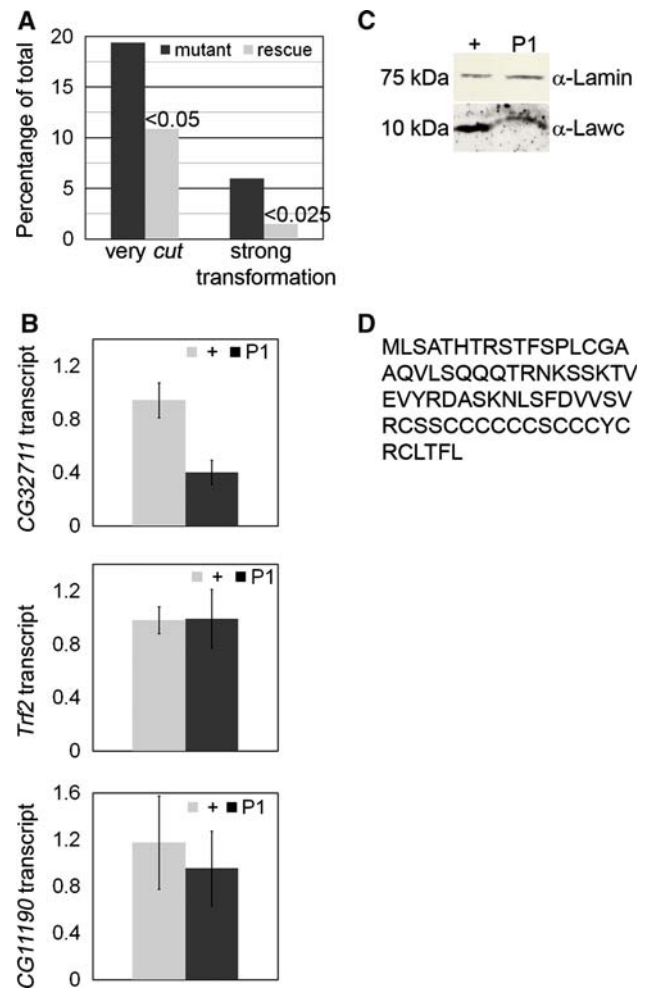
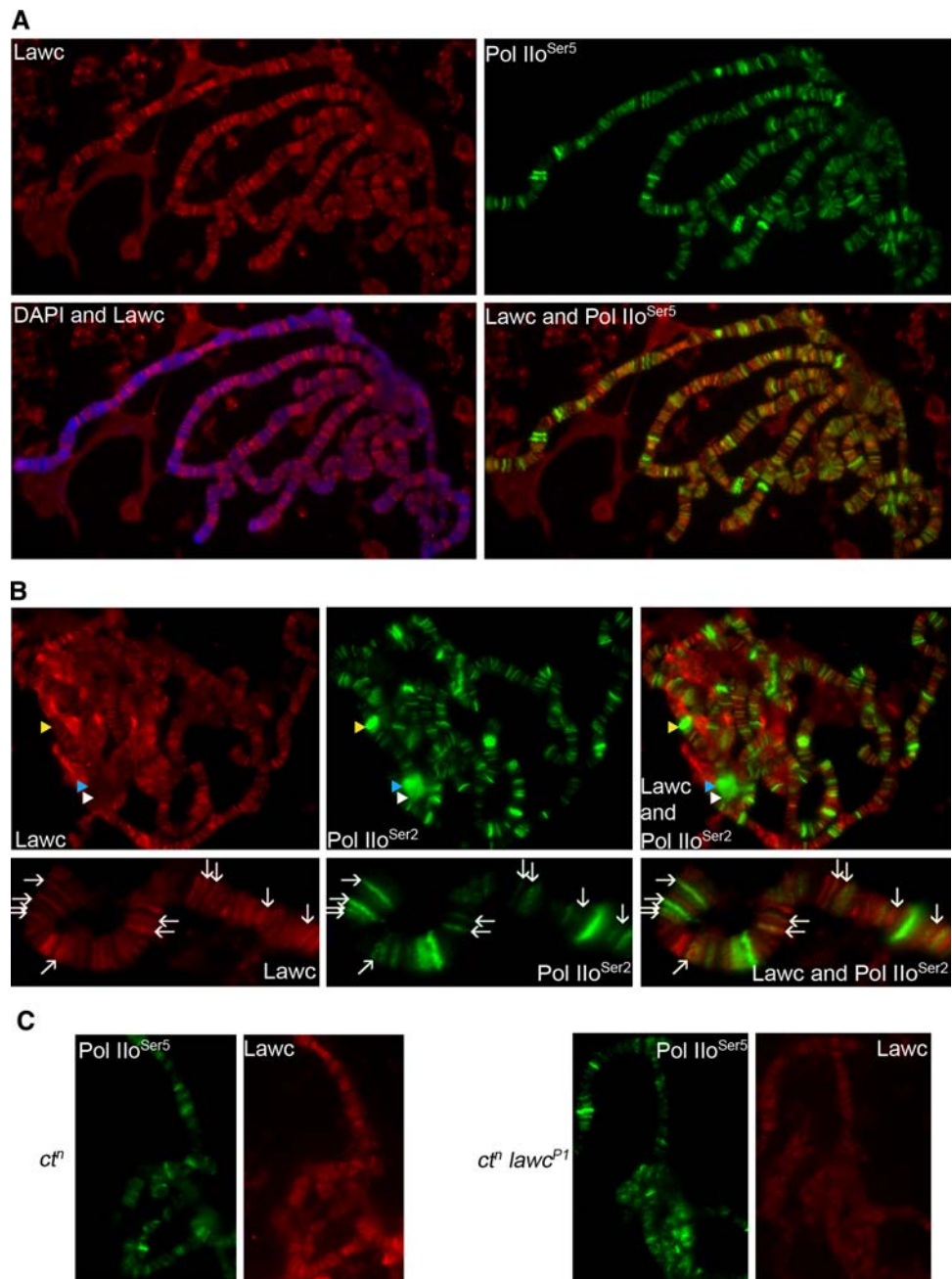


