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Recruitment of the ATP-dependent chromatin remodeler dMi-2 to the transcribed region of active heat shock genes

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ABSTRACT

The ATP-dependent chromatin remodeler dMi-2 can play both positive and negative roles in gene transcription. Recently, we have shown that dMi-2 is recruited to the *hsp70* gene in a heat shock-dependent manner, and is required to achieve high transcript levels. Here, we use chromatin immunoprecipitation sequencing (ChIP-Seq) to identify other chromatin regions displaying increased dMi-2 binding upon heat shock and to characterize the distribution of dMi-2 over heat shock genes. We show that dMi-2 is recruited to the body of at least seven heat shock genes. Interestingly, dMi-2 binding extends several hundred base pairs beyond the polyadenylation site into the region where transcriptional termination occurs. We find that dMi-2 does not associate with the entire nucleosome-depleted *hsp70* locus 87A. Rather, dMi-2 binding is restricted to transcribed regions. Our results suggest that dMi-2 distribution over active heat shock genes are determined by transcriptional activity.

INTRODUCTION

ATP-dependent nucleosome remodelers are important facilitators of gene activity. They participate in nucleosome eviction and assembly, nucleosome mobilization and histone variant exchange (1). By doing so, they modulate the DNA access of RNA polymerase II (RNAP II) and transcriptions factors and generate conditions conducive for transcription.

Members of the CHD family of nucleosome remodelers co-localize with RNAP II and elongation factors on many active genes (2–6). CHD1 and Kismet have been suggested to support transcriptional elongation by remodelling nucleosome structure (4–6). This view is supported by the finding that yeast CHD1 physically interacts with several elongation factors on actively transcribed genes, including Paf1, Spt4-Spt5 and Spt16-Pob3 (4). Moreover, in *Drosophila*, loss of Kismet function results in reduced levels of elongating RNAP II, elongation factor SPT6 and CHD1 at many actively transcribed regions of polytene chromosomes (5,6). Here, Kismet acts at a step after pTEF-b recruitment, and is required for association of histone methyltransferases with active genes (6).

The molecular mechanisms of CHD remodeler recruitment to active genes are not well understood. Human CHD1 binds H3K4me3 via its double chromodomains *in vitro* (7,8). Accordingly, it has been suggested that this interaction is important for targeting CHD1, and associated factors, to active chromatin *in vivo* (8,9). However, deletion of the chromodomains of *Drosophila* CHD1 does not impact its association with actively transcribed regions of polytene chromosomes, suggesting that H3K4me3 recognition does not play a significant role in this case (2). In addition, Kismet chromodomains do not bind methylated histone peptides *in vitro* and Kismet association with polytene chromosomes is not affected by loss of histone methyltransferases Trx and Ash1 (6).

We have used *hsp70* heat shock genes as a model system to study by what parameters chromatin association of dMi-2 is governed. dMi-2 is recruited to heat shock-activated *hsp70* genes, and is required for their full activation in flies (10). dMi-2 appears to occupy several regions within the body of the *hsp70* gene. However, it is not known if dMi-2 covers the *hsp70* gene completely, if it is evenly distributed or displays preferences for the 5′- or 3′-ends.

Actively transcribed *hsp70* loci are extensively poly-ADP-ribosylated (11,12). Binding of dMi-2 to *hsp70* in S2 cells is reduced in the presence of a small

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molecule poly ADP ribose polymerase (PARP) inhibitor (10). In addition, dMi-2 binds to PAR *in vitro* and possesses several PAR-binding motifs suggesting that dMi-2 recruitment to *hsp70* involves a direct interaction with the PAR polymer (10). Moreover, dMi-2 binds nascent *hsp70* transcripts and can interact both with DNA and RNA *in vitro* (10,13). Based on these results, we have proposed that dMi-2 is initially recruited to the *hsp70* locus when this becomes PARylated shortly after heat shock (HS). Once transcription has been activated, dMi-2 engages with nascent transcripts. However, the relative contributions of PAR, DNA and RNA binding to dMi-2 chromatin association and distribution across genes are not well defined.

Histone PARylation within the *hsp70* locus is believed to contribute to the rapid nucleosome loss that occurs within the first 2 min of heat shock (11,12). Interestingly, nucleosome loss at *hsp70* loci is not restricted to the *hsp70* transcription units but extends several kilobases up- and down-stream. It is limited on either side by silencer elements (scs and scs′). Nucleosome depletion across the *hsp70* locus increases the access of RNAP II and transcription factors for DNA and their concerted action results in the production of thousands of *hsp70* RNA molecules per nucleus. It is currently not known if dMi-2 binding is elevated across the entire PARylated *hsp70* locus or if dMi-2 binding is restricted to those regions that are actively transcribed.

In addition to *hsp70*, the expression of two other HS genes (*hsp26* and *hsp83*) is affected in transgenic flies expressing reduced levels of dMi-2 (10). This raises the possibility that all HS genes require dMi-2 for full activation and that dMi-2 physically associates with other HS genes during the HS response.

In this study, we extend our analysis of HS-regulated dMi-2-chromatin interaction by addressing several key questions. First, we have used chromatin immunoprecipitation sequencing (ChIP-Seq) to obtain a high resolution, genome-wide dMi-2 binding profile in both untreated and heat-shocked S2 cells. Through this global approach, we have identified seven regions which exhibit strong, HS-induced enrichment of dMi-2 binding. In addition to *hsp70* genes, these regions harbour six additional HS genes. Inspection of ChIP-Seq profiles revealed that dMi-2 associates with the body of these HS genes. A more detailed analysis of dMi-2 distribution showed that dMi-2 binding closely follows nascent RNA production. Importantly, dMi-2 binding extends several hundred base pairs beyond polyadenylation sites into regions where transcriptional termination occurs. We have analysed dMi-2 binding within the PARylated *hsp70* locus 87A and find that dMi-2 recruitment is restricted to actively transcribed regions. These results suggest that RNA synthesis, rather than a general increase in DNA accessibility by PARylation and nucleosome depletion, determines the distribution of dMi-2 at active HS loci. However, ChIP-Seq and RT-qPCR analysis of dMi-2 binding to genes that are constitutively transcribed at high levels, and are induced by other forms of stress indicates that strong transcriptional activity is not sufficient to accumulate dMi-2. Together, our results allow us to identify transcription as the key parameter that determines the distribution of dMi-2 over active HS genes.

