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RESEARCH COMMUNICATION

Phosphorylation of histone H3 during transcriptional activation depends on promoter structure

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Covalent modifications of histone N-terminal tails are required for the proper assembly and activation of the general transcription factors at promoters. Here, we analyze histone acetylation and phosphorylation in *Drosophila* transgenes activated by the yeast Gal4 transcriptional activator in the context of different promoters. We show that, independent of the promoter, transcription does not correlate with acetylation of either H3-Lys 14 or H4-Lys 8. Histone H3 associated with the DNA of Gal4-induced transcribing transgenes driven by the *Drosophila* Hsp70 promoter is hyperphosphorylated at Ser 10 during transcription. Surprisingly, histone H3 at Gal4-induced transgenes driven by the P element Transposase promoter is not hyperphosphorylated. The data suggest that transcription occurs without acetylated H4 and H3 in both transgenes in *Drosophila* polytene chromosomes. Instead, phosphorylation of H3 is linked to transcription and can be modulated by the structure of the promoter.

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Posttranslational covalent modifications of N-terminal tails of the core histones at the nucleosome play an important role in determining chromatin structure, which is an essential component of the control of nuclear biology (Jenuwein and Allis 2001). The expression of a large number of genes from organisms across the phylogenetic scale correlates with histone modifications such as acetylation, methylation, phosphorylation, and ubiquitination in the nucleosomes surrounding the promoter of the gene (Roth et al. 2001; Berger 2002; Fry and Peterson 2002; Sun and Allis 2002). Acetylation of histones H3 and H4 N-terminal tails has been broadly associated with activation of transcription. Histone acetyltransferases (HATs) are found in a variety of coactivators and transcription factor complexes, whereas histone deacetylases (HDACs) are present in protein complexes with a repressive function (Roth et al. 2001). H3 methylation at Lys 9 has the opposite effect and therefore is found in regions where transcription is repressed by chromatin

structure (Rea et al. 2000; Bannister et al. 2001). Phosphorylation of H3 at Ser 10 has also been correlated with activation of transcription in yeast, mammals, and *Drosophila* (Cheung et al. 2000; Lo et al. 2000; Nowak and Corces 2000; Lo et al. 2001; Thomson et al. 2001; Li et al. 2002; Strelkov and Davie 2002).

Two nonexclusive models have been postulated to explain the role of histone N-terminal tail modification in transcription. The first one suggests that histone modifications have an effect on the relative charge of the histone tails, rendering a more open or closed chromatin state that determines the accessibility of transcription factors to the core promoter. The second model, termed the histone code hypothesis, predicts that a combination of covalent modifications of histone tails functions as a target for the specific binding of effector proteins (Turner 2000; Jenuwein and Allis 2001). Binding of these proteins will ultimately determine the transcriptional state of the chromatin. The histone code hypothesis is gaining support following the realization that well characterized proteins with functions involving chromatin structure and control of gene expression contain domains that selectively bind covalently modified histone N-tails. For example, proteins containing a bromodomain specifically bind lysine-acetylated histone tails. Bromodomain proteins include many transcriptional regulators such as several HATs and important components of the transcription machinery such as TAF250 (Jacobson et al. 2000). In addition, proteins containing a chromodomain specifically bind methylated histone tails. For example, HP1 is a chromodomain-containing protein involved in the formation of heterochromatin and in the silencing of gene expression by binding methylated H3 at Lys 9 (Bannister et al. 2001).

It is not clear whether H3 phosphorylation at Ser 10 also functions as a recognition site for specific binding of chromatin-associated proteins. In vivo and in vitro data suggest that acetylation of H3 at Lys 14 and phosphorylation at Ser 10 are coupled and that H3 acetylation by yeast GCN5 is enhanced by phosphorylation at Ser 10 (Cheung et al. 2000; Lo et al. 2000). This association correlates with transcription activation and is supported as well by structural data showing a specific interaction between arginine 164 of GCN5 and the phosphorylated Ser 10 of the H3 N-terminal tail. Other results, however, question the general implication of this model when applied to other organisms; for example, the dramatic changes in H3 phosphorylation that take place during the heat shock response in *Drosophila* are not followed by equivalent changes in histone acetylation (Nowak and Corces 2000). The histone H3 N-terminal tails at the *Drosophila* heat shock genes become hyperphosphorylated at Ser 10 upon induction of transcription by heat shock. Ser 10-phosphorylated H3 is also observed under normal conditions at many other sites in polytene chromosomes, including actively transcribing regions such as ecdysone-induced puffs. In addition, phosphorylated histone H3 disappears from all nonheat shock *loci* during the heat shock response, coinciding with the shutdown of transcription of most genes, further supporting the hypothesis that H3 phosphorylation may have a central role in the control of gene expression in *Drosophila*. Except for a weak acetylation signal in some of the heat

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shock puffs, the global changes observed in H3 phosphorylation after heat shock are not followed by detectable equivalent changes in H3 or H4 acetylation (Nowak and Corces 2000).

