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ORIGINAL PAPER

The dissociable RPB4 subunit of RNA Pol II has vital functions in *Drosophila*

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Abstract RNA polymerase II (Pol II) is composed of a ten subunit core and a two subunit dissociable subcomplex comprising the fourth and seventh largest subunits, RPB4 and RPB7. The evolutionary highly conserved RPB4/7 heterodimer is positioned in the Pol II such that it can make contact with various factors involved in RNA biogenesis and is believed to play roles both during the process of transcription and post-transcription. A detailed analysis of RPB4/7 function in a multicellular eukaryote, however, is lacking partly because of the lack of a suitable genetic system. Here, we describe generation and initial analysis of *Drosophila Rpb4* mutants. In the fly, RPB4 is a product of a bicistronic gene together with the ATAC histone acetyltransferase complex constituent ADA2a. *DmAda2a* and *DmRpb4* are expressed during fly development at different levels. The structure of mature mRNA forms suggests that the production of DmADA2a and DmRPB4-specific mRNAs is ensured by alternative splicing. Genetic analysis indicates that both DmRPB4 and DmADA2a play essential roles, because their absence results in lethality in early and late larval stages, respectively. Upon stress of high temperature or nutritional starvation, the levels of RPB4 and ADA2a messages change differently. RPB4 colocalizes with Pol II to several sites on polytene chromosomes, however, at selected locus, the abundances of Pol II and RPB4 vary greatly. Our data suggest no tight functional link between DmADA2a and DmRPB4, and reveal differences in the abundances of Pol II core subunits and RPB4 localized at specific regions on polytene chromosomes, supporting the suggested role of RPB4 outside of transcription-engaged Pol II complexes.

Keywords RNA polymerase II · ADA2 · Stress response · Transcription · *Drosophila*

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Introduction

The RPB4 subunit of *Drosophila* RNA polymerase II is encoded by a bicistronic operon, *CG33520*, located on the right arm of the third chromosome (Muratoglu et al. 2003; Pankotai et al. 2005). In addition to the 150-amino acid Pol II subunit, this gene also encodes the 550-amino acid transcriptional adapter protein ADA2a. The organization of the complex transcription unit raises several questions on the structural and functional relationship of the two products. On the other hand, the unusual gene organization also hinders efforts to analyze RPB4 and ADA2a functions individually. In *S. cerevisiae* both RPB4 and ADA2 are believed to play roles in transcription under stress conditions, it is, therefore, an intriguing question whether the expression of the two proteins from the same transcriptional unit in the fly is related to their functional interconnection. In an attempt to answer these questions, we generated genetic constructs permitting the analysis of *Drosophila Rpb4* function and report here a first set of data obtained on the expression, function and localization of *Drosophila* RPB4.

Eukaryotic RNA polymerases are multi-subunit enzymes comprised a ten subunit core and a dissociable

heterodimer of the RPB4 and RPB7 subunits (Bushnell and Kornberg 2003). The RPB4/7 heterodimer is positioned in the complex such that it can make contacts with other polymerase subunits, the nascent RNA, and factors associated with the transcription complex at both the initiation and elongation phases (Armache et al. 2005; Meka et al. 2005). Among others, RPB4 interacts with FCP1, the phosphatase responsible for the dynamic changes of the phosphorylation state of the Pol II large subunit carboxyl terminal domain (CTD) during transcription (Kimura et al. 2002). Therefore, it is no surprise that the RPB4/7 subcomplex plays roles in different steps of the RNA biosynthesis partly, by modifying Pol II activity and partly, by recruiting components of the RNA-processing machinery [for a review see: (Choder 2004)]. Several reports have been published on the involvement of RPB4 in transcription initiation under normal and various stress conditions (Miyao et al. 2001; Pillai et al. 2001, 2003), in transcription elongation and termination (Verma-Gaur et al. 2008), and also in mRNA processing and export (Farago et al. 2003; Runner et al. 2008). Furthermore, RPB4 is shown to play a role in the decay of specific classes of mRNAs (Goler-Baron et al. 2008; Lotan et al. 2005), and as well in transcription coupled DNA repair (Li and Smerdon 2002).

The majority of data accumulated on RPB4 function to this day is from analysis of yeast systems. In *S. pompe Rpb4* is an essential gene (Sakurai et al. 1999), while in *S. cerevisiae* RPB4 seems to be required only for the transcription of specific genes under extreme conditions (Pillai et al. 2001). Thus, the analysis of RPB4 and RPB4/7 functions in higher eukaryotes is clear of interest from several aspects of transcription and related processes. However, a suitable model for this so far is unavailable.

Here, we describe a genetic dissection of the *CG33520* transcription unit and show that similarly to ADA2a, RPB4 is also essential in *Drosophila*. Our data suggest that the differential expression of the two proteins from one primary transcript is achieved by alternative splicing. This mechanism results in significant alterations in the ratio of *Ada2a* and *Rpb4*-specific mRNAs at specific stages of development. *Ada2a* and *Rpb4* mRNA levels change differently following heat shock and nutritional starvation as well. Furthermore, we show that the RPB4 protein is associated with Pol II at intensely transcribed chromosomal regions, but at specific sites the abundances of Pol II and RPB4 varies greatly.