MATERIALS AND METHODS

S2 cell culture

Drosophila melanogaster S2 cells were maintained in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% FCS. Heat shock treatment of S2 cells was carried out as described before (10). To activate the metallothionein A gene, S2 cells were treated with 100 μM $CdCl_2$ for 90 min. In order to stably integrate an expression vector containing a cDNA under control of the *hsp70* promoter, S2 cells were co-transfected with pPuro and pCMep1 as described previously (13). pCMep1 was created by inserting a cDNA encoding dMep1 into pCMycDam (14).

ChIP

Chromatin immunoprecipitations were carried out, as described previously, using the dMi-2-C antibody and IgG as a control (10,15). Primer sequences are available upon request. Triplicate mean values of percentage input DNA and standard deviations are plotted.

ChIP-Seq

Sequencing libraries were prepared with the Illumina ChIP-Seq DNA Sample Prep kit according to Illumina's instructions. Libraries were sequenced on the Genome Analyzer IIx following the manufacturer's protocols and analysed with Bowtie [version 0.12.7, (16)] and MACS [version 1.4.0rc2, (17)]. Sequence reads (36 bp) were approximately counted and deduplicated using a bloom filter (collision probability 10^{-8}) and aligned to the *D. melanogaster* genome sequence provided by Ensembl revision 62 allowing at most two mismatches (−n 2) with a mismatch quality sum of 70 (−e 70) and restricting to exactly one mapped location (−m 1 −k 1). We considered at most three reads with identical starting position and strand direction in all downstream analysis for dMi-2, two for IgG. This lead to 9.532.789 aligned reads for the dMi2 heat-shocked sample, 8.756.412 for the non-heat-shocked sample and 2.610.348 reads for the IgG controls pooled after sequencing.

Peak calling was performed separately for both conditions against the pooled IgG background with MACS default parameters except for 'mfold', which was set to 6–30. The resulting genomic regions (HS: 9485, NHS 6644) were combined, with overlapping regions merged into one, yielding 9386 putative dMi-2 binding regions. To assess differential enrichment independent of whether MACS identified a peak in both data sets, we utilized DESeq (18) with the size parameter set to the number of aligned reads. A region was assigned to just the condition with the higher read count if DESeq reported an adjusted $P \leq 0.05$.

RNA isolation and RT-qPCR

Total RNA was isolated from S2 cells with 1 ml of TRizol (Life Technologies) as described by the manufacturer. One microgram of RNA was reverse transcribed by incubation with 0.3 µg of random primers (Invitrogen) and 100 U of M-MLV reverse transcriptase (Invitrogen). cDNA synthesis was performed according to the manufacturer's protocol. cDNA was analysed by qPCR using Absolute SybrGreen Mix (Thermo Fisher) and the Mx3000P real-time detection system (Agilent). The primer sequences used in RT-qPCR are available on request. All amplifications were performed in triplicate using 0.6 µl of cDNA per reaction. Triplicate mean values were calculated according to the $\Delta\Delta C_t$ quantification method using *rp49* gene transcription as reference for normalization. Relative mRNA levels in untreated S2 cells were set to 1 and other values were expressed relative to this.

RESULTS

Heat shock does not result in a major redistribution of dMi-2

During HS, RNAP II and elongation factors loose association with many actively transcribed genes and strongly accumulate at several HS loci (19–22). As a consequence, expression of many genes is down-regulated, whereas transcription of HS genes is strongly stimulated. This dramatic redistribution of RNAP II and elongation factors can be visualized by indirect immunofluorescence of polytene chromosomes. An antibody recognizing the elongating form of RNAP II (RNAP II^{ser2P}) stains more than 100 transcriptionally active regions of polytene chromosomes at normal temperatures (Figure 1A, upper panel). In contrast, less than 10 strongly staining regions are revealed by the same antibody on polytene chromosomes after 20 min of HS (Figure 1A, lower panel). These regions include the *hsp70* containing 87 A and 87C loci.

We have previously shown that the ATP-dependent nucleosome remodeler dMi-2 accumulates at these loci during HS (10). Visually comparing the banding patterns produced by a dMi-2-specific antibody on control and HS-treated polytene chromosomes showed a general decrease in dMi-2 signal intensity in some polytene chromosome preparations (Figure 1B, compare upper and lower panels). However, we did not observe a dramatic decrease in the number of dMi-2 bands comparable to that observed for RNAP II. Nevertheless, strong dMi-2 binding to the activated 87A and 87C loci was clearly visible [Figure 1B; (10)]. However, this assay is hampered by low resolution and loss of dMi-2 binding from many sites might be easily overlooked. In addition, it is difficult to accurately quantify a potential reduction of dMi-2 binding to individual bands.

We, therefore, used ChIP-Seq to determine genome-wide binding patterns of dMi-2 in S2 cells at high resolution. Chromatin from cells that were subjected to a 20 min HS and from cells that were kept under normal conditions (NHS) was immunoprecipitated with a dMi-2-specific antibody (15). We have previously demonstrated that this antibody detects the HS-dependent association of dMi-2 with *hsp70* genes in Kc cells in ChIP assays