To gain further insights into the role of H3 phosphorylation during transcription in *Drosophila* and its correlation with acetylation, we addressed the question of whether phosphorylation and acetylation at specific residues of the histone N-terminal tails are associated with transcription of transgenes ectopically activated by the Gal4 transcriptional activator. In yeast, the acidic domains of the widely used exogenous transcriptional activators Gal4 and VP16 recruit the SAGA complex to the promoter of the gene (Bhaumik and Green 2001; Larschan and Winston 2001). Because complexes equivalent to SAGA are also found in humans and in *Drosophila*, where they contain PCAF, a protein homologous to the GCN5 present in the yeast's SAGA (Aoyagi and Wassarman 2000), we asked whether transcriptional activation by Gal4 in *Drosophila* involves the same type of covalent histone tail modifications as in yeast. The results suggest that phosphorylation and not acetylation of H3 accompanies transcriptional activation of these transgenes, and this modification is not determined by the nature of the transcriptional activator but rather by the structure of the core promoter itself.

Results and Discussion

Gal4-induced transcription of Hsp70 promoter-driven transgenes is associated with H3 hyperphosphorylation at Ser 10

Immunostaining using specific antibodies has shown that a large number of sites in *Drosophila* polytene chromosomes contain phosphorylated histone H3 at Ser 10. The same experiments show that activation of transcription by the heat shock factor (HSF) at the heat shock genes induces high levels of H3 phosphorylation at the site of transcription (Nowak and Corces 2000). We first addressed the question of whether H3 phosphorylation at the *Hsp70* promoter depends exclusively on HSF or can take place by activation of transcription mediated by any other transcription factor. A simple way to address this question is to analyze histone H3 phosphorylation at nucleosomes linked to the DNA of transcribing transgenes that use the TATA box promoter of the *Hsp70* gene but lack the HSF binding sites. We thus tested whether transcription of a GFP reporter gene under the control of Gal4–UAS sites fused to the promoter region of the *Hsp70* gene was associated with H3 phosphorylation. To this end we crossed flies transformed with the *pUAST–Hsp70:GFP* transgene (Fig. 1a) with flies carrying a transgene encoding Gal4 under the control of the *Hsp70* promoter (*Hsp70:Gal4*). This Gal4 transgene is constitutively expressed in salivary glands, and therefore no heat shock was necessary to induce expression of Gal4. Transcription of the GFP gene induced by Gal4 was monitored by Western blot analysis and by observing the salivary glands under a dissecting fluorescence microscope (Fig. 1d,e). Only larvae carrying both transgenes (*pUAST–Hsp70:GFP* and *Hsp70:Gal4*) show GFP fluorescence (e.g., see Fig. 1e); the levels of GFP expression were quantitated more precisely by Western analysis of GFP protein produced in larvae carrying each trans-

gene (Fig. 1d). Antibodies specific against phosphorylated H3 at Ser 10 were then used to perform immunostaining experiments on polytene chromosome spreads. In order to determine whether the *pUAST–Hsp70:GFP* transgene colocalized with phosphorylated H3 histones, the same spreads were coimmunostained using monoclonal antibodies specific against the DNA binding domain of the Gal4 protein, which should label only the locus occupied by the transgene containing Gal4–UAS DNA sequences. Results show that, upon activation of transcription by Gal4, phosphorylated H3 histones are found in the *pUAST–Hsp70:GFP* transgene (Fig. 2). These results suggest that the *Hsp70* promoter plus an exogenous transcriptional activator are sufficient to induce H3 phosphorylation at Ser 10 during transcription, indicating that HSF is not unique in the induction of H3 phosphorylation. In addition, the results indicate the presence of phosphorylated H3 in most polytene chromosome interbands and not in bands. We also found an almost perfect correlation between the presence of phosphorylated H3 at Ser 10 and the GAGA protein (data not shown). Since GAGA is a general transcriptional activator (Granok et al. 1995) and strong evidence indicates that transcription in polytene chromosomes occurs in interbands and not in bands, where chromatin is highly compacted (Sass 1982; Weeks et al. 1993), these observations suggest that histone phosphorylation is a landmark for actively transcribing regions in *Drosophila* polytene chromosomes.