Materials and methods

Fly stocks and genetic analysis

Drosophila melanogaster strains were maintained at 25°C on standard medium. The origin and description of stocks used in this work are *Rpb4* RNAi (31237R1 NIG-FLY Stock Center), daughterless-GAL4 (w[1118]; P3) (BL8641) and c147-Gal4 (BL-6979) (Bloomington Stock Center), UAS-EGFP-RPB3 (Yao et al. 2006). d^{189} is a short deficiency in the regulatory region of CG33520 (Pankotai et al. 2005).

Plasmid constructs pCaSpeR4-DtlAda2aRpb4 and pCaSpeR4-DtlAda2a used to generate transgenes *P[Dtl-Ada2a-Rpb4]*) and *P[Dtl-Ada2a]*) have been described (Pankotai et al. 2005). To construct the P[RPB4c] transgene, a full-length *Rpb4* cDNA fragment was generated by RT-PCR amplification using *D. melanogaster* embryo RNA template and primers RPB4F: GCTAGGATCCC CGTGGATATGGTGGAT and RPB4R: CGATGTCGAC TTAGTATTGTAAGCTGCGTTTAGT.

To facilitate genetic analysis, the d^{189} deficiency and the *P[Dtl-Ada2a]* transgene were recombined into one chromosome. The recombinants were tested for the presence of d^{189} deficiency by PCR and the strain obtained (labeled as $Rpb4^{d189}$), was kept over *TM6cTb,Sb* balancer. To study the lethal phase $Rpb4^{d189}$ was transferred into *yw* genetic background. The genotype was: *yw/yw; +/+; P[Dtl-Ada2a]-*d^{189}*/TM3y*$^{+}$. To test the rescue ability of the *Rpb4* cDNA, two strains were generated; in the first one, the daughterless driver (*da-GAL4)* and $Rpb4^{d189}$ were combined onto one chromosome by genetic recombination. The recombinants were identified based on their darker eye color resulting from the presence of two copies of the *miniwhite* gene on P-elements, and the presence of d^{189} deficiency. In the second strain, the P[RPB4c] transgene and d^{189} deficiency were recombined onto one chromosome. From the crosses of these strains rescued animals arose with *w/w; +/+; P[Dtl-Ada2a]-*d^{189}*-DaGAL4/*d^{189}*-P[Rpb4c]* genotype.

Immunostaining

Localization of the RPB4 was studied on polytene chromosome spreads obtained from salivary glands of third instar wandering larvae. Anti-dRPB4 antibodies were raised in rabbits against the RPB4-specific peptide EDE-ELRQILDDIGTKRSLQY. The specificity of the antibody was demonstrated by its interaction with *D. melanogaster* RPB4 expressed in bacteria. For this DmRPB4 cDNA was inserted into pET21 and expressed in BL21 cells following standard protocol. For the detection of RPB4 expression, wild-type and *Rpb4* L1 larvae (250 each) were crushed in buffer containing, EDTA, DTT, and protease inhibitor mix, the obtained extracts were cleared by centrifugation, protein concentrations determined by Bradford, and equal amounts of protein samples were loaded onto 12% SDS-PAGE. For immunoblots, RPB4-specific Ab was used in 1:1,000 dilutions. Mouse antibody H14, which is specific

for Ser5-phosphorylated form of Pol II large subunit CTD, was from Covance Research Products. Anti-TM3 2,2,7-trimethylguanosine (TMG) monoclonal antibody Ab-1, was obtained from Oncogene, anti-GFP-specific polyclonal Ab (ab13970) was obtained from Abcam. Primary antibodies were diluted 1:300 (Pol II), 1:200 (RPB4 and GFP) and 1:50 (TMG). Secondary antibodies were AlexaFluor488-conjugated goat-anti-mouse IgG, AlexaFluor555-conjugated goat-anti-rabbit IgG (Molecular Probes) and FITC-conjugate donkey anti-chicken IgY (Fitzgerald Industries). Secondary antibodies were used at 1:500 dilutions. Stained polytene chromosome spreads were examined with an NIKON Eclipes 80i microscope, and photos were taken with a Retiga 4000R camera using identical settings for mutant and control samples.

RT-PCR analysis

For studying the structure of *Ada2a-Rpb4* messages, the following primers were used in RT-PCR reactions:

F1: GAACCCCGTGGATATGGTGG,
R1: CATGTGGCACACCGATTGGC,
R2: CTGCATCAGCAAGCTTCGCG,
F2: TATGCTGTTGGAGTCTCTGC,
R3: CTAAACGCAGCTTACAATACTAA

Template RNA was obtained from S2 cells or from wild-type *D. melanogaster* larvae using RNeasy Mini Kit (Qiagen). For the quantitative determination of *Rpb4, Ada2a* and *hsp70* mRNA levels total RNAs were isolated from w^{1118} or *Rpb4* minus animals at developmental stages indicated in the Figures using RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesised from 1 μg RNA using TaqMan Reverse Transcription Reagent (ABI). Quantitative RT-PCR (Q-RT-PCR) was performed (ABI, 7500 Real Time PCR System) using primers specific for the respective cDNAs and for 18S rRNA as internal control. Specific products were detected by the incorporation of SYBR Green fluorescent dye (< I > Power SYBR Green PCR Master Mix, ABI). C_T values were set against a calibration curve. The $\Delta\Delta C_T$ method was used for the calculation of the relative abundances (Johnson et al. 2000). The primers were 18S forward: GCCAGCTAGCAATTGGGTGTA, 18S reverse: CCGGAGCCCAAAAAGCTT, *hsp70*F AG GGTCAGATCCACGACATC, *hsp70*R CGTCTGGGTTG ATGGATAGG, and F1, R1 and R2 as above.

