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ORIGINAL ARTICLE

Effects of histone deacetylase inhibitors on transcriptional regulation of the *hsp70* gene in *Drosophila*

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Histone acetyltransferases/deacetylases contribute to the activation or inactivation of transcription by modifying the structure of chromatin. Here we examined the effects of histone deacetylase inhibitors (HDIs), trichostatin A, and sodium butyrate on *hsp70* gene transcriptional regulation in *Drosophila*. The chromatin immunoprecipitation assays revealed that HDI treatments induced the hyperacetylation of histone H3 at the promoter and the transcribing regions of *hsp70* gene, increased the accessibility of heat-shock factor to target heat-shock element, and promoted the RNA polymerase II-mediated transcription. Moreover, the quantitative real-time PCR confirmed that the HDI-induced hyperacetylation of histone H3 enhanced both the basal and the inducible expression of *hsp70* mRNA level. In addition, the acetylation level of histone H3 at the promoter exhibited a fluctuated change upon the time of heat shock. These experimental data implicated a causal link between histone acetylation and enhanced transcription initiation of *hsp70* gene in *Drosophila*.

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Introduction

Chromatin remodeling and histone modifications have emerged as important mechanisms of the control of gene expression. Chromatin is a dynamic structure that modulates the access of regulatory factors to DNA, and dynamic changes in chromatin structure are directly influenced by post-translational modifications of the amino-terminal tails of core histones [1]. The tails of histones are susceptible to a wide range of post-translational modifications, including acetylation, phosphorylation, and methylation [2]. The "histone code" theory proposes that the post-translational modifications of histone tails are an epigenetic chromatin marking system that can regulate on-off states of transcription [3]. Hyperacetylation of histones is generally associated with transcriptionally active chromatin, whereas the inactive chromatin region is enriched in deacetylated histones [4, 5]. Furthermore, specific DNA binding transcription factors recruit histone acetyltransferases (HATs) and histone deacetylases (HDACs) to promoters leading to the activation or repression of transcription [6, 7]. Treatment of organisms with histone deacetylases inhibitors (HDIs) is known to increase histone acetylation and consequently cause an altered chromatin structure at nucleosomal level, in particular by leading to histone hyperacetylation in specific sites. These alterations promote or inhibit the transcription of a large number of genes [8, 9].

The regulation of *hsp70* gene is a complex and precise mechanism. The *hsp70* gene may be expressed both at a low basal level under normal growth conditions and at a high induced level after heat shock. Under non-heat-shock conditions, the promoter sequences of *hsp70* are occupied by at least three transcription factors, i.e., GAGA factor (GAF), TATA-binding protein, and RNA polymerase (pol) II [10]. Upon the heat shock, the inducible expression of *hsp70* is mediated by the interaction of the heat-shock factors (HSFs) with heat-shock elements (HSEs), which are

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Abbreviations: HDI (histone deacetylase inhibitor); Hsp (heat shock protein); TSA (trichostatin A); BuA (sodium butyrate); HAT (histone acetyltransferase); HDAC (histone deacetylase); ChIP (chromatin immunoprecipitation)
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located in the 5′-upstream region of *hsp70* gene [11]. In addition, the association of some elongation factors, e.g. FACT, Spt5, Spt6, p-TEFb, etc., are necessary for RNA pol II escaping the paused state and elongating through the entire *hsp70* gene [12-14].

Many studies have demonstrated that treatments of HDIs can result in histone hyperacetylation and remodeling of chromatin structure. Trichostatin A (TSA) was shown to strongly induce hyperacetylation of histone H3 [15], and sodium butyrate (BuA) caused histone hyperacetylation [16] by suppressing HDAC activity. It has been demonstrated that the inducible transcription of *hsp70* and *hsp30* were enhanced by HDI treatments during *Xenopus* development [17]. In addition, we have previously shown that when the third-instar larvae of *Drosophila* were fed with HDIs, a much-enlarged "puff" structure at the polytene chromosome band of 87A-C, where the *hsp70* gene is located, was observed [18]. This implicated a correlation between histone acetylation and *hsp70* activation, since the size of a particular "puff" is thought to be positively related with the intensity of transcription of the corresponding gene. These data suggest that histone acetylation may play an important role in *hsp70* gene transcriptional regulation. However, the basic molecular mechanisms of histone acetylation-mediated *hsp70* gene regulation are not fully understood and still remain a subject of current research interests.

In this study, by using HDI feeding of *Drosophila* larvae and chromatin immunoprecipitation (ChIP) assay, we showed that HDIs enhanced the acetylation level of histone H3 at the promoter and transcribing regions of *hsp70* gene, promoted the association of HSF with HSE, and facilitated RNA pol II transcription elongation. On the other hand, heat shock decreased the acetylation level at the promoter, but increased the acetylation level at the transcribing regions of *hsp70*. During heat shock, the acetylation level of histone H3 at the promoter exhibited a fluctuated change upon the time of heat-shock treatments. These results suggest that histone acetylation was involved in a precise and complex regulatory mechanism of both transcription initiation and elongation of *hsp70* gene in *Drosophila*.

Materials and Methods

HDI treatments and heat-shock induction of larvae

Wild-type strain of *Drosophila melanogaster* Canton-S was used, and larvae were collected from the standard cornmeal/yeast/agar medium and cultured for 6 h in physiological brine (130 mM NaCl, 4.7 mM KCl, and 1.9 mM $CaCl_2$) with or without HDIs. The HDIs used were TSA and BuA, and they were added at final concentrations of 10 μM and 10 mM, respectively. Then, some larvae were heat shocked at 37 ºC for 30 min. For the analyses of changes in acetylation level at *hsp70* promoter, larvae were induced at 37 ºC for 30 s, 1 min, 2 min, 5 min, 10 min, 20 min, and 30 min, respectively. Larvae sampled for real-time quantitative PCR analysis were frozen and stored at –80 ºC. Larvae collected for ChIP were crosslinked for 30 min in 4% formaldehyde, rinsed three times in physiological brine, and stored at –80 ºC.