Fig. 1 The *lawc* gene corresponds to *CG32711*. **a** Wing and arista phenotypes of *ctⁿ lawc^{P1}* (mutant) and *ctⁿ lawc^{P1}; P[CG32711 rescue construct]* (rescue) flies are shown with statistical significance represented by α values indicated above each rescue bar. **b** Transcript levels obtained by qRT-PCR from three separate larval extracts of each genotype (hemizygous *ctⁿ* (+) and hemizygous *ctⁿ lawc^{P1}* (P1)) were normalized to *Act5C*. One + extract was set as the reference and $\Delta\Delta C_t$ values for each extract were calculated and then averaged. Graphs report results with *C32711*, *Trf2*, and *CG11190* primers. **c** Western analysis of *ctⁿ* (+) and *ctⁿ lawc^{P1}* (P1) third instar larvae using α -Lawc antibodies confirms the reduction in *CG32711/lawc* gene expression detected by qRT-PCR. α -Lamin was used as a loading control. **d** Predicted amino acid sequence of the Lawc protein

Lawc localizes to the interbands of polytene chromosomes and partially co-localizes with Pol IIo

In order to gain additional insights into the possible role of *Lawc* in chromatin structure and gene regulation, we determined its distribution on polytene chromosomes by immunofluorescence microscopy. Antibodies against *Lawc* detect a protein present at nearly every interband on polytene chromosomes, including puffs (Fig. 2a), whereas the preimmune serum does not recognize any protein (data not

Fig. 2 Lawc localizes to the interbands of polytene chromosomes and partially co-localizes with Pol IIo. **a** Polytene chromosomes from wild-type third instar larvae were incubated with α -Lawc, α -Pol IIo^{Ser5} (H14) and DAPI (blue). **b** Polytene chromosomes from wild-type third instar larvae were incubated with α -Lawc and α -Pol IIo^{Ser2} (H5). Arrowheads point to 74EF (white), 75B (blue), and 78D (yellow) puffs. In order to allow for a more detailed examination of α -Lawc and H5 staining patterns, an enlarged and rotated version of a portion of a chromosome from each image is represented immediately below the image of the full spread. Arrows indicate some regions of Lawc-Pol IIo^{Ser2} co-localization. **c** Polytene chromosomes from *ctⁿ* and *ctⁿ lawc^{P1}* larvae incubated with α -Lawc and α -Pol IIo^{Ser5} antibodies



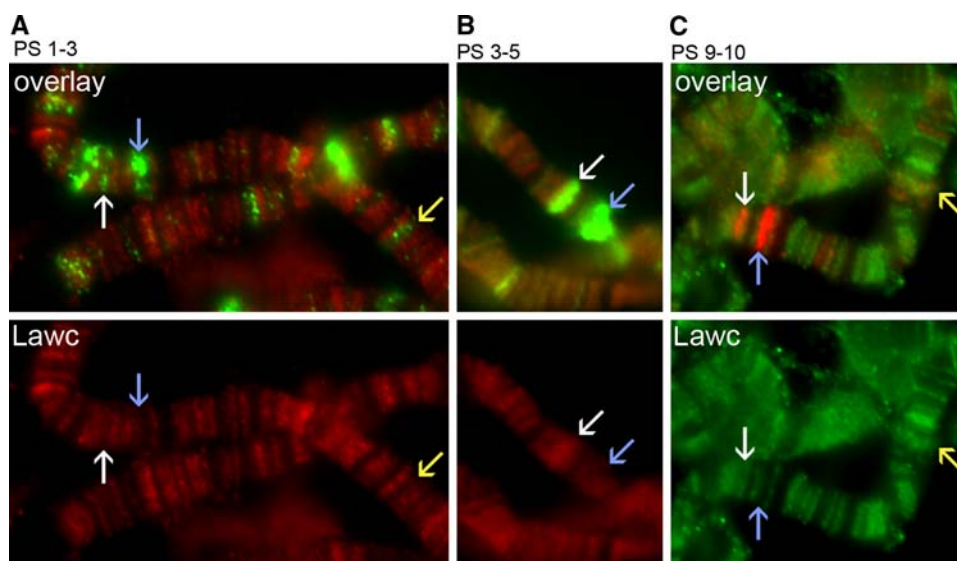
shown). Interbands are regions of open chromatin and contain genes that are actively transcribed in polytene cells as well as Pol IIo phosphorylated in the Ser2 and Ser5 residues of the CTD repeat. Lawc partially co-localizes with Pol IIo^{Ser5} and Pol IIo^{Ser2} (Fig. 2a, b). A reduction of Lawc protein was observed in chromosomes from *lawc^{P1}* mutant larvae but levels of Pol IIo^{Ser5} appear to be unaffected (Fig. 2c). Although Lawc co-localizes with the initiating/early elongating form of Pol II (Pol IIo^{Ser5}) quite well, we noted that the intensity of Lawc signal on chromosomes often does not correlate with the intensity of Pol IIo at the same location. This tendency is more apparent when the