Heat shock and nutritional stress

For determining the hsp70 expression, *Rpb4* and control L1 larvae (300 each) were placed to 37°C for 60 min, then collected and processed for RNA preparation. For induction of temperature stress response, w^{1118} animals at second instar larval stage or at developmental stages indicated were incubated twice at 37°C, each time for 1 h, at 2 and 8 h after the experimental zero point. For induction of starvation response, second instar w^{1118} larvae were placed on filter paper on a Petri dish soaked either with PBS or PBS and yeast paste as control. Total RNA extraction was performed at the indicated time points. RNA preparations were done using triplicate biological samples and PCR amplifications were done in duplicates.

Results

The CG33520 transcription unit encodes DmRPB4 and DmADA2a

Protein blast searches using putative amino acid sequences corresponding to ORFs of the *D. melanogaster CG33520* transcription unit revealed similarities to two proteins designated in databases as homologs of the yeast and human ADA2a and RPB4. Alignments of ADA2a- and RPB4-related sequences and the exon–intron structure of *CG33520* indicate that the two proteins share the first exon, while the 2–4th and 5–6th exons are ADA2a and RPB4 specific, respectively (Fig. 1a). In the fourth exon, a translational stop codon closes the ADA2 open reading frame. Thus, the structure of the *CG33520* suggests that two proteins are produced from one transcription unit. They can be generated by different mechanisms: one possibility is that the use of alternative poly(A) addition sites serves as a switch between ADA2a and RPB4 production; another is that the switch between ADA2a- and RPB4-specific mRNA productions occurs by regulated splicing of a primary transcript containing the coding regions for both proteins. mRNA containing only the shared (1st exon) and the RPB4-specific sequences (5th and 6th exons) would exist in both cases, and indeed this mRNA form can be detected in total mRNA samples obtained from either animals or S2 cells. To identify existing mRNA forms, we amplified specific ADA2a-RPB4 regions using primers as indicated in Fig. 1a on cDNA templates obtained from third instar larvae. The nucleotide sequences of the products obtained using the F1/R1 and F1/R2 primer pairs confirmed the presence of two mRNA forms. Significantly, RT-PCR amplification using the F2/R3 primer pairs also gave rise to a product. This corresponded to a spliced RNA form that lacks both the *III.* (ADA2a-specific) and *IV.* (RPB4-specific) introns. The presence of this cDNA indicates the existence of mRNAs containing completely spliced ADA2a message together with RPB4-specific sequences (Fig. 1a). This indicates that the switch between ADA2a and RPB4 mRNA formation takes place by splice acceptor site selection.

The unusual structure of the *CG33520* transcription unit and that it encodes two proteins prompted us to investigate whether this gene organization is unique in *D. melanogaster* or is also found in other species. Comparisons of the related protein and nucleotide sequences revealed that in the 12 *Drosophila* species for which sequence data are available the *Ada2a* and *Rpb4* coding regions are present in similar organization as in *D. melanogaster*. A similar gene organization in other organisms, however, cannot be recognized. Thus, encoding *Ada2a* and *Rpb4* within one transcription unit seems to be a unique feature of *Drosophilidae*.

Genetic dissection of the *CG33520* transcription unit

Obtaining an *Rpb4* allele for the in vivo analysis of *Drosophila* RPB4 function poses difficulties because of the complex structure of the *CG33520* transcription unit. Moreover, *CG33520* is so close to its 5′ neighbor gene *CG31241* that the regulatory region of this latter is partly within the *Ada2-Rpb4* coding region. *CG31241* itself is a complex transcription unit, encoding two proteins of different functions (Komonyi et al. 2005). In light of these, we generated *Rpb4* alleles by constructing first functionally null genotype for both *Ada2a, Rpb4*, and for cistrons determined by *CG31241*, and then reintroducing specific functions into this by transgenes. Earlier we reported the isolation of a small deletion d^{189}, which removes the promoters of both *CG33520* and *CG31241* (Fig. 2a). d^{189} can be considered to be a null allele for all genes of the two transcription units (Pankotai et al. 2005; Papai et al. 2005). Homozygous d^{189} mutants die in L1 stage. Since a 7.1-Kb genomic fragment corresponding to the *CG33520* and *CG31241* coding and regulatory regions (Fig. 2a, transgene P[Dtl-Ada2a-Rpb4])) rescues the lethality of d^{189} homozygous animals, the lethality clearly results from the loss of a function(s) encoded by one or both of the two transcription units. The rescue also proves that there is no second site lethal mutation(s) on the d^{189} chromosome. A small deletion within the *Rpb4*-coding region in the 7.1-Kb genomic fragment (Fig. 2a, transgene P[Dtl-Ada2a]) abolishes its capacity to complement the L1 lethality caused by the d^{189} deficiency. This indicates that the L1 lethality of d^{189} animals is the result of the loss of RPB4 function. A direct proof of this conclusion is that the expression of RPB4 from a second transgene results in a complete rescue. P[RPB4c] contains the *Rpb4* coding region under the control of the GAL4 responsive UAS promoter. d^{189} homozygous animals that carry both P[Dtl-Ada2a]) and P[RPB4c] transgenes develop like wild types if a ubiquitous driver such as *da-GAL4* ensures GAL4 production. A description of the crosses resulting in genotypes permitting the analysis of the function of particular transgene combinations is shown in Fig. 2b. These data prove without doubts that the RPB4 subunit of Pol II is essential in *Drosophila*, since it lack results in lethality in an early stage of development. The d^{189} deficiency and transgenes corresponding to the *CG33520* and *CG31241* transcription units represent appropriate tools for the in vivo study of RPB4 function.