Chromatin immunoprecipitation

The larvae were treated and crosslinked as described above. Nuclear extracts were obtained as described by Giancotti *et al*. [19] from 4% formaldehyde crosslinked larvae, and were sonicated on ice to shear the DNA into fragments with lengths between 200 and 800 bp. ChIP assay was performed using a ChIP kit supplied by the Upstate Biotechnology (Lake Placid, NY, USA) following the manufacturer's instructions. Before antibody precipitation, 10% of the chromatin solution was removed, crosslinking was reversed, and serial dilutions (10%, 1%, and 0.1% input) of purified DNA were analyzed by PCR to ensure that equal amounts of starting materials were used for each precipitation. The amounts of antibodies used per immunoprecipitation were as follows: anti-acetyl-histone H3, 5 μl (The Upstate Biotechnology, Lake Placid, NY, USA); anti-GAF, 4 μl (a gift from Dr C Wu, Laboratory of Biochemistry NCI, NIH); anti-HSF, 2 μl (a gift from Dr JT Lis, Department of Molecular Biology and Genetics, College of Agriculture and life Sciences, Cornell University); and anti-RNA pol II, 25 μl (Santa Cruza, CA, USA). Immunoprecipitated chromatin was then assayed by semiquantitative PCR using primers specific to sequences at the *hsp70* locus. Approximately 2% of the immunoprecipitated material was used for PCR amplification. Amplification mix (25 μl) contained 2 μl of the immunoprecipitated template, 400 nM DNA primers, and 12.5 μl Premix DNA Taq. PCR reaction was carried out under the following conditions: 94 ºC for 5 min; 94 ºC for 30 s, 55 ºC for 1 min, and 72 ºC for 1 min for 24 cycles, followed by an extension at 72 ºC for 10 min. Coordinates of the primers are given relative to the transcription start site as follows: S1, –684 to –475 (5′-ccc gct aag tga gtc ctg-3′ and 5′-gaa ttt agt tgc gac tta tta ttt tac-3′); S2, -252 to -9 (5′-tag aat ccc aaa aca aac tg-3′ and 5′-cgt ata cga agc gcc tct a-3′); S3, +38 to +243 (5′-cga aag cta agc aaa taa ac-3′ and 5′-cat tgt gtg tga gtt ctt ctt t-3′); and S4, +1704 to +2144 (5′-gcc aag gag atg agc acg-3′ and 5′-tcc aga gta gcc gcc aaa-3′). The quantitative estimation of the PCR products was accomplished by photodensitometric analysis of the bands in agarose gel after electrophoresis, and results were analyzed by using the *t*-test for the significance of differences and are given as means ± S.D.

Real-time quantitative PCR analysis of hsp70 mRNA

RNA was isolated from larvae using the RNA extraction kit supplied by Promega. The reverse transcription reaction was performed by using an RT system (Promega) following the manufacturer's instructions. Quantification of mRNA was performed using an ABI PRISM® 7000 sequence Detection System (PE Applied Biosystems, Weiterstadt) and SYBR® Green (TaKaRa) as a double-stranded DNA-specific fluorescent dye. A housekeeping gene, *Rp49* (ribosomal protein 49), was used as the internal control for standardizing *hsp70* mRNA expression.

Amplification mix (25 μl) contained 2 μl cDNA solution, 12.5 μl SYBR® premix Ex Taq™, 0.5 μl ROX Reference Dye, and 200 nM DNA primers. Amplification primers for *hsp70* were 5′-agc cgt gcc agg ttt g-3′ and 5′-cgt tcg ccc tca tac a-3′ and for *rp49* were 5′-agc act tca tcc gcc acc-3′ and 5′-atc tcg ccg cag taa acg-3′. Samples were amplified by a PCR program of 40 cycles of 10s at 95 ºC, 15s at 55 ºC, and 1 min at 72 ºC. The C_t (threshold cycle) was defined as the

number of cycles required for the fluorescence signal to exceed the detection threshold. Data were analyzed by using the $2^{-\Delta\Delta C_t}$ method, which is a convenient way to analyze the relative changes in gene expression [20].

Results

HDI treatments increased the acetylation level of histone H3 at both the promoter and transcribed regions of hsp70 gene

In our previous study, we found that both TSA and BuA were capable of affecting the chromatin structure at the site (87A-C) where the *hsp70* gene is located along the polytene chromosome in *Drosophila* [18]. Here, we further examined the positions of HDI-induced histone acetylation across the *hsp70* gene in an attempt to explore the mechanisms of acetylation-mediated *hsp* gene activation. We performed ChIP analyses on larval nuclear extracts using the antibody against acetylated histone H3 (AcH3, Figure 1A). The immunoprecipitated DNA was isolated and analyzed by PCR using primers that amplify the promoter, 5′- and 3′-transcribing fragments of the *hsp70* gene, respectively (Figure 1B, fragments S2, S3, and S4). As a control for immunoprecipitation of specific DNA, primers that span the region far upstream of *hsp70* promoter (Figure 1B, fragment S1) were also used in ChIP analysis. The ChIP results revealed that treatments of both HDIs (TSA and BuA) not only elevated the acetylation level of histone H3 at the promoter (Figure 2, S2, lanes TSA and BuA) but also resulted in hyperacetylation of histone H3 at the transcribing regions of *hsp70* (Figure 2, S3 and S4, lane TSA and BuA). In contrast, it can be seen from Figure 2, S1 that HDI treatments did not change the AcH3 level at the far upstream region of *hsp70*. These data suggested that TSA and BuA affected *hsp70* transcription by specifically enhancing the acetylation level of nucleosomes that were situated at the *hsp70* gene locus. The data also implicate that histone acetylation modification not only played a role during the modulation of nucleosomal configuration at *hsp70* promoter but may also be involved in the transcriptional elongation of the gene. On the other hand, we found that after heat shocking for 30 min, the acetylation level of histone H3 decreased at the *hsp70* promoter, but increased at the transcriptional regions of *hsp70* (Figure 2, S2 and S4, lane HS). These results further confirmed that a close correlation existed between the histone acetylation modification and *hsp70* transcription.