co-localization between Lawc and elongating Pol IIo^{Ser2} is closely examined. Lawc seems to co-localize with faint Pol IIo^{Ser2} bands best. Bright Pol IIo^{Ser2} bands tend to correspond to regions containing little or no Lawc protein. Nevertheless, the co-localization between active Pol II and Lawc suggest an involvement of this protein in some aspect of the transcription process.

Lawc is recruited to puffs after transcription has begun

Lawc is present at the 74EF and 75B ecdysone puffs but does not co-localize completely with active Pol IIo (Fig. 2a, b).

Fig. 3 Lawc is recruited to ecdysone puffs after transcription has begun. **a–c** Polytene chromosomes from wild-type third instar larvae were incubated with α -Lawc and **a** α -Pol Ilo^{Ser2} (H5) (green), **b** H14 (green), or **c** α -EcR (red). The puff stage (PS) of each larva was determined as described in the text. The white arrows point to 74EF, the blue arrows indicate 75B and the yellow arrows point to 78D



To address this apparent discrepancy, we considered the possibility that Lawc is recruited to active genes at a precise stage during the transcription process, and that loci at which active Pol Ilo and Lawc fail to co-localize are in a different stage of the transcription cycle. To test this hypothesis, we took advantage of the fact that transcription of certain 20E-responsive genes has been extensively characterized and the timing of transcription at these loci can be examined in polytene chromosomes. We used puff staging to determine the transcriptional status of genes at various 20E-responsive loci (Ashburner 1972; Dworniczak et al. 1983). There are three types of ecdysone puffs: intermolt, early, and late. At puff stage (PS) 1, the intermolt puffs are visible. At this time, a 20E pulse occurs, which will turn intermolt genes off and early genes on. Consequently, at PS 2 the intermolt puffs are no longer visible and the first sign of puffing at early gene loci can be observed. Eventually the early genes become fully active and the larva has large puffs at early gene loci, but no late puffs; this corresponds to PS 3–5. The Ashburner model proposes that products of the early genes provide negative feedback to inactivate early genes and, in addition, activate the late genes (Ashburner 1990). Therefore, when puffs are present at both early and late gene loci, the early genes are in the process of turning off while the late genes have just been activated (PS 6–8). Visible early and late puffs allow for the identification of a PS 6–8 larva and provide a way to observe how chromatin at early gene loci is changing in response to transcriptional cessation. The presence of only late puffs indicates that the larva is in PS 9–10.

The 74EF early puff contains two 20E-responsive isoforms: *E74A* and the shorter *E74B*. *E74A* is an early gene and *E74B* can be considered an intermolt gene (Huet et al. 1993; Karim and Thummel 1992). While some transcript from *E74A* can be detected at PS 2, transcript levels seem

to plateau at PS 4, when 74EF puff size peaks, at levels well over two-fold that of PS 2 (Huet et al. 1993; Karim and Thummel 1992). The 75B early puff has two early gene isoforms, *E75A* and the shorter, less abundant *E75B*, and one intermolt isoform, *E75C*. The *E75C* transcript is barely detectable, even at PS 1, while the *E75A* early transcript shows the same transcription dynamics as *E74A* (Huet et al. 1993; Karim and Thummel 1992).

We examined the presence of Lawc at 20E-responsive loci during various puff stages. We find Lawc to be absent from the early puff (74EF and 75B) and late puff loci (such as 78D) at PS 1–3 while some Pol Ilo^{Ser2} is present (Fig. 3a). Lawc and Pol Ilo^{Ser5} are present in early puffs at PS 3–5 (Fig. 3b), suggesting that Lawc is present at 20E-responsive loci during the transcriptional peak of *E74A* and *E75A* and, therefore, that the recruitment of Lawc is downstream of at least one round of transcription by Pol Ilo. Although Lawc is present during PS 3–5, we noted that the α -Lawc signal is not always intense (Fig. 3b). After PS8, the early genes are repressed by the EcR and neither Lawc nor Pol Ilo^{Ser2} are present (Fig. 3c and data not shown). Consistent with the timing observed at early puffs, Lawc is at the visibly open late puff 78D after PS 8 (Fig. 3c).