Co-expression of ADA2a and RPB4

Because RPB4 has been shown to play a role in stress response in *S. cerevisiae* and multiprotein complexes containing ADA2 proteins are also involved in stress

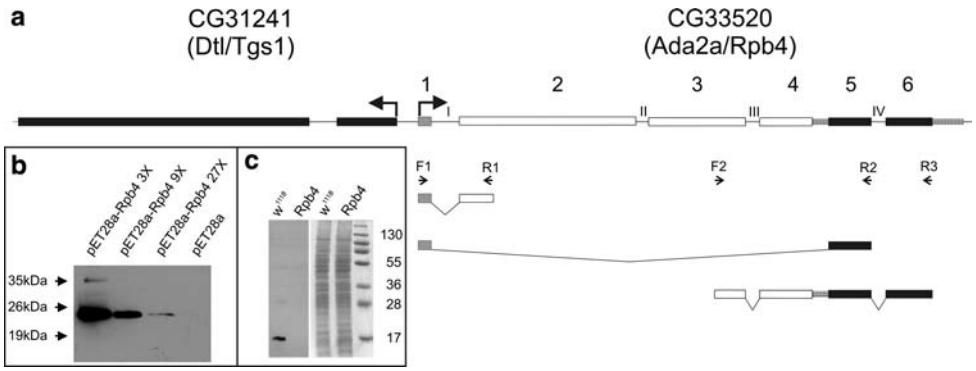

Fig. 1 **a** The exon–intron structure of *CG33520* gene. Introns are indicated by *lines* and *roman numbers*, exons by *boxes* (*white Ada2a*, *black Rpb4*, *gray* shared) and *Arabic numbers*. The position of primers used to detect specific mRNA forms, and the PCR product obtained by specific primer pairs are shown. **b** Dilutions of DmRPB4 expressing *E. coli* extracts were separated on 15% SDS-PAGE and immunoblotted using polyclonal sera raised again a DmRPB4-specific peptide (Note that because of the presence of the HIS-tag the RPB4 protein migrates slower than expected. Compare with the image in part **c**.). **c** Total extracts of control (w^{1118}) and *Rpb4* L1 larvae were separated on 12% SDS-PAGE and immunoblotted with RPB4-specific antibody. On the *right* image of CCB-stained gel on which comparable amount of L1 extracts were separated