Influence of HDI-induced histone H3 hyperacetylation on binding of transcription factors to specific sequences

HSF and GAF are two critical transcription factors of heat-shock genes. Next, we wanted to know whether HDI-induced histone H3 hyperacetylation influenced the binding of these two transcription factors to their corresponding *cis*-elements. We crosslinked and immunoprecipitated chromatin with HSF and GAF antibodies in larval nuclear extracts with or without HDI treatments, PCR-analyzed the immunoprecipitated DNA, and the results were shown in Figure 3. A low level of HSF that was associated with the *hsp70* promoter prior to heat-shock and HDI treatments was seen (Figure 3, S2, lane Crtl). Following heat-shock and HDI treatments, the level of bound HSF increased at the promoter region (Figure 3, S2, lanes HS, TSA, TSAHS, BuA, and BuAHS). Meanwhile, HSF level at the 5′-transcribing region dropped moderately compared with that at the promoter, and was absent from the 3′-end of the gene (Figure 3, S3 and S4). This result was in accordance with what would be expected, since no HSEs have been known to reside within the transcription regions (Figure 3, S3 and S4), whereas the 5′-transcribing region was near to the first HSE element, and would be easily detected considering the size range of the chromatin (200–800 bp) generated by sonication in ChIP assays. For the level of bound GAF, it can be seen from Figure 3 that prior to heat-shock and HDI treatments, contents of GAF associated with the *hsp70* promoter, and 5′- and 3′-transcribing regions were all maintained at a medium level (Figure 3, S2, S3, and S4, lane Crtl). This may implicate that GAF is necessary for both transcription initiation and elongation of hsp70. However, following heat-shock and HDI treatments, the intensity of GAF association to its *cis*-element remained unchanged (Figure 3, S2, S3, and S4, lanes HS, TSA, TSAHS, BuA, and BuAHS), further suggesting that histone acetylation and GAF acted at different points in the process of *hsp70* transcription.

Change of histone H3 acetylation level at the promoter of hsp70 gene during heat shock

We have shown that the hyperacetylation of histone H3 enhanced the binding ability of HSF to HSE, implicating a positive role of histone acetylation in transcriptional activation of *hsp70* gene. We then further examined the change of H3 acetylation level (AcH3) during the heat shock. We performed ChIP analysis of larval nuclear extracts sampled at several different time points following heat-shock activation, in an attempt to track the temporal changes of AcH3 level at the promoter. As shown in Figure 4, the proportions of AcH3 at the promoter showed a drop-raise-drop change. At 2 min of heat shock, the content of the AcH3 declined to the lowest level, then as the time of heat shock prolonged, the AcH3 level increased gradually, and at 30 min of heat shock, the content of the AcH3 began to decrease again (Figure 4A and 4B). Meanwhile, real-time RT-PCR analysis indicated that the mRNA expression of *hsp70* reached a peak of approximate 150-fold activation