To examine whether this temporal recruitment of Lawc is general or specific to ecdysone puffs, we studied the distribution of Lawc in relation to the formation of heat-shock puffs. These puffs are located at 87A and 87C, they contain multiple copies of the *Hsp70* heat-shock protein-coding gene, and they form shortly after activation by heat shock (37°). Combined fluorescent in situ hybridization (FISH) and immunofluorescence was used to demonstrate that Lawc is not present at the 87C locus before heat shock (Fig. 4a). It is, however, present at 87A (Fig. 4a). It has been demonstrated that, while there is no Pol Ilo^{Ser2} at 87C prior to heat shock, Pol Ilo^{Ser2} is present at 87A (Ivaldi

et al. 2007). Therefore, it is likely that there is another gene at or near 87A that is being transcribed before heat shock and it may be more appropriate to make conclusions based on the heat-shock response at 87C. Strong transcriptional induction of the *Drosophila Hsp70* genes is rapid following exposure to elevated temperatures. In just 2 min of heat-shock treatment, the amount of *Hsp70* transcript is increased about 150-fold in larvae (Zhao et al. 2006). After 3 min of heat shock, the *Hsp70* genes residing at 87C are active but Lawc is not present (Fig. 4b). However, after 10 min, Lawc is also present at the heat-shock puffs (Fig. 4b). Ten minutes corresponds to the peak of Pol IIo recruitment (Yao et al. 2006). FISH experiments demonstrate that once 87C has recovered from heat shock, Lawc is not present at the locus (Fig. 4c). The timing of Lawc recruitment at heat-shock genes seems very similar to that of 20E-responsive genes: Lawc is not present before or immediately following transcription initiation, becomes abundant after at least one full round of transcription, and disappears when transcription has ceased.

The amount of Pol IIo^{Ser2} increases in the *lawc*^{P1} mutant but transcript levels do not

The results discussed above suggest that Lawc is recruited after genes are transcriptionally active and at the peak of RNA production. To further examine the role of Lawc in the transcription process, we determined the levels of initiating Pol IIo (Pol IIo^{Ser5}) and elongating Pol IIo (Pol IIo^{Ser2}) in the *lawc*^{P1} mutant by western analysis. The results show that *lawc* mutants have a higher amount of Pol IIo^{Ser2} than the control, while Pol IIo^{Ser5} remains unaffected (Fig. 5a). Since Lawc co-localizes with Pol IIo, responds to changes in the transcriptional status of 20E and heat shock-responsive genes, and has an effect on phosphorylation of the CTD of Pol IIo, we conclude that Lawc is involved in some aspect of the transcription process, although it is not clear that the increase in Pol IIo^{Ser2} accumulation actually results in productive transcription.

To address this issue, we began a search for genes whose expression is altered in the *lawc*^{P1} mutant background. Although it is possible that genes at 75B or 74EF are affected, these genes would be difficult to analyze in *lawc* mutants because puff stages progress quickly and the expression of these genes could not be directly compared among larvae unless the animals were puff staged cytogenetically, which would make the isolation of enough starting material for gene expression analysis very laborious. However, the effect of the *lawc*^{P1} mutation on *Hsp70* transcription is testable. qRT-PCR was performed with *Hsp70* primers on mutant and control larvae incubated at 37° for 20 or 60 min. We were unable to detect a difference during either of these conditions (data not shown). Since *lawc*^{P1}

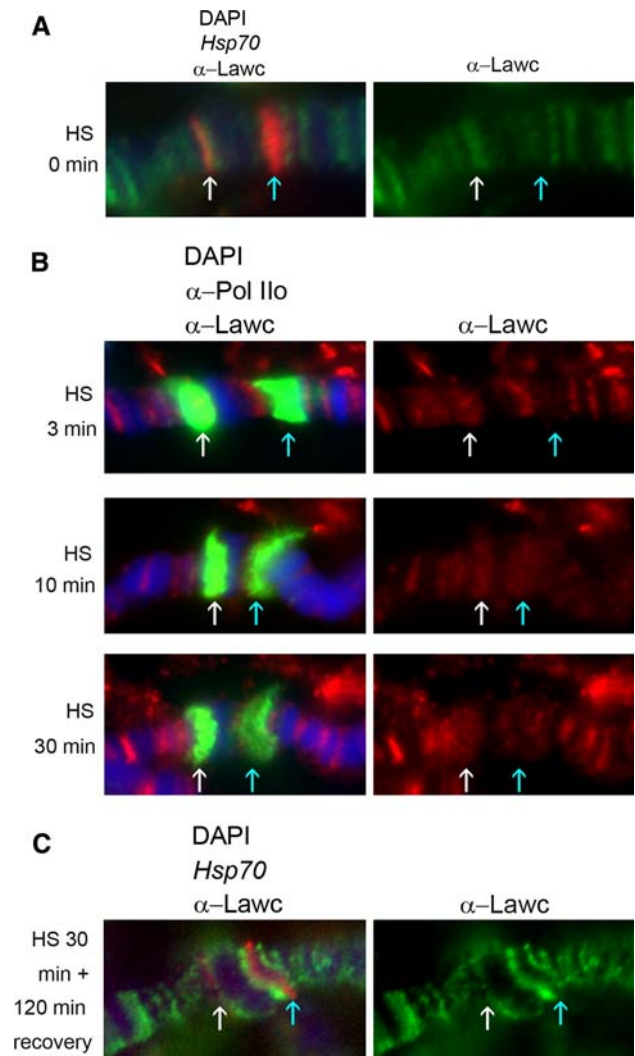


Fig. 4 Lawc is recruited to heat-shock puffs after transcription has begun. Images are shown with 87A on the left (white arrow) and 87C on the right (blue arrow). **a** Combined FISH-immunofluorescence was performed on wild-type polytene chromosomes with DAPI (blue), an *Hsp70* probe (red), and α -Lawc. **b** Wild-type larvae were placed at 37° (HS) for the indicated amount of time. Polytene chromosomes were incubated with DAPI (blue), α -Lawc, and α -Pol IIo (green). **c** The experiment was carried out as in **a** except larvae were exposed to 37° and then allowed to recover at 25°