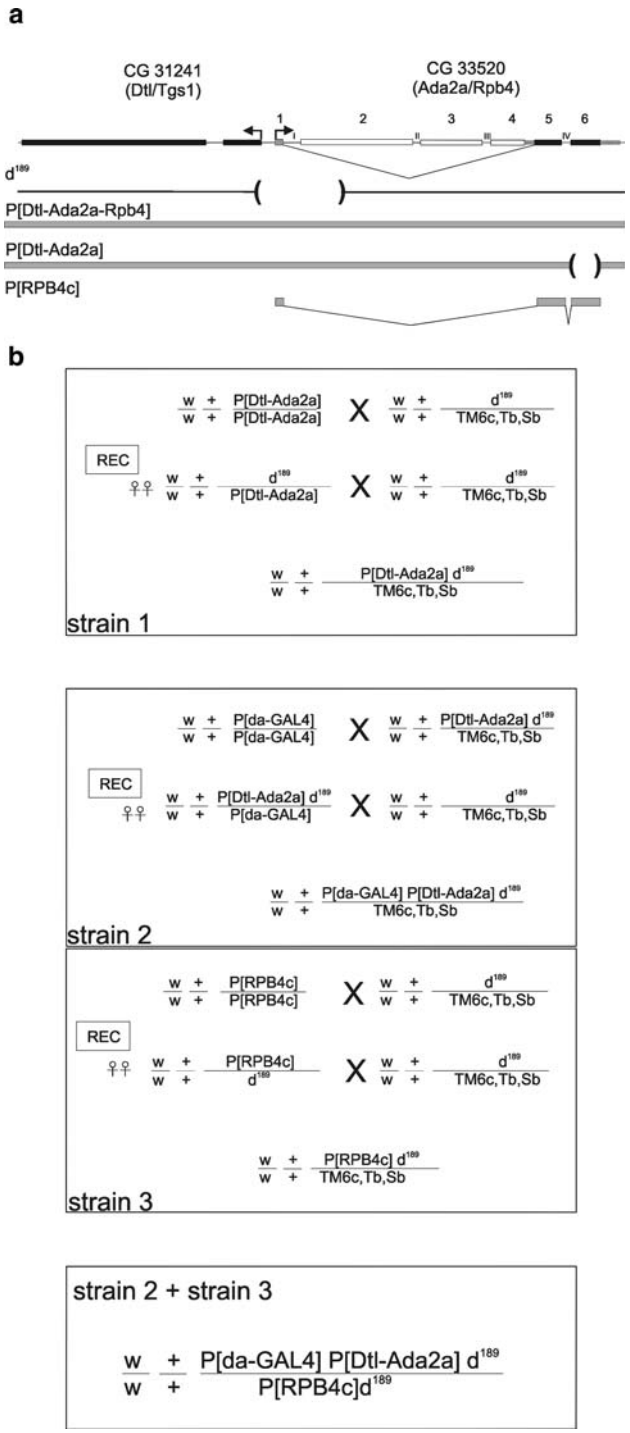

Fig. 2 **a** The position of d^{189} deficiency and the structure of transgenes used for the functional analysis of *CG33520*. **b** Outline of the crosses used in the genetic analysis of *CG33520* functions

response (Nagy and Tora 2007), we were interested whether their expression is coordinated in *Drosophila*. We used quantitative RT-PCR to determine *Ada2a* and *Rpb4*-specific mRNA levels at normal and stress conditions. The *Ada2a* mRNA level is nearly constant during development (Fig. 3). In contrast, the *Rpb4*-specific message is present in high quantity in early embryos, but its expression decreases significantly in the later developmental stages. After heat shock, the level of *Ada2a* mRNA decreases in L2–L3 stages. In contrast to that the *Rpb4* mRNA level does not change significantly following heat stress (Fig. 3). In L2 stage larvae, upon heat shock the level of *Ada2a*-specific message decreases while the amount of *Rpb4*-specific message does not, or rather increases (Figs. 3, 4).

In second instar larvae, upon starvation the level of ADA2a mRNA first decreases to 50% of its normal value, then it is returns to the wild-type level (Fig. 4). Under similar conditions, the *Rpb4* mRNA level drops and remains low for at least 4 h (Fig. 4). We observed similar changes in the mRNA level in S2 cells by semiquantitative PCR, as well (data not shown).

DmRPB4 co-localizes with Pol II at actively transcribed polytene chromosomal regions

To determine whether RPB4 is present in all or only in some of the transcribing Pol II complexes, we studied RPB4 and Pol II localization on polytene chromosomes. We co-immunostained salivary gland chromosome spreads from third instar larvae with antibodies specific for RPB4 and Pol II large subunit. The DmRPB4-specific antibody was generated against a 12 amino acid peptide present in the C terminal region of the protein. Immunoblots of bacterially expressed DmRPB4 and immunoblots of total protein extracts of wild-type first instar larvae display a single immunoreactive band corresponding to the expected size of RPB4 (Fig. 1b, c). In contrast to that in total extracts of *Rpb4* L1 larvae, none or a much reduced level of RPB4 can be detected. Significantly, the antibody that we used is highly specific for RPB4, and does not show unspecific cross reactivity with other proteins present in *Drosophila* total protein extracts. For Pol II, we used Ab H14, which recognizes the Ser5-P CTD of Pol II large subunit. The presence of this CTD modification is characteristic for actively transcribing Pol II complexes. Wild-type chromosomes stained with RPB4 antibody display strong signal at numerous chromosomal sites (Fig. 5a). A comparison of the staining patterns obtained with the DNA-specific dye DAPI and anti-RPB4 antibodies indicates that the RPB4-specific signal is localized at interbands, where the DNA is in a less-compacted chromatin structure. No specific staining is observable at the heterochromatic chromocenter region. This suggests that, in accordance with the expectation, RPB4 is localized at transcriptionally active chromosomal regions. Co-staining for phosphorylated Pol II and RPB4 shows that at the sites of strong RPB4-specific signal Pol II localization is also detectable (Fig. 5a). These sites include puffs corresponding to known ecdysone-induced genes,

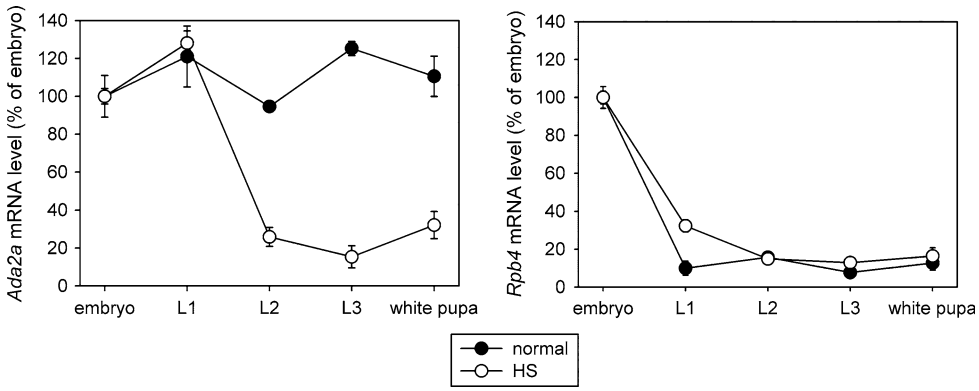

Fig. 3 The expression of *Ada2a* and *Rpb4*-specific mRNA are different during development and respond differently to heat shock. *Ada2a* and *Rpb4* mRNA levels were determined by quantitative RT-PCR in animals at the indicated developmental stages kept under normal conditions or exposed to heat shock. The mRNA levels at specific stages are expressed as percentages of that in embryo stages