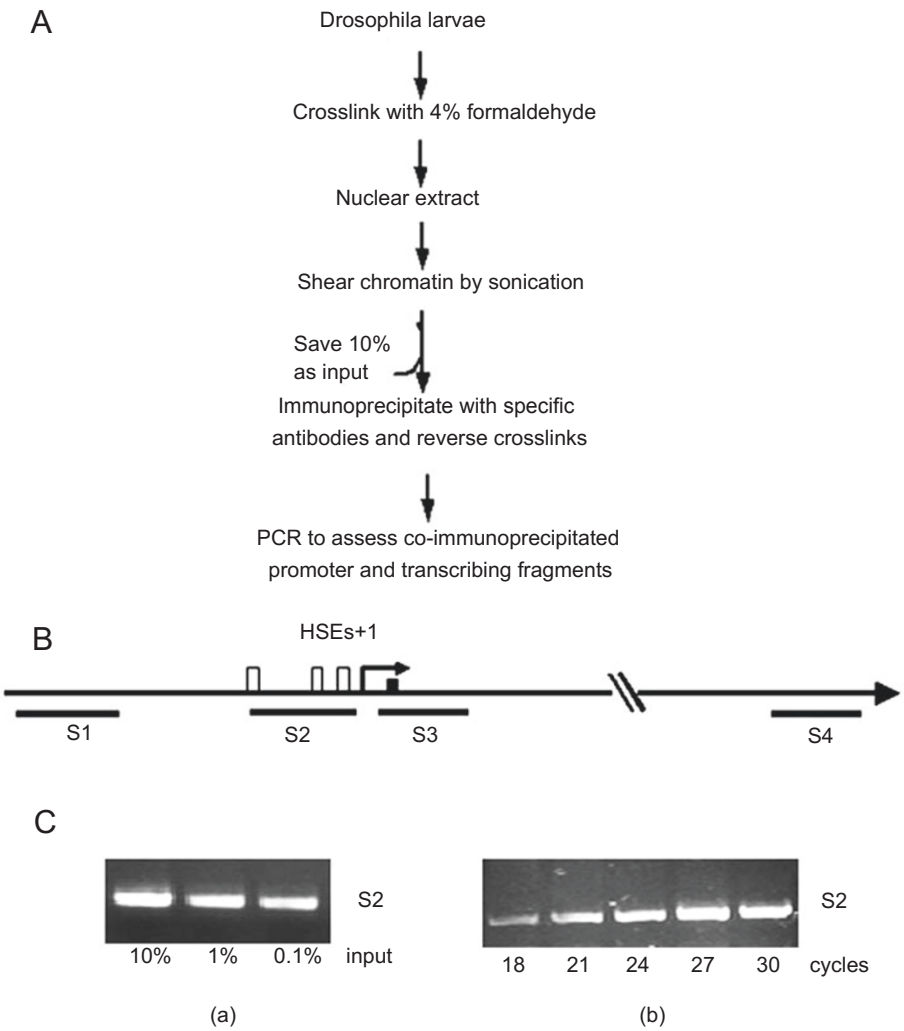

Figure 1 **(A)** Protocol of the ChIP assay. *Drosophila* larvae were treated as described in "Materials and methods" before ChIP assay. **(B)** Schematic presentation of the structure of *hsp70* gene. Open boxes indicate HSE sites, solid box denotes the site of paused pol II, and +1 points the transcription start site. The bold underlines span the PCR-amplified fragments representing the far upstream of the promoter (S1), the promoter (S2), the 5′-transcribing fragment (S3), and the 3′-transcribing fragment (S4) of hsp70 gene, respectively. **(C)** Determination of the linear range of semiquantitative PCR amplification using the promoter (S2) primers. (a) Analysis of input content under the condition of 30 cycles, and 1% input was used to determine the amounts of starting precipitable materials. (b) Analysis of amplification cycle number with 1% input template, and for next experiments, the templates were amplified for 24 cycles in each PCR reaction.

within 2 min, whereas at 5 min of heat shock, the mRNA expression of *hsp70* declined greatly, and as the time of heat shock prolonged, the mRNA level raised again (Figure 4C). This phenomenon was consistent with the negative feedback regulation mechanism of *hsp70*.

HDI-induced histone H3 hyperacetylation enhanced transcription of hsp70 by RNA pol II

Experimental data presented above demonstrated that histone acetylation modification participated in the transcriptional regulation of *hsp70* gene, and it promoted the binding of HSF to HSE. Finally, we tested the influence of HDI-induced histone H3 hyperacetylation on the association of RNA pol II with *hsp70* gene by using ChIP assay with anti-pol II antibody. As expected, RNA pol II was detected at the proximal promoter and the 5′-transcribing region in non-induced larvae (Figure 5, S2 and S3, lane Crtl). After heat-shock and HDI treatments, an increase in the amount of RNA pol II recruited to the 5′-transcribing region was seen, and this could be detected through to the