does not affect *Hsp70* transcription, we carried out microarray analysis to examine differences in transcription between the *ctⁿ lawc*^{P1} mutant and the *ctⁿ* control. Several genes were then selected for further testing.

Alterations in gene expression in *ctⁿ lawc*^{P1} mutants could be a direct effect of the absence of Lawc protein or an indirect effect caused by changes in the levels of Lawc-regulated proteins. Therefore, we first performed combined fluorescent in situ hybridization (FISH) and immunofluorescence analysis using Lawc antibodies on polytene chromosomes from wild-type and *ctⁿ* larvae to eliminate genes that do not co-localize with Lawc and therefore are not

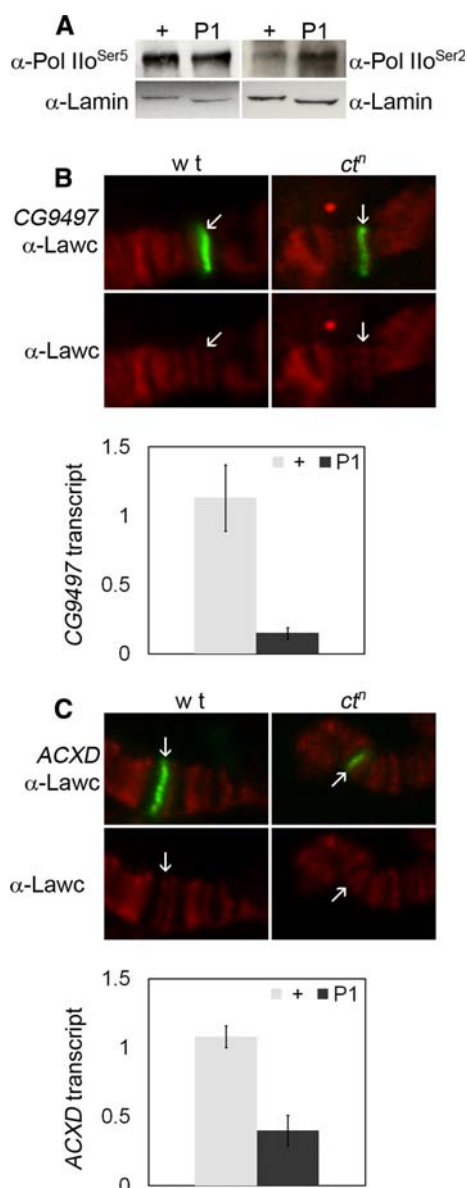


Fig. 5 The amount of Pol Ilo^{Ser2} is augmented in the *lawc^{P1}* mutant but transcript levels are not. **a** Western analysis on hemizygous *ctⁿ* (+) and hemizygous *ctⁿ lawc^{P1}* (P1) third instar larvae with H14 and H5 antibodies shows an increase of the Pol Ilo^{Ser2} form in the *ctⁿ lawc^{P1}* mutants. α -Lamin was used as a loading control. **b**, **c** Combined FISH-immunofluorescence with α -Lawc was performed on polytene chromosomes from wild-type male third instar larvae (wt) or *ctⁿ* third instar male larvae. FISH probes (green) were made to either **b** *CG9497* or **c** *ACXD*. Graphs depict the result of qRT-PCR experiments comparing **b** *CG9497* and **c** *ACXD* transcript levels in hemizygous *ctⁿ* (+) and hemizygous *ctⁿ lawc^{P1}* (P1) third instar larval salivary glands. Relative transcript levels are calculated as described for Fig. 1

likely to be directly affected by the *lawc^{P1}* mutation in this tissue. We examined seven genes, three found to be over-expressed and four under-expressed in the *lawc^{P1}* mutant microarray. We observed no difference in Lawc localization in *ctⁿ* larvae when compared to wild-type (Fig. 5b, c). Only two of the genes examined, *CG9497* and *ACXD*, co-localize

with Lawc (Fig. 5b, c). These two genes are then likely to be direct targets of Lawc regulation. Since the polytene chromosomes were obtained from salivary gland preparations, qRT-PCR was performed to confirm transcript mis-expression in *lawc^{P1}* salivary glands. The amount of *CG9497* transcript is reduced 87% in the mutant ($\alpha < 0.01$) while *ACXD* is reduced 63% relative to the control ($\alpha < 0.02$) (Fig. 4b, c). These results are similar to the values obtained by microarray analysis (data not shown). qRT-PCR analysis of both genes revealed that they are infrequently transcribed even in the control background (data not shown). The *CG32301* gene is located close to *ACXD* (Crosby et al. 2007) and *CG32301* transcript levels are also reduced in the *lawc^{P1}* mutant (data not shown). Likewise, the only affected gene near *CG9497* is *CG9500* (Crosby et al. 2007), and its expression is also reduced (data not shown). No other genes located within 20 kb in either direction of *CG9497* or *ACXD* were found to have obviously altered expression in the microarray studies. Therefore, the presence of these neighboring genes should not confound the conclusion that *lawc* mutants have decreased expression of some genes.