We then asked whether the recruitment of factors controlling H3 phosphorylation depends on the structure of the promoter. The *Hsp70* promoter contains a consensus TATA box, plus an Initiator and downstream sequences that may influence the transcriptional activity of the

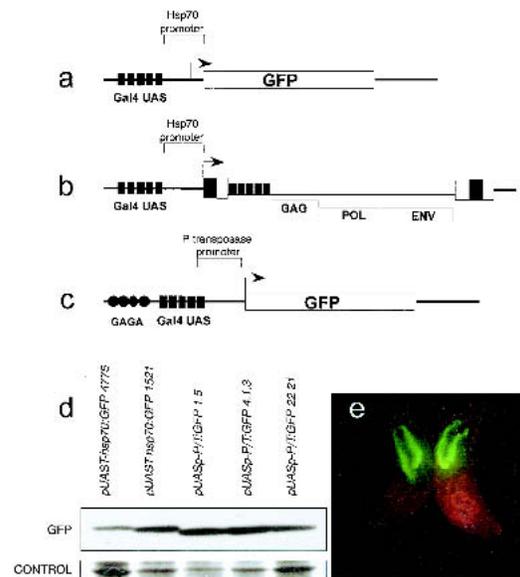


Figure 1. Structure of reporter genes used for analysis of histone modifications and expression analysis. (a) *pUAST–Hsp70:GFP*. (b) *pUAST–Hsp70:gypsy*. (c) *pUASp–P/T:GFP*. (d) Western analysis of GFP expression in *pUAST–Hsp70:GFP* and *pUASp–P/T:GFP* in *Hsp70:Gal4* larvae. Numbers in the names of the transgenes indicate different independent lines. Loading controls correspond to Actin proteins as determined by molecular weight after staining of the blotted membrane with Coomassie blue. (e) An example of GFP fluorescence in salivary glands in larvae expressing a *pUAST–Hsp70:GFP* transgene.

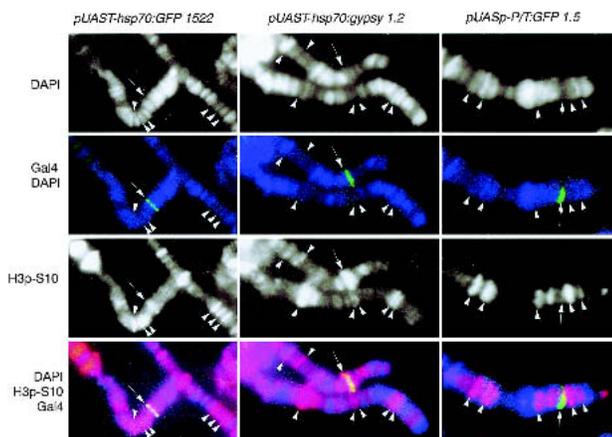


Figure 2. Correlation between histone H3 phosphorylated at Ser 10 and expression of various transgenes. Phosphorylated H3-Ser 10 is shown in red, Gal4 in green, and DAPI in blue. Arrows point to the localization of each transgene. Arrowheads show colocalization of polytene chromosome interbands (no DAPI staining) and phosphorylated H3.