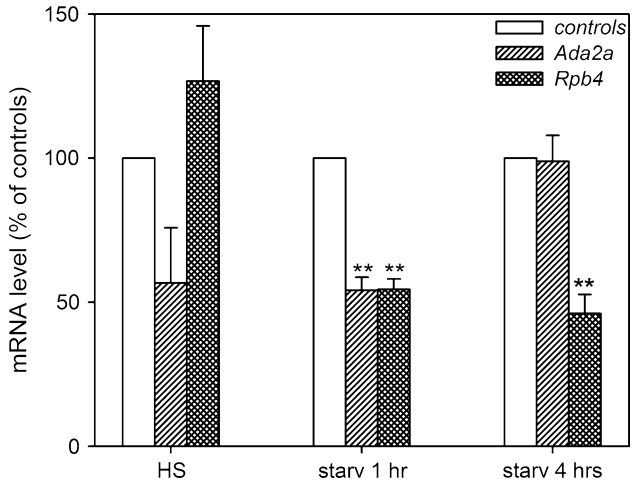

Fig. 4 The level of *Ada2a* and *Rpb4* mRNAs change differently under heat shock and nutritional starvation in second instar larvae. The *Ada2a* and *Rpb4* mRNA levels were determined after heat shock or after 1 or 4 h of starvation by Q-RT-PCR. Specific mRNA levels were compared with untreated controls in each category

which are transcriptionally active at this stage of development, and also to interbands, which are sites of less intensive transcription. Surprisingly, at several sites, a strong polymerase-specific signal is detectable in the lack of RPB4-specific staining, or conversely RPB4 is detectable in the absence of Pol II (Fig. 5a, green and red arrows, respectively). On chromosomes obtained from larvae that were heat shocked for 30 min at 37°C the strong transcriptional activation of heat-shock genes at several chromosomal sites is visible in the forms of puffs (Fig. 5a, white triangles). In the puffs, the accumulation of RPB4-specific signal is clearly visible.

The detection of RPB4 in heat-shock puffs prompted us to determine if the heat-shock response was affected by *Rpb4* mutation. For this, wild-type control and *Rpb4* L1 animals were collected, exposed to 60 min 37°C heat stress and total RNA was extracted. Surprisingly, quantitative RT-PCR detection did not indicate a significant difference in *hsp70* message induction between wild type and *Rpb4* samples. We repeatedly detected a 500–800-fold increase in *hsp70* mRNA level both in control and *Rpb4* samples upon heat stress, while the changes in the level of *Ada2a* message in the same samples were less than twofold (data not shown).

In an attempt to determine whether RPB4 localizes to those sites where splicing is in progress we stained polytene chromosomes with TMG-specific antibodies, that recognize the cap structure of spliceosomal snRNAs. As shown in Fig. 5b, no perfect co-localization of the two signals can be determined: at several bands where hardly any RPB4 signal is detectable intensive staining with TMG Ab indicates splicing of the nascent RNA (green arrow), while at other sites very strong RPB4- and little TMG-specific signal is detectable (red arrow).

The strikingly different pattern of RBP4 and Pol II signals at specific polytene chromosome regions was surprising in particular, since recent genome-wide analyses using ChIP-on-chip techniques found RPB4 and the RPB4/7 complex co-localized with RPB3 (Jasiak et al. 2008; Verma-Gaur et al. 2008). To resolve discrepancies between data obtained by others using ChIP-on-chip and our data generated by polytene chromosome staining, we found it interesting to compare the chromosomal localization of *Drosophila* RPB4 and RPB3 directly. For these experiments, we used *Drosophila* line that express an EGFP-RPB3 under the control of a c147-GAL4 driver (Yao et al. 2006) and co-stained polytene chromosome spreads with anti-EGFP (Abcam) and anti-RBP4 antibodies. As shown in Fig. 5c, the staining patterns we obtained are very similar to those we detected using RPB1 (H14) together with RPB4 antibodies. In short, a specific and well-reproducible pattern can be detected both the RPB3 and RPB4-stained samples. The overlap, however, between the two signals is only partial. Similarly to RPB1, the RPB3 signal is detectable at several positions together with none or very low RPB4 signal. At other sites, a high occupancy of RPB4 is detectable in the lack of significant levels of RPB3 localization (Fig. 5c). Immunostaining with a third Pol II-specific antibody (7G5) which recognizes the large