Lawc interacts with the nuclear proteasome

The results discussed so far suggest a role for the Lawc protein in transcription. Examination of the sequence of this protein does not reveal the presence of conserved domains that could give insights into the function of Lawc. In order to gain a better understanding of the role of the Lawc protein in transcription, we performed a yeast-two hybrid screen to try to identify proteins that interact with Lawc. A fusion protein containing full-length Lawc and the LexA DNA-binding domain served as the bait. Yeast-two hybrid hits were chosen for sequencing after they exhibited growth on selective media, demonstrated high β -galactosidase expression, and failed to grow on selective media in the presence of only the empty LexA DNA-binding domain (Table 1).

One of the proteins found to interact with Lawc is dREG γ , a component of the nuclear proteasome that was found multiple times in the screen (Table 1). In order to confirm that Lawc interacts with the REG γ proteasome in flies, we made use of a mutation in the *Pros26* gene. *Pros26* is a component of the 20S catalytic core (Covi et al. 1999), which is common to both the standard and REG γ proteasomes. We examined the *lawc* phenotype of flies hemizygous for *lawc^{P1}* and heterozygous for *Pros26 pb p^p* and found that *Pros26 pb p^p* significantly enhances the arista-to-leg transformation beyond that of the *ctⁿ lawc^{P1}*; *pb p^p*/+ control ($\alpha < 0.005$) (Fig. 6). The *Pros26* mutation does not affect the phenotype of *ctⁿ* flies (data not shown). This result suggests that mutations in a proteasome component

Table 1 Results of a yeast two-hybrid screen to identify Lawc interacting proteins

Hit	Predicted/known function	Frequency
BAP111	Brahma remodeling complex	1
CG4857-PB	Unknown	1
CG7466-PA	Signal transduction	1
CG11674-PA	Spliceosomal complex	1
Delta	Notch receptor ligand	8
Dlc90F	Cytoskeleton	1
dREG γ	Nuclear proteasome	4
Fur2	Tyrosine kinase signal transduction	2
Hsp26	Protein chaperone	5
Mob1	Unknown	1
N	Signal transduction	1
Pvfl	Growth factor	3
Socs16D	Cytokine mediated signaling	2
Y1	Receptor-mediated endocytosis, translation	2

A LexA-Lawc fusion protein was tested for the ability to interact with components of a *Drosophila* embryonic cDNA library. The above interactions (hits) were detected. The predicted/known function is listed as reported on Flybase (Crosby et al. 2007). Frequency refers to the number of times each protein was pulled out of the screen

genetically interact with *lawc* mutations, suggesting that the two proteins act in the same pathway.

Although it has previously been reported that dREG γ is a nuclear protein, the presence of this protein on chromosomes has not been demonstrated. We find that dREG γ is also present in the interbands of polytene chromosomes and that it extensively co-localizes with Pol IIo (Fig. 7). Therefore the dREG γ protein may also be involved in transcription. In addition, Lawc and dREG γ have very similar distribution patterns on polytene chromosomes. In fact, dREG γ localizes to puffs at the same puff stage as Lawc (Fig. 7c), suggesting that the two proteins may play similar roles in the transcription process. However, dREG γ can also be found at these loci during the first puff stages and is recruited to actively transcribed genes before Lawc (Fig. 7c). Likewise, dREG γ is present at the 87A and 87C heat-shock loci both before the arrival of Lawc and after Lawc is recruited (Fig. 7d). The observed interactions between Lawc and dREG γ and the temporal order in which the two proteins are recruited to transcriptionally active genes suggest that Lawc may play a role in the regulation of the activity of the dREG γ proteasome.

Discussion

The *lawc* gene has been shown to be a member of the *trxG*, suggesting a function of the encoded product in chromatin-