gene (Lee et al. 1992; Wu et al. 2001). To test whether downstream sequences have a role in H3 phosphorylation, we fused the origin of transcription containing the Initiator and downstream sequences of the *gypsy* retrotransposon (Arkhipova et al. 1986), to the upstream promoter region of *Hsp70* containing the TATA box (Fig. 1b). The *pUAST-Hsp70:gypsy* transgene contains the same TATA box from *Hsp70* as *pUAST-Hsp70:GFP* but differs from this transgene in the sequences downstream of the original transcription. These sequences correspond to those of the *gypsy* retrovirus and include regions R and U5 of the LTR plus binding sites for the Suppressor of Hairy wing protein [Su(Hw)] and all sequences encoding GAG, POL, and ENV proteins (Fig. 1b). Immunostaining experiments using antibodies raised against the *gypsy* Envelope protein (ENV) show that *gypsy* is actively expressed on salivary glands from larvae carrying the *pUAST-Hsp70:gypsy* and the *pUAST:Gal4* transgenes, whereas no ENV protein was found in larvae not expressing Gal4 (data not shown). This result indicates that *gypsy* is actually transcribed from the *pUAST-Hsp70:gypsy* transgene and not from *gypsy* copies elsewhere in the genome. Immunolocalization experiments to detect phosphorylated H3 were performed with *pUAST-Hsp70:gypsy*, and results were identical to those obtained with the *pUAS-Hsp70:GFP* transgene. H3 is always heavily phosphorylated at the site where *pUAST-Hsp70:gypsy* and the Gal4 protein are present (Fig. 2). The conclusion of this experiment is that phosphorylation of H3 at Ser 10 at the *Hsp70* promoter is not dependent on specific sequences located downstream of the initiation of transcription.

Phosphorylation of H3 might require binding of TBP at the core promoter

To further investigate the effect of promoter structure on histone H3 phosphorylation, we analyzed a promoter completely unrelated to the *Hsp70* promoter. We found a candidate in the P Transposase promoter (P/T) present in the *pUASp* transformation vector developed by P. Rorth

(1998). The transcription start site of the P Transposase promoter has been determined experimentally to be nucleotide 87 of the P element sequence, with the putative TATA box (tatactact) found at -28 (O'Hare and Rubin 1983). The presence of a C at position 5 of the TATA box is highly unusual and has not been observed in any of the 900 characterized promoters described in the eukaryotic promoter database (EPD). In addition, it has been shown experimentally that the presence of a C at this position will disrupt the interaction with the TATA binding protein (TBP; Patikoglou et al. 1999; Praz et al. 2002). The P Transposase promoter also lacks Initiator and DPE elements. These structural characteristics might explain the ability of the P transposase promoter to drive transcription in the germ line, whereas the *Hsp70* promoter is not expressed in these cells (Rorth 1998). We constructed a reporter gene containing the promoter region of the *Drosophila* P element Transposase fused to the coding region of the GFP gene (*pUASp-P/T:GFP*; Fig. 1c). Surprisingly, when we analyzed histone modifications on transcribing *pUASp-P/T:GFP* transgenes, we failed to detect H3 phosphorylation at the sites of insertion (Fig. 2). Because these results could be attributed to position effects associated with a particular transgene, we repeated the same experiments with two additional *pUASp-P/T:GFP* and *pUAST-Hsp70:GFP* transgenes inserted in different positions in the genome (see Materials and Methods). In all additional lines, transcription of *pUAST-Hsp70:GFP* transgenes also correlated with hyperphosphorylation of H3, whereas chromatin associated with all *pUASp-P/T:GFP* transgenes showed nondetectable H3 phosphorylation (data for all additional lines not shown). Because higher levels of H3 phosphorylation may correlate only with high levels of transcription, it is possible that the differences in the level of H3 phosphorylation observed between *Hsp70* and P transposase promoters were in fact due to differences in promoter strength. To test this possibility, we performed a Western blot analysis comparing GFP levels in third-instar larvae carrying *pUASp-P/T:GFP* or *pUAST-Hsp70:GFP* transgenes in the presence of Gal4 expressed from the same *Hsp70:Gal4* transgene. Results show that the amount of GFP does not correlate with the amount of H3 phosphorylation observed, suggesting that differences in H3 phosphorylation between promoters are due to factors other than the strength with which promoters activate transcription (Fig. 1d).

The *Hsp70* promoter contains a consensus TATA box that binds TBP. Because TFIID complexes can be assembled without the participation of TBP (Verrijzer 2001), we asked whether the P Transposase promoter actually recruits TBP. To this end, we performed coimmunolocalization experiments using antibodies against TBP and Gal4 in both *pUASp-P/T:GFP* and *pUAST-Hsp70:GFP* transgenes. The results suggest that TBP binds to the *Hsp70* promoter but is missing from the P Transposase promoter (Fig. 3), implying that the assembly of general transcription factors may follow different biochemical pathways in terms of chromatin structure, depending on specific signals at the core promoter. In addition, promoter-specific differences in H3 phosphorylation also suggest that the kinase responsible for such phosphorylation might be recruited by components associated with general transcription factors at the core promoter and is independent of the nature of the transcriptional activator. The factor that recruits such a ki-