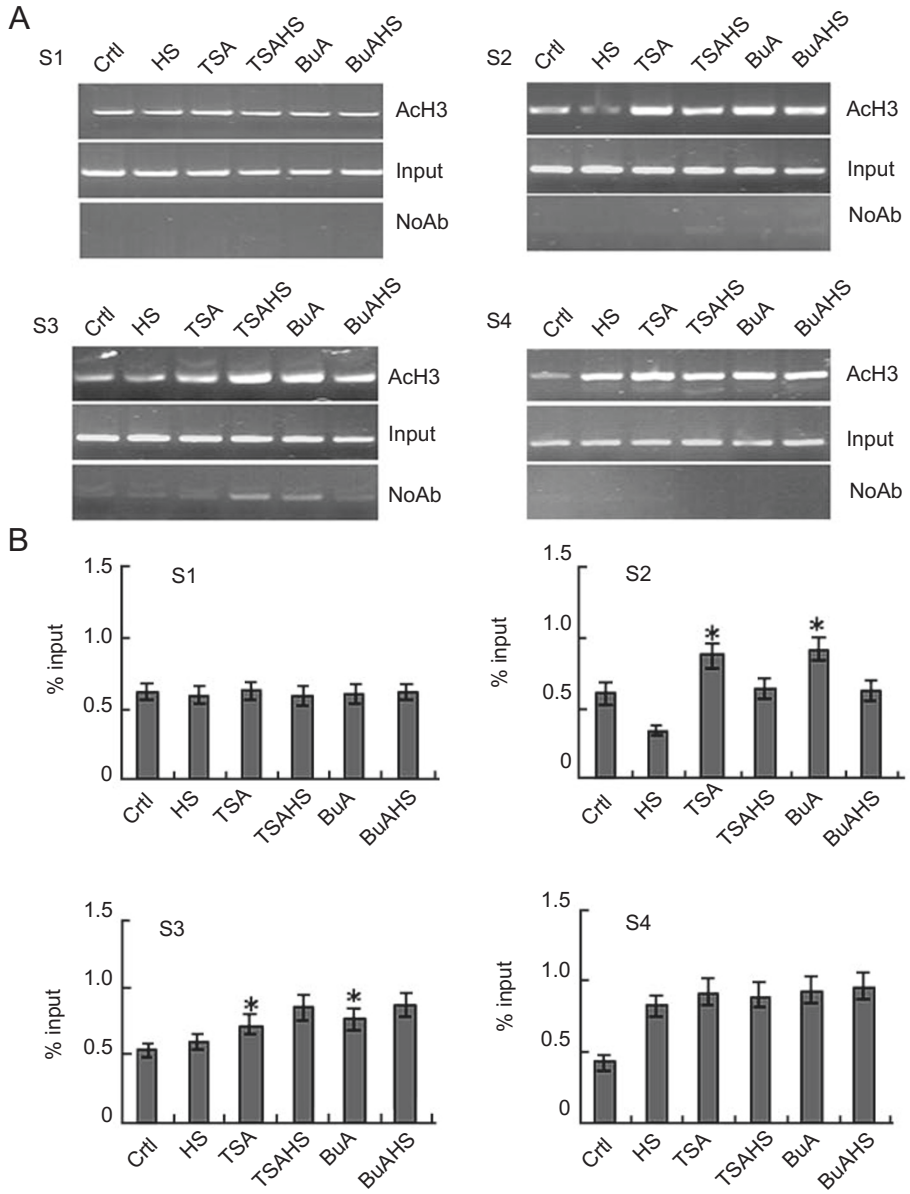

Figure 2 Influence of HDI and heat-shock treatments on acetylation level of histone H3 located at promoter and transcribing regions of *hsp70*. **(A)** Results of PCR analysis of the crosslinked and immunoprecipitated DNA on ethidium bromide (EB)-stained 2% agarose gels, corresponding to amplifications of the far upstream of the promoter (S1), the promoter (S2), the 5′-transcribing fragment (S3), and the 3'-transcribing fragment (S4) of *hsp70*, respectively. Larvae were treated as described in "Materials and methods". ChIP assays of the sonicated and crosslinked chromatin fragments were performed using antibody against AcH3 and pre-immune serum (NoAb). In PCR reactions, 1% of input DNA, which has been confirmed to lie within the linear range of amplification (**Figure 1C**), was used to determine the amounts of starting materials that were used for each precipitation. **(B)** Quantitative analysis of EB-stained bands of PCR products corresponding to S1, S2, S3, and S4, respectively. Values on the y-axis represent the amount of DNA immunoprecipitated as a percentage of total input DNA. Experiments were performed in triplicates, and standard deviations are shown. **P*<0.05 versus untreated control. CON, control without any treatment; HS, 37 °C heat shock induced for 30 min; TSA, 10 μM TSA treatment for 6 h; TSAHS, 10 μM TSA treatment for 6 h prior to 30 min 37 °C heat shock; BuA, 10 mM BuA treatment for 6 h; and BuAHS, 10 mM BuA treatment for 6 h prior to 30 min 37 °C heat shock.

3′-end of the gene (Figure 5, S3 and S4, lanes HS, TSA, TSAHS, BuA, and BuAHS). This implicated that the HDI-induced histone H3 hyperacetylation facilitated the recruitment of RNA pol II, and may stimulate transcriptional