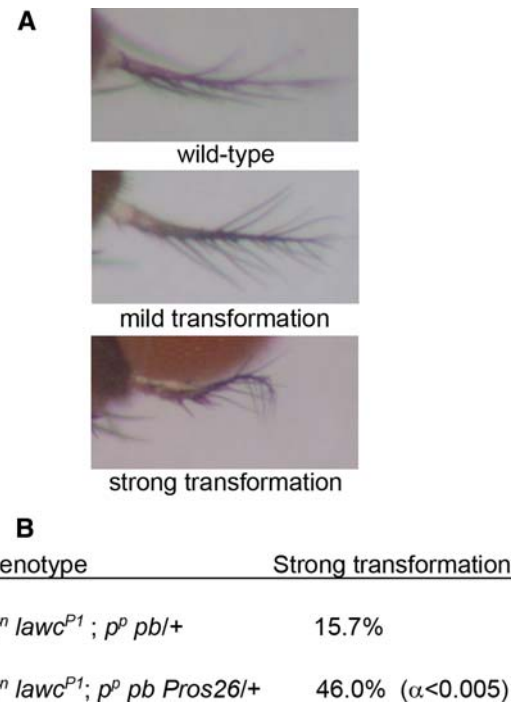
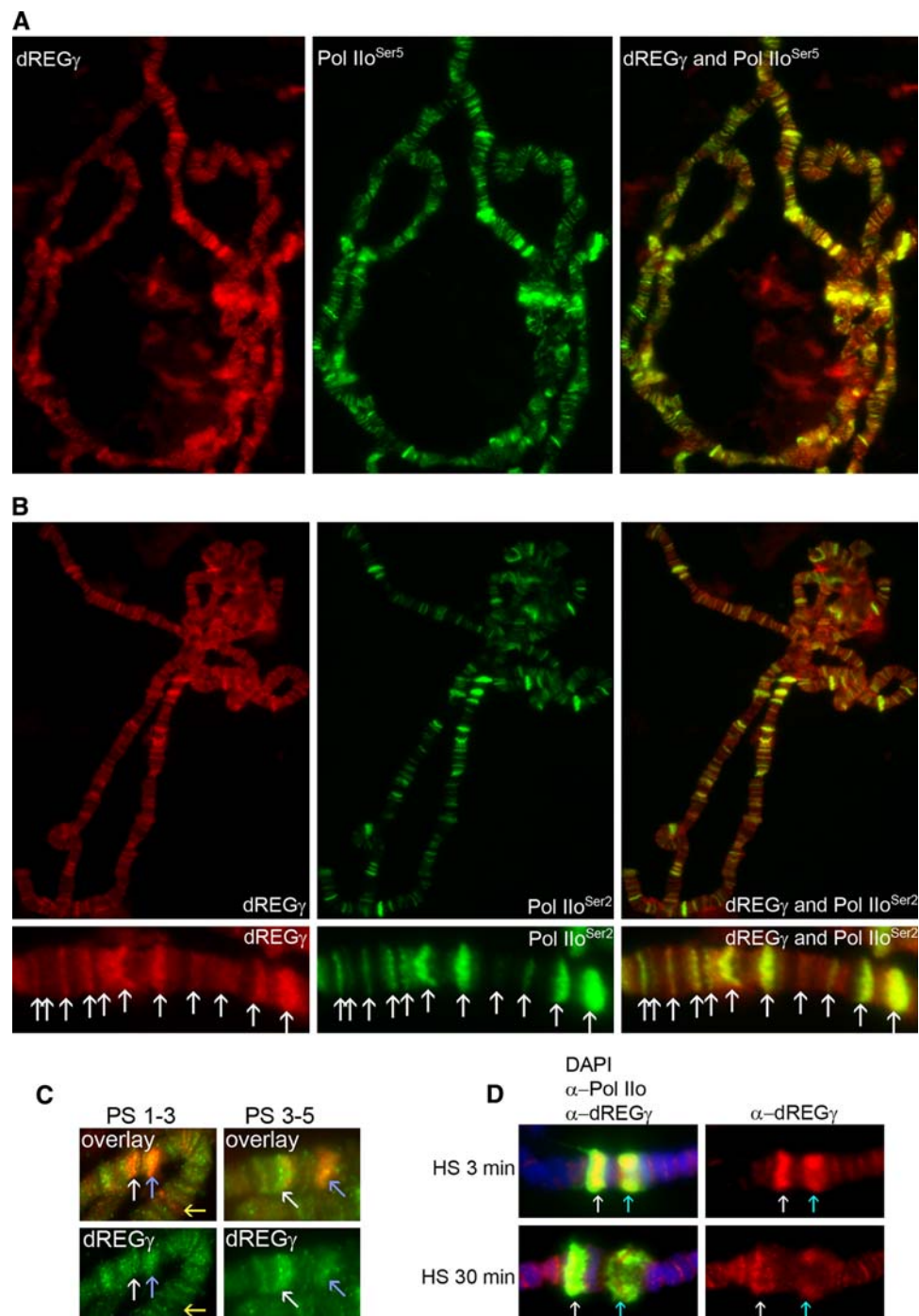


Fig. 6 Lawc interacts with the nuclear proteasome. **a** Photographs illustrate differences in the arista phenotype as scored in **b**. Note that the mild transformation consists of a subtle thickening of the arista base and that the strong transformation results in a much thicker arista that is mis-shaped. **b** The α value reported is the result of a test of statistical significance between *ctⁿ lawc^{P1}; Pros26 p^p pbl/+* and *ctⁿ lawc^{P1}; p^p pbl/+*

based transcriptional processes. Nevertheless, nothing is currently known about the nature of the Lawc protein or the mechanisms by which it may affect transcription. Here we show that *lawc* corresponds to the uncharacterized gene *CG32711* and encodes a small Cys-rich protein present at sites of active transcription. Interestingly, Lawc interacts with the nuclear proteasome genetically as well as in the yeast two hybrid assay. We find that Lawc is recruited to transcribed genes after at least one round of transcription has taken place. Based on these observations, we propose a model suggesting that Lawc regulates the activity of the nuclear proteasome to control the degradation of unknown factors involved in transcription elongation. Future work should address the specific molecular mechanisms by which the Lawc protein regulates gene expression.