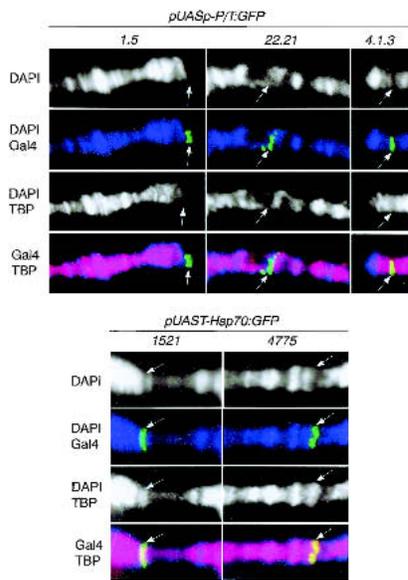


Figure 3. Analysis of TBP localization in various transgenes. TBP staining is shown in red, DAPI in blue, and Gal4 in green. Arrows show the position of the transgene in different stocks.

nase must be directly dependent on the presence of the TBP protein. In *Drosophila*, TRF1 and TRF2 are two known general transcription factors found in salivary glands that can replace the function of TBP in transcription initiation at certain promoters (Hansen et al. 1997; Rabenstein et al. 1999; Freiman et al. 2001). It follows from our data that the assembly of the preinitiation complex by different components of TFIID may also determine changes in the chromatin structure at the transcribing gene. Finding these components, in particular the kinase responsible for the phosphorylation of histone H3, might be an important key to better understand the complexity of transcription regulation in higher eukaryotes.

Transcription of Gal4-activated transgenes is independent of acetylation of histone H4 at Lys 8 and histone H3 at Lys 14

Because the genomic distribution of acetylated histones is apparently unaltered after the dramatic changes in gene transcription that take place during the heat shock response (Nowak and Corces 2000), we decided to determine whether transcription correlates with acetylation of specific histones in the transgenes analyzed above. We first used antibodies specific against acetylated histone H4 at Lys 8, which has been correlated with actively transcribing chromatin (Strahl and Allis 2000). Immunostaining of polytene chromosome spreads utilizing the same procedures as those described above were performed. Sites of acetylated H4 at Lys 8 do not colocalize with Gal4 labeling, which corresponds to the sites of the actively transcribing GFP transgenes in all of the promoters assayed (Fig. 4).

Because Gal4 probably recruits the SAGA complex to the promoters of the transgenes, we decided to also test whether there is a functional link in *Drosophila* between H3 phosphorylation and H3 acetylation, as occurs in yeast (Lo et al. 2000). Analysis of the amino acid sequence of GCN5 from distantly related organisms such

as yeast, *Drosophila*, and humans shows a striking conservation of the arginine at position 164, suggesting that this protein has retained its ability to recognize phosphorylated H3-Ser 10 across the phylogenetic scale. In *Xenopus* oocytes, for example, the activation of transcription by the thyroid hormone receptor also correlates with an increase of H3 phosphorylated at Ser 10 and acetylated at Lys 14, suggesting that acetylation and phosphorylation are also coupled during transcription activation in higher eukaryotes (Li et al. 2002). If transcriptional activation by Gal4 in *Drosophila* also involves the recruitment of the SAGA complex to the promoter, the *Drosophila* GCN5 (dPCAF) protein may have a role in the histone modifications undergone by the GFP transgenes used in the present study. We therefore performed immunostaining experiments using antibodies specific against acetylated H3 at Lys 14, the amino acid target for the yeast acetyl transferase GCN5. Because *pUAST-Hsp70:GFP* transgenes are hyperphosphorylated and *pUASp-P/T:GFP* transgenes are not, one can argue that dPCAF might be involved in the activation of transcription of the former, through acetylation of H3 at Lys 14, but not in the activation of transcription of the latter. Surprisingly, H3 is not acetylated at detectable levels in any of the two transgenes (Fig. 5). These results suggest that acetylation of H4 at Lys 8 or H3 at Lys 14 is not required for activation of transcription in either of the transgenes used in this study. Whether dPCAF is recruited by Gal4 to the promoter of the transgenes cannot be determined with the data presented here. However, even if dPCAF is recruited to the promoter of the Gal4-UAS transgenes, we can conclude that the function of H3 phosphorylation observed here is independent of a requirement for phosphorylation of Ser 10 as a prerequisite to enhance acetylation of Lys 14.