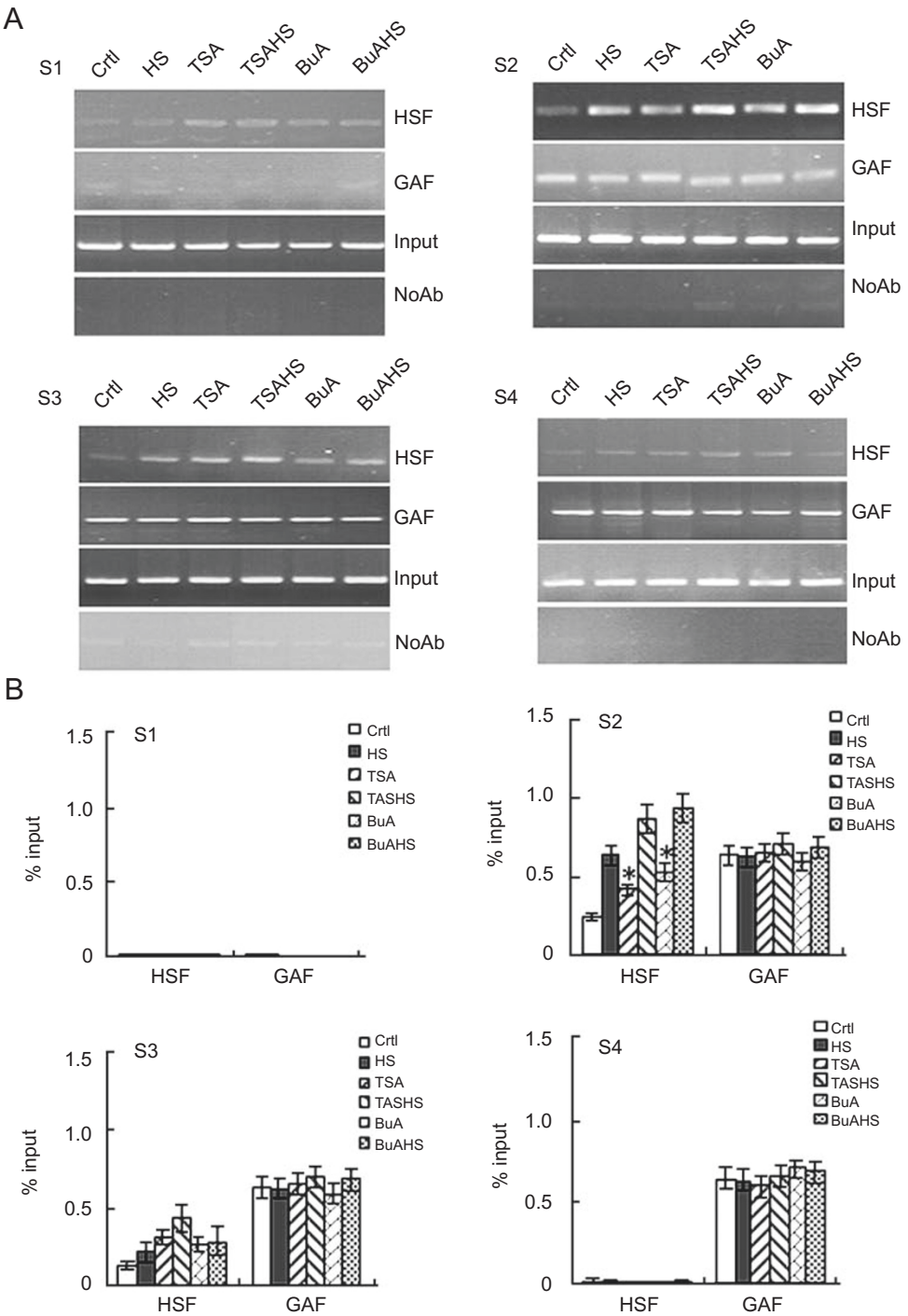

Figure 3 Influence of HDIs on binding of transcription factors to specific sequences. ChIP assays were performed as indicated in "Materials and Methods" with antibodies specific to HSF and GAF, as well as with pre-immune serum (NoAb). The immunoprecipitated DNA was analyzed for the presence of *hsp70* sequences (S1, far upstream of promoter; S2, promoter; S3, 5′-transcribing region; S4, 3′-transcribing region). **(A)** PCR analysis of crosslinked and immunoprecipitated DNA on EB-stained 2% agarose gels. **(B)** Quantitative analysis of EB-stained bands of PCR products. Methods for treating larvae and the amounts of input materials are as described in **Figure 2**. Experiments were performed in triplicates, and standard deviations are shown. **P*<0.05 versus untreated control. The abbreviations used are defined in **Figure 2**.

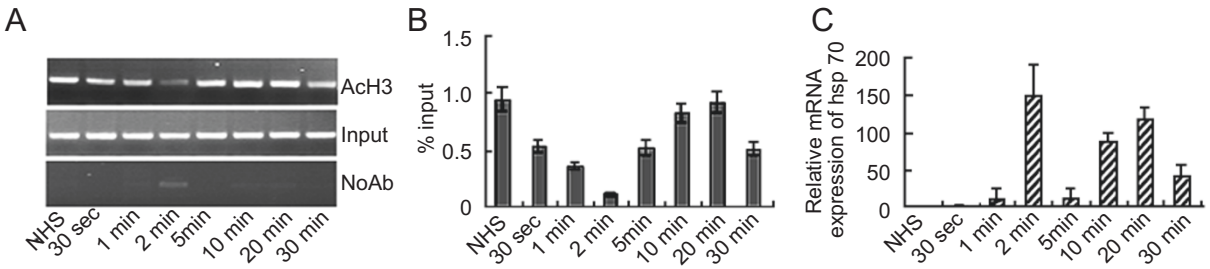

Figure 4 Changes in histone H3 acetylation level at *hsp70* promoter and in the inducible expression of *hsp70* following heat shock. **(A)** ChIP analysis with antibody specific to AcH3 on EB-stained agarose gels, and the results of quantitative analysis were shown in **(B)**. *Drosophila* larvae were heat induced at 37 ºC for 30 s, 1 min, 2 min, 5 min, 10 min, 20 min, and 30 min, respectively, before crosslinking and immunoprecipitation. The amounts of the input materials are as described in **Figure 2**. Experiments were performed in triplicates, and standard deviations are shown. **(C)** Results of real-time RT-PCR showing the expression of *hsp70* following heat shock. Total RNA was isolated from *Drosophila* larvae, which were either non-heat shocked or heat induced at 37 ºC for different time. Quantitative real-time RT-PCR was performed to determine the *hsp70* mRNA expression levels. The relative mRNA expression was defined by using the $2^{-\Delta\Delta C_t}$ method [20] to analyze the data. The real-time RT-PCR reactions were performed three times, and bars represent means ± S.D..

elongation through the entire *hsp70* gene. In addition, we isolated the total RNA from larvae with or without HDI treatments, and performed quantitative real-time PCR to analyze the *hsp70* mRNA expression. The results showed that both TSA and BuA were able to increase the basal and inducible transcription of *hsp70*, and HDI treatment and heat shock could act synergistically to raise the transcription intensity of *hsp70* (Figure 6). This result is in accordance with the above-mentioned changes in recruitment of RNA pol II on *hsp70* gene (Figure 5, S3 and S4). Data presented here clearly indicate that HDIs can enhance the basal and inducible expression of *hsp70* in *D. larvae*.

Discussion

The correlation between histone acetylation and hsp70 gene expression regulation

Distinct from the tissue-specific and housekeeping genes, *hsp70* is among a group of genes that are poised to transcribe but require an external stimulus to induce transcription. Binding sites for GAF, HSF, and other promoter elements are required to generate an accessible promoter structure in non-heat-shocked cells [21], and the binding of RNA pol II to the promoter determines the inducible expression of *hsp70* [22]. Heat shock causes the trimerization and activation of HSF, and rapidly promotes *hsp70* transcription. During these processes, whether the nucleosomal histone modifications occur around the *hsp70* promoter is still a disputed issue. Labrador and Corces [23] demonstrated that Gal 4-induced transcription of *hsp70* promoter-driven transgenes was associated with H3 hyperphosphorylation at Ser10 residue, but independent of the acetylation of H4 at Lys8 and H3 at Lys14. While studies in the mouse showed that histone acetylation was associated with *hsp70* induction, there has been evidence that H3 phosphorylation can be targeted to *hsp70* nucleosomes when the gene was not induced [24]. On the other hand, Smith *et al*. [25] indicated that a novel chromatin remodeling complex, TAC1, which is a component of histone methyltransferase Trx and histone acetyltransferase CBP, was recruited to *hsp70* locus after heat stress, and it modified nucleosomal histone H3 in the 5′-coding region of *hsp70*. Our results revealed that HDI treatments could specifically induce H3 hyperacetylation at *hsp70* locus (Figure 2), and enhanced the expression of *hsp70* (Figure 6). Moreover, HDI-induced H3 hyperacetylation increased the HSF binding to HSE (Figure 3), promoted the association of RNA pol II with 5'-coding region, and the downstream region of *hsp70* (Figure 5). These results suggested that histone acetylation modification plays an important role in both transcription initiation and elongation of the *hsp70* gene.