Based on the observation of genetic interactions between *lawc^{P1}* and a *Trf2* allele, Kopytova et al. reported that *lawc^{P1}* must be a mutation in *Trf2* (Kopytova et al. 2006). However, data presented here indicates that *lawc^{P1}* does not affect *Trf2* transcript levels and the observed genetic interactions between *lawc* and *Trf2* could be due to the participation of both proteins in different aspects of the transcription process. Here we demonstrate that Lawc is a nuclear protein that is present in the interbands of polytene

Fig. 7 dREG γ has a localization pattern on polytene chromosomes similar to that of Lawc. **a** Polytene chromosomes from wild-type third instar larvae were incubated with α -dREG γ , and Pol Ilo^{Ser5}. **b** Polytene chromosomes from wild-type third instar larvae were incubated with α -dREG γ , and α -Pol Ilo^{Ser2}. In order to allow for a more detailed examination of α -Lawc and Pol Ilo^{Ser2} staining patterns, an enlarged and rotated version of a portion of a chromosome from each image is represented immediately below the image of the full spread. *Arrows* indicate regions of dREG γ -Pol Ilo^{Ser2} co-localization. **c** Polytene chromosomes from wild-type third instar larvae were incubated with α -dREG γ and α -EcR (*red*). The puff stage (PS) of each larva was determined. The *white arrows* point to 74EF and the *blue arrows* indicate 75B, while the *yellow arrows* point to 78D. **d** Images are shown with 87A on the left (*white arrow*) and 87C on the right (*blue*). Wild-type larvae were placed at 37° (HS) for the indicated amount of time. Polytene chromosomes were incubated with DAPI (*blue*), α -dREG γ and α -Pol Ilo (*green*)



chromosomes, where it co-localizes with active Pol II, suggesting a role for Lawc in the regulation of transcription. We find that Lawc enters chromosome loci dependent on the transcription status of the residing genes. Puff-staging data place Lawc downstream of at least one round of transcription by Pol IIo and upstream of recruitment of the SIN3 corepressor after PS 6 (Pile and Wassarman 2000). Lawc appears to enter chromosome puffs during gene expression peaks and reduced levels of Lawc lead to excess Pol Ilo^{Ser2}. Based on these data, it could be hypothesized

that Lawc is involved in turning genes off, since lack of Lawc protein in *lawc* mutants appears to result in higher levels of elongating Pol II. Nevertheless, genes identified as targets of Lawc, such as *CG9497* and *ACXD*, are transcriptionally down-regulated in the *lawc*^{P1} mutant background. Given these results, it is unlikely that Lawc is involved solely in repression of transcription.

An important insight in elucidating the function of Lawc is the finding that this protein interacts with the nuclear proteasome. The dREG γ component of the nuclear proteasome

co-localizes with Pol Ito and is present at the same actively transcribed and 20E-responsive loci as Lawc. Although the role of the 26S proteasome in transcription is better established, there has been one study providing evidence that the REG γ proteasome can regulate transcription (Li et al. 2006). Therefore, it is possible that the effect of Lawc on transcription is mediated by the nuclear proteasome and, therefore, Lawc may regulate the degradation of an unknown component of the transcription machinery. Based on the timing of the arrival of Lawc to actively transcribed puffs, at least one round of transcription may have occurred before Lawc recruitment. Since Lawc appears to enter actively transcribed genes after Pol Ito^{Ser2} phosphorylation during the first round of transcription, Lawc may not be required for any events prior to the generation of Pol Ito^{Ser2}. However, at the same time, mutations in the *lawc* gene result in an increase of Pol Ito^{Ser2} levels. These results could be explained if Lawc is not involved in the first elongation event, but in subsequent events after the pioneering Pol Ito^{Ser2} has crossed the template. Since this pioneering Pol Ito^{Ser2} and its associated elongation factors have altered the chromatin environment (reviewed in Reinberg and Sims 2006), it is possible that this chromatin change is responsible for the recruitment of the Lawc protein, either directly or indirectly. It has been demonstrated that highly transcribed genes do not rely heavily on the reestablishment of chromatin, presumably because Pol Ito^{Ser2} density is so high that cryptic promoters are hidden from Pol Ito (Li et al. 2007). Perhaps new chromatin components, such as histone H3.3, are recruited to these genes but they do not have a chance to become stably incorporated into the template until transcription slows. It is possible that Lawc would be recruited to these genes at this point. The model predicts that Lawc would be most abundant at genes with a low Pol Ito^{Ser2} density and this is consistent with the observation that Lawc intensity does not correlate with that of Pol Ito in polytene chromosomes. In addition, genes affected in the *lawc*^{P1} mutant (*ACXD* and *CG9497*) are normally expressed at low levels while unaffected genes (*Trf2*, *CG11190*, *Hsp70* and *Act5C* genes) are highly expressed. Additional work will be required to provide answers to these questions.

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