In support of this observation, we found that H3 phosphorylated at Ser 10 is always localized in interbands (see Fig. 2), whereas acetylated H4 and H3 always localize in the bands (Figs. 4, 5). Because transcription in polytene chromosomes localizes mostly to the interbands, these results further support the idea that histone phosphorylation, and not acetylation, is widely associated with active transcription in *Drosophila*. In addition to the dis-

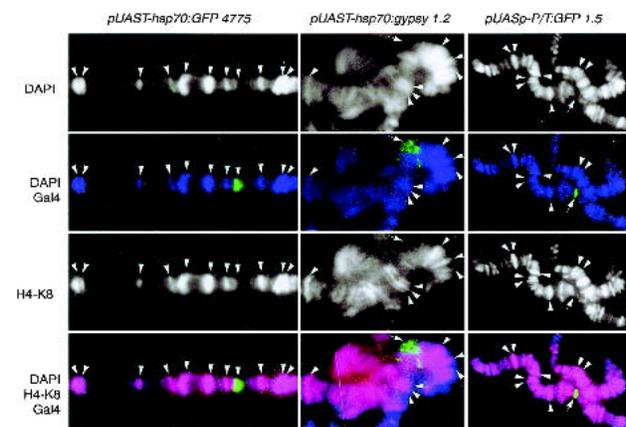


Figure 4. Acetylated H4 at Lys 8 does not colocalize with actively transcribed transgenes. Acetylated H4 at Lys 8 is shown in red, Gal4 in green, and DAPI in blue. Arrows show localization of each transgene. Arrowheads show colocalization of polytene chromosome bands and acetylated H4 at Lys 8.

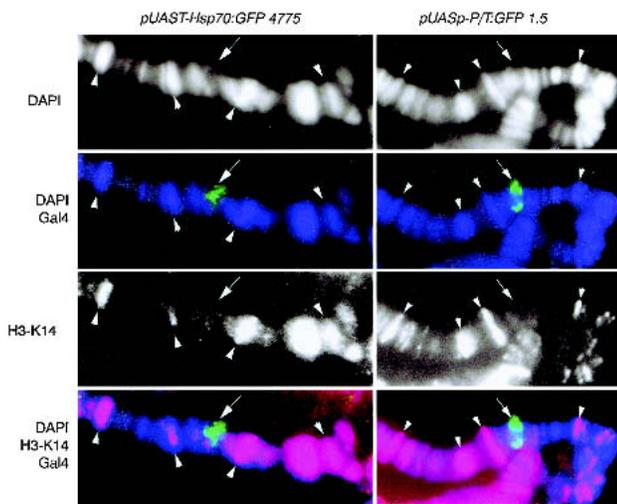


Figure 5. Acetylated H3 at Lys 14 does not colocalize with transcribing transgenes or with polytene chromosome interbands. Acetylated H3 at Lys 14 is shown in red, Gal4 in green, and DAPI in blue. Arrows show insertion sites of the transgene. Arrowheads show colocalization of acetylated H3 at Lys 14 only with polytene chromosome bands.

tribution of acetylated histones H3 at Lys 14 and H4 at Lys 8 described in this work, identical polytene band-interband distribution has been found for polyacetylated H3, acetylated H4-Lys 5, and acetylated H4-Lys 12 (Pile and Wassarman 2000). In light of the large amount of evidence suggesting a role for histone acetylation in transcription activation, Pile and Wassarman (2000) interpreted these results as suggesting that immunolocalization on polytene chromosomes might not be sufficiently sensitive to detect changes in histone acetylation after transcription initiation, and that the pattern of histone acetylation along the chromosome might be a reflection of background acetylation, only visible in polytene bands where chromatin is highly compacted. Evidence against this possibility comes from the observation that acetylated H3 at Lys 14 (Fig. 5) and polyacetylated H3 (data not shown) are not present in all bands, whereas acetylated H4 at Lys 8 is present in almost all polytene bands (Fig. 4). This unequal distribution indicates that, at least for some acetylated histones, the polytene chromosome distribution reflects differences in chromatin organization and not only in chromatin compaction. This observation suggests that the lack of histone acetylation at sites of active transcription is not a technical artifact and might, in fact, be a reflection of the absence of acetylated histones in these regions. Alternatively, the lack of acetylated histones at sites of Gal4-induced transcription could indicate that histone acetylation is only transient during the initiation of transcription and is not required at subsequent steps.