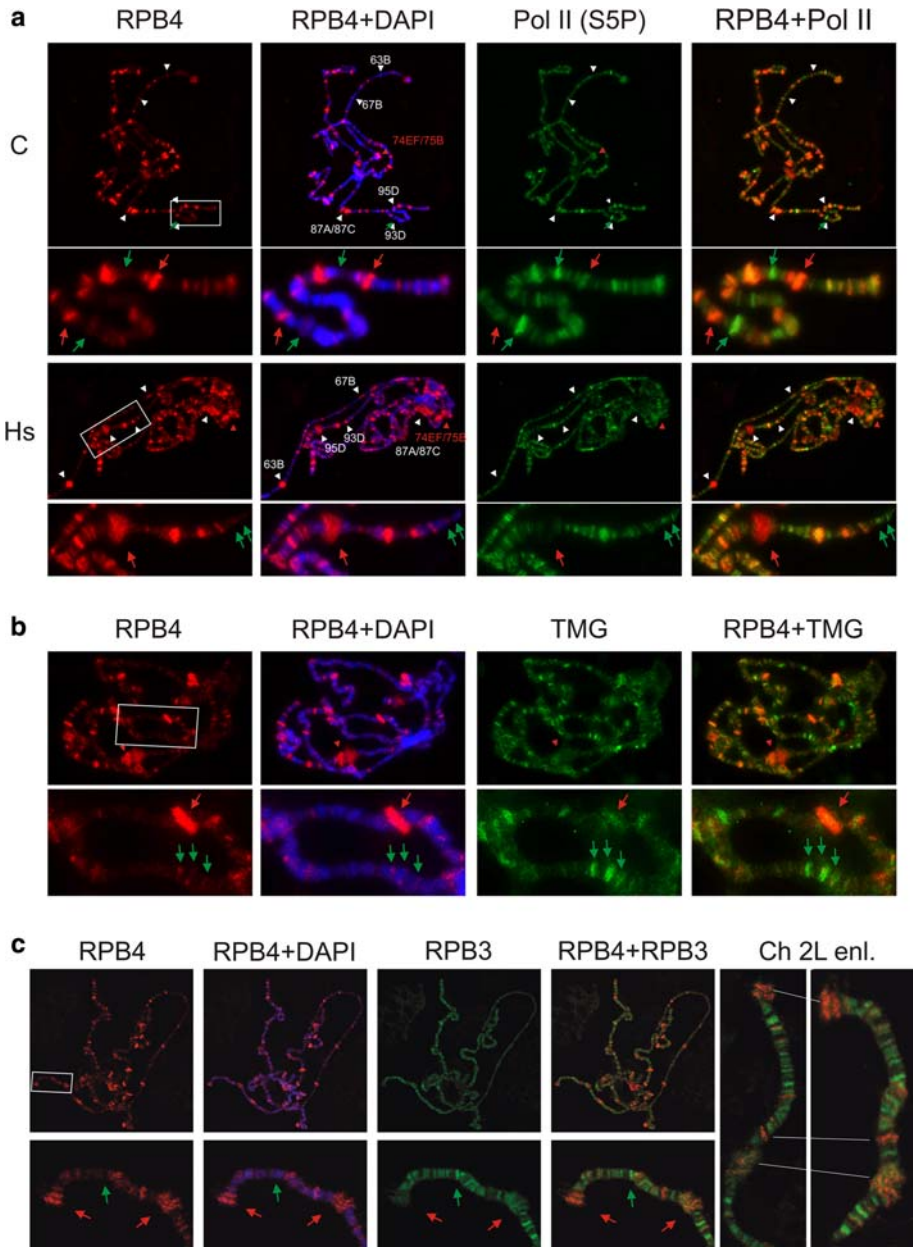

Fig. 5 Detection of RPB4 binding to polytene chromosomes. Polytene chromosome spreads obtained from salivary glands of w^{1118} third instar larvae were stained with antibodies specific for RPB4, Ser5-phosphorylated, TMG, RPB3 or with DAPI as indicated on the *top*. Images of co-stained chromosomes are shown in single channels and as merged pictures. *C* and *Hs* are control and heat-shocked chromosomes prepared without and immediately after 30 min of heat shock. Enlarged regions are labeled by *boxes*. *Arrows* point to positions where heat-shock puffs are formed (*white*), and positions displaying strong signals of Pol II (**a**, **c**) or TMG (**b**) (*green*) and RPB4 (*red*), respectively, in the absence of the other. On the *bottom right* identical regions of two 2L chromosome arms are shown to demonstrate the highly reproducible staining pattern

subunit irrespective of its phosphorylation status resulted in very similar results (data not shown).

Discussion

In *S. cerevisiae*, the RPB4 subunit of Pol II is believed to be a non-essential component of the transcription machinery that is involved in several steps of mRNA biosynthesis and plays specific roles under stress conditions (Choder 2004; Sampath and Sadhale 2005). We found that in *D. melanogaster* RPB4 is essential for normal development, and in its absence development stops at an early phase. In the fly, RPB4 is the product of a bicistronic gene that also encodes the transcriptional adaptor ADA2a. ADA2a null mutants die at a late stage of larval development. The L1 lethality of RPB4 null animals in contrast to the L3 lethal phenotype of ADA2a mutants might be a result of the differential expression of the two proteins or can indicate a more rapid turnover of RPB4 than that of ADA2a.

Encoding RPB4 and ADA2a within one transcription unit seems to be unique for *Drosophilidae*. This gene organization prompts the question: how are the two proteins generated from one transcription unit? Recently, it has been reported that the loss of scRPB4 resulted in an alteration in the polyadenylation site usage at the RNA14 gene (Runner et al. 2008). It was, therefore, interesting to

test if the production of RPB4 itself is regulated by alternative polyA site selection. The structure of identifiable mRNA forms does not suggest this idea. More likely, the generation of two different mRNAs from the same transcription unit is achieved by alternative splice site selection.