Alteration of chromatin structure at hsp70 promoter upon induction

Acetylation of lysine residues in core histone tails reduces the positive charge, weakening electrostatic interactions with DNA, and other nucleosomes [26, 27]. This modification affects the higher-order structure of chromatin, giving rise to a chromatin conformation that is more prone to the access to transcription factors [28, 29]. However, the extent to which *hsp70* promoter chromatin structure is remodeled upon transcriptional induction still remains unclear so far. Alexander and David found that the chromatin showed a dynamic alteration at the *hsp12*,

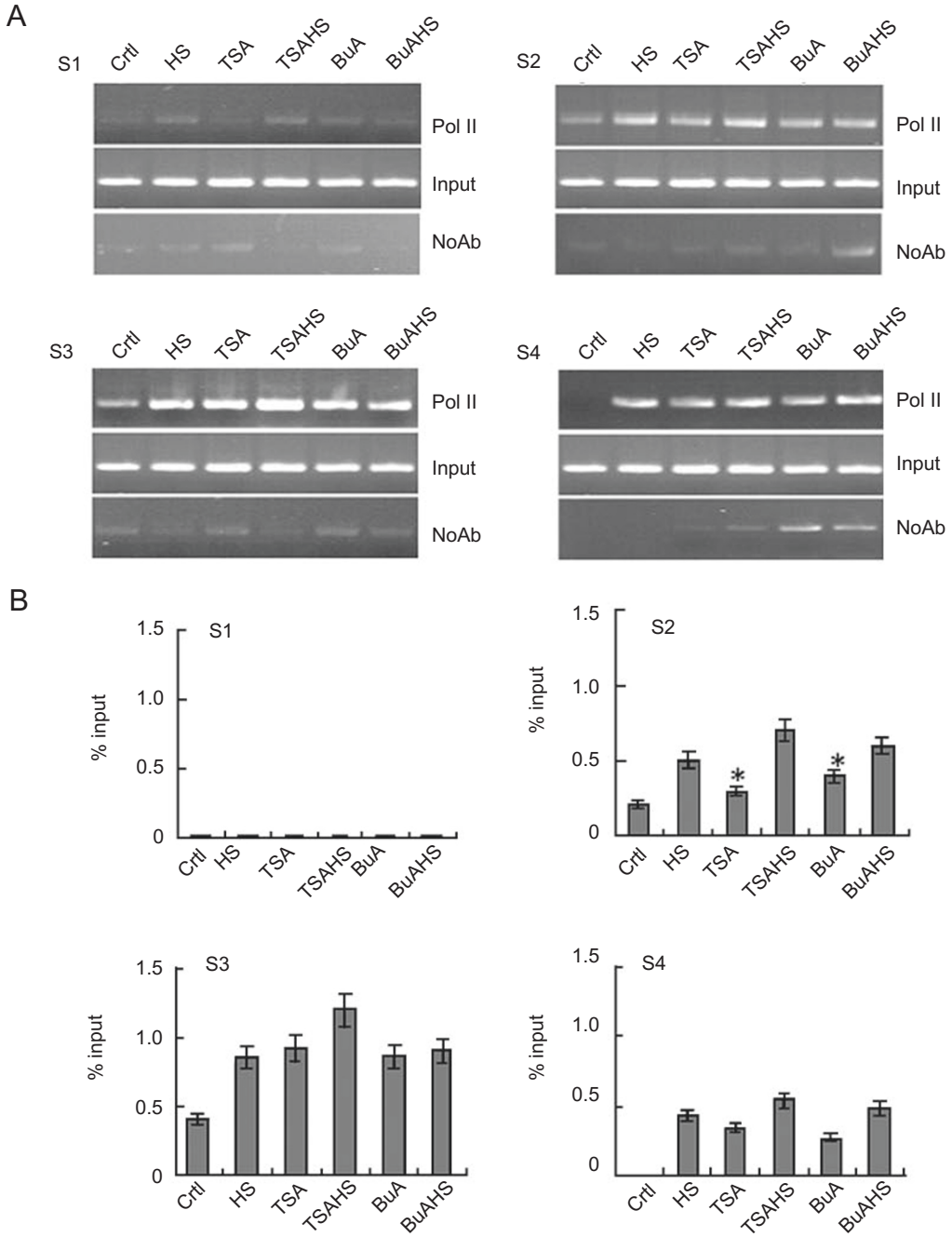

Figure 5 HDI-induced H3 hyperacetylation enhanced the transcription of *hsp70* by RNA pol II. **(A)** PCR analysis of the crosslinked and immunoprecipitated DNA on EB-stained agarose gels. **(B)** Quantitative analysis of EB-stained bands of PCR products. Panels S1, S2, S3, and S4 show the association of RNA pol II with the far upstream of the promoter (S1), the promoter (S2), the 5′ -transcribing fragment (S3), and the 3′ -transcribing fragment (S4) of *hsp70*, respectively. RNA pol II was only detected at the transcribing regions (S3 and S4), and after HDI and heat-shock treatments, the association of pol II increased. Methods for treating larvae and the amounts of the input materials are as described in **Figure 2**. Experiments were performed in triplicates, and standard deviations are shown. **P*<0.05 versus untreated control. The abbreviations used are defined in **Figure 2**.