The discrepancy between results in yeast and vertebrates and in *Drosophila* argues in favor of two separate roles for H3 phosphorylation. One role might correspond to the observed general association of transcription with H3 phosphorylation in polytene chromosomes, which seems to be independent of acetylation. In support of this observation, histone phosphorylation and histone acetylation are independently regulated during the activation of transcription of *c-fos* and *c-jun* in mouse (Thomson et al. 2001). The second role might be related to the con-

servation of arginine 164 in GCN5, which would serve as a recognition site for the binding of PCAF and subsequent acetylation of Lys 14, as occurs in yeast and in genes activated by the thyroid hormone receptor. An interesting possibility for a role of H3 phosphorylation is suggested by recent reports indicating that the histone H3.3 variant replaces H3 in regions of active transcription in *Drosophila* (Ahmad and Henikoff 2002a,b). It is possible that the H3 phosphorylation we observe is mechanistically linked to the replacement of H3 in *Drosophila*, and therefore that the same mechanism is not employed by yeast, which lacks histone H3 (Ahmad and Henikoff 2002b).

Materials and methods

Transgenes and transgenic flies

pUAST-Hsp70:GFP transgenes were obtained from the Bloomington *Drosophila* stock center. The stock numbers used in this work were 4775 (chromosome 2), 1521 (chromosome 2), 1522 (chromosome 3), and 5193 (chromosome 1). In order to obtain the *pUAST-Hsp70:gypsy* transgene, the *PUAST* transformation vector, containing the core promoter of the *Hsp70* gene (Brand and Perrimon 1993), was used to clone the origin of transcription of *gypsy* by inserting a DNA fragment, starting at the nucleotide 234 position of the retrovirus, into the origin of transcription of the *Hsp70* promoter. This construct removes the initiator element and all downstream sequences from the *Hsp70* promoter and leaves intact the Initiator element from *gypsy* and the sequences upstream of the *Hsp70* transcription initiation site in *pUAST*. In the second transgene, *pUASp-P/T:GFP*, the GFP DNA sequence was inserted into the *NotI* site of the *pUASp* vector (Rorth 1998). Each plasmid was microinjected into *y w; P(Δ2-3)/TM6* embryos, and flies carrying the transgene inserted in the *TM6* (*pUAST-Hsp70:GFP1.5*) chromosome were selected by standard procedures (Robertson et al. 1988). To obtain new insertion sites, both *Drosophila* stocks were crossed to *y w; P(Δ2-3)/TM6*. New insertions in the X (*pUAST-Hsp70:GFP4.1.3*) and in the second chromosome (*pUAST-Hsp70:GFP 22.21*) were recovered. The *Hsp70:Gal4* transgene was obtained from Dr. Allen Shearn (Department of Biology, Johns Hopkins University, Baltimore, MD).

Immunocytochemistry and Western blot analysis

Western analysis was carried out by standard procedures using SuperSignal West Pico Chemiluminescent substrate from Pierce for detection. Protein extracts were prepared from equal amounts of larvae carrying different transgenes expressing GFP in their salivary glands and loaded in the gels. After immunodetection, membranes were stained with Coomassie blue to further control for loading of the samples. Immunolocalization of proteins on polytene chromosomes was as described (Harrison et al. 1993). Anti-Ser 10 phosphohistone H3 antibodies were obtained from Dr. David Allis (Department of Biochemistry and Molecular Genetics, University of Virginia H.S.C., Charlottesville, VA) and Upstate Biotechnology, and anti-Lys 8 acetyl H4 and anti-Lys 14 acetyl H3 antibodies were obtained from Upstate Biotechnology. Anti TBP antibodies were provided by Dr. James T. Kadonaga (Section of Molecular Biology, University of California, San Diego). Anti-Gal4 DBD monoclonal antibody was obtained from Santa Cruz Biotechnology. Antibodies against *gypsy* Envelope protein were previously obtained in our laboratory (Song et al. 1997). Proteins were visualized using FITC- or Texas red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories); DNA was stained with DAPI and chromosomes were examined using a Zeiss Axiophot microscope and a Photometrics cooled CCD camera (Roper Scientific).

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