Co-expression of the two proteins from one transcription unit is intriguing considering a possible functional interconnection between them. In the case of RPB4 and ADA2 this possibility is particularly attractive as histone-modifying complexes which harbor ADA2 factors are involved in transcriptional responses to physiological and environmental stress (Nagy and Tora 2007), and yeast RPB4 has been implicated in the transcriptional regulation of stress response (Bourbonnais et al. 2001; Pillai et al. 2003). By determining the levels of specific messages with quantitative RT-PCR, we found that in embryos the levels of both mRNAs are high. At the beginning of L1 stage, the level of the *Rpb4* message drops sharply and remains low throughout larval development. The time of the sharp decrease in *Rpb4* mRNA level coincides with the lethal phase of *Rpb4* null mutants. In contrast, under normal condition the level of *Ada2a* message is relatively high in larval stages. Under stress conditions, we observed specific changes in the levels of *Ada2a*- and *Rpb4*-specific messages. Upon starvation, after an initial drop, the *Ada2a* mRNA level recovered to the wild-type level, while the *Rpb4*-specific message remained low for an extended period. This could be related to the role of ADA2-containing complexes in cellular metabolism. Heat stress resulted in a different response in the levels of the two messages: after heat stress the level of *Ada2a* message was decreased, the expression of *Rpb4*, however, was not affected.

In *S. cerevisiae*, RPB4 affects expression of a small number of genes under normal growth conditions and also in stress response (Pillai et al. 2003). One study estimated that despite that RPB4 is present in high excess compared with the other Pol II subunits in yeast cells; only about 20% of the Pol II complexes contain the RPB4/7 dimer. On the other hand, recent data obtained by ChIP-on-chip technique indicate that RPB4 is recruited on coding regions of most transcriptionally active genes. Based on these observations, a role for RPB4 in transcription elongation was suggested (Jasiak et al. 2008; Verma-Gaur et al. 2008). On immunostained polytene chromosomes, we detected RPB4 co-localization with CTD-phosphorylated Pol II at numerous sites. These sites correspond to puffs, or interband regions, which are believed to contain chromatin in transcriptionally active structure. Following high-temperature stress, RPB4 localizes to heat-shock puffs. Nonetheless, we did not detect a decrease in *hsp70* mRNA induction following heat stress in *Rpb4* larvae when compared with wild-type controls. Intriguingly, at many sites the staining intensities by RPB4 and Pol II-specific antibodies are very different and a smaller number of chromosomal locations Pol II localization can be seen clearly in the absence of RPB4. This suggests that in a *Drosophila* polytene tissue, active Pol II complexes, which do not contain RPB4, are assembled on specific genes. Conversely, RPB4 is localized at sites where Pol II is present only in low abundance or not at all. These observations are seemingly in contradiction with the recent data by others on the genome-wide co-localization of RPB4 with subunits of the Pol II complex (Jasiak et al. 2008; Verma-Gaur et al. 2008). In these ChIP-on-chip experiments, an RPB4 occupancy lower than that of RPB3 was observed consistently and it depended on the length of the transcription unit. A possible reason for the contradictory results of ours and others could be the lower sensitivity of the assay we used and the different lengths of transcription units on the polytene chromosomes. Perhaps, more important is, however, that the ChIP-on-chip assay detects protein complexes that are in close proximity to DNA and are therefore crosslinked effectively. On the polytene staining less tightly bound proteins might be detected as well. It is, therefore, conceivable that a fraction of the RPB4 is in association with complexes assembling for RNA maturation and/or transport and this fraction is not detected by chromatin immunoprecipitation. Lastly, polytene cells and chromosomes might represent a specific environment in which the occupancy of specific factors differs from that seen on de-condensed chromatin of diploid nuclei. In particular, export of transcripts generated on many copies of polytene templates might require an RPB4 level significantly higher than it is detectable in diploid cells. Two independent observations in particular might give grounds to the reasoning outlined above: First, the level of *S. pombe Rpb4* was found to be sevenfold higher than that of *Rpb7* (Kimura et al. 2001). Moreover, in sedimentation experiments *Rpb4* appeared to be associated with complexes smaller than Pol II. These observations suggest that *Rpb4* may have functions independently of *Rpb7* and outside the context of Pol II. Second, yeast *Rpb4* mutations fully functional in transcription, but effecting post-transcriptional events have been identified (Farago et al. 2003). Based on the analysis of these mutants and several other observations, a role of RPB4 in transcript export has been suggested. In light of these data our observation of differences in the density of Pol II and RPB4-containing complexes on the *Drosophila* polytene chromosome might reflect different functions RPB4 plays in transcript initiation, elongation, maturation and transport.

The constructs, we generated for the in vivo study of RPB4 functions represents the first *Rpb4* allele in a multicellular eukaryote. A transgene-producing DmRPB4-specific siRNA is also available in stock centers. We found

that silencing of the RPB4 production by this transgene causes early L2 larval lethality (data not shown). The phenotypes of *Rpb4* null and *Rpb4* silenced animals thus correlate well.

Our data presented here prove the essential function of *Rpb4* at the beginning of the *D. melanogaster* development. The transgenes and genotypes we describe represent tools by which specific roles of this essential factor can be explored.

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