hsp26, and *hsp82* promoters in *Saccharomyces cerevisiae* [30]. The results from this and our previous study [18] indicated that upon transcriptional induction, a significant chromatin alteration at the promoter of *hsp70* gene oc-

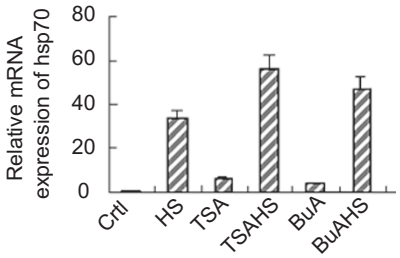

Figure 6 HDI treatments increased both the basal and the inducible expression of *hsp70* mRNA. Total RNA was isolated from *Drosophila* larvae, which were cultured and treated as described in "Materials and Methods". Quantitative real-time RT-PCR was performed to determine the *hsp70* mRNA expression levels. The relative mRNA expression was defined by using the $2^{-\Delta\Delta C_t}$ method [20] to analyze the data. The real-time RT-PCR reactions were performed three times, and bars represent means ± S.D.. The abbreviations used are defined in **Figure 2**.

curred in *Drosophila melanogaster*. As shown in Figure 4, the content of AcH3 was drastically reduced at *hsp70* promoter following a 2-min heat shock. These histone re-arrangements were dynamic, since after 20 min of heat shock, an increase of histone H3 acetylation was detected, conversely as the heat shock further prolonged, AcH3 level reduced again (Figure 4).

Under stress conditions, HSF was rapidly recruited to the *hsp70* promoter, and reached saturating levels after 75 s heat shock [14]. At the same time, the *hsp70* gene was quickly induced and reached an approximate 150-fold activation within 2 min (Figure 4C, HS, 2 min). On the other hand, the accumulation of induced HSP70 protein down-regulated *hsp70* gene as a repressive *trans*-acting factor; HSP may interact with the activated HSF to interfere with the interaction between HSF and HSE and further down-regulate transcription of *hsp70* gene [31]. Therefore, we speculate that when the larvae were heat shocked, the activated HSF began to bind to the HSEs and occupied the positions of nucleosomes along the gene, resulting in a declined AcH3 level at these positions, as revealed by the ChIP analysis in this study (Figure 4A and 4B, HS, 2 min). In the meantime, the *hsp70* mRNA was induced abundantly (Figure 4C, HS, 2 min). Along with the prolonged time of heat shock, a large quantity of HSP70 protein was accumulated and tended to bind to the activated HSF. This led to the reassembly of nucleosomes at the HSEs, which subsequently caused the resume of acetylation level of H3 (Figure 4A and 4B, HS, 5 min) [35], resulting in the decrease of hsp70 expression (Figuer 4C, HS, 5 min). It appears that a reverse correlation existed between histone content at *hsp70* promoter and the transcriptional activity of the gene.

HDI-induced histone H3 hyperacetylation facilitated the access of regulatory factors and RNA pol II

Acetylation modification of histone affects the association of transcription factors with the their specific regulatory elements; reversely, the bound transcription factors may recruit the histone acetyltransferase to the promoter and to modify histones [7, 32, 33]. In this report, we demonstrated that HDI-induced histone hyperacetylation increased the binding affinity of HSF, but not that of GAF, to chromatin template (Figure 3). GAF exerts its function at the level of chromatin structure, helping to maintain the promoter in an open conformation [21, 34, 35]. Unlike the transcription activator HSF, GAF cannot directly activate the transcription. It can actually disrupt higher-order structure of chromatin, and ensure the correct assembly of transcription machinery involving TFIID and pol II [36, 37]. Histone acetylation modification and GAF act at different points in the transcriptional process. It is possible that GAF disrupts the higher-order structure of chromatin and recruits histone acetyltransferases to facilitate the association of other transcription factors, as well as the basal transcription machinery to the promoter. Moreover, GAF was detected at the transcribing regions of *hsp70* gene (Figure 3, S3 and S4), implicating that GAF could be involved in remodeling chromatin structure downstream of the promoter. Meanwhile, HSF stimulates transcription by releasing the paused RNA pol II into elongation process and determines the expression intensity in response to temperature [38]. HSF can interact with TFIID, and this may change the interaction between TFIID and RNA pol II, allowing the paused RNA pol II to escape from the nucleosome barrier and embark into elongation [39]. The inducible transcription of *hsp70* is mainly dependent on the HSF recruitment, and HDI treatments increased the binding level of HSF and further enhanced the *hsp70* mRNA expression. In this study, we performed ChIP assay using anti-pol II antibody and the results showed that both heat-shock and HDI treatments were able to enhance the association of RNA pol II with the 5′-transcribed region (Figure 5, S3), and to promote the transcriptional elongation (Figure 5, S4). The elevated acetylation level may lead to the alteration of the nucleosome structure downstream of the promoter and offer an easier access of elongation factors to specific DNA sequences.

To conclude, information arising from this study support that the regulation of the *hsp70* gene is a complex mechanism, in which histone acetylation plays a critical role in both transcription initiation and elongation. Nevertheless, whether the other forms of histone modifications (e.g., histone phosphorylation and methylation, etc.) may also be involved in a precise transcription regulation of this gene, and their correlation with histone acetylation, are

yet to be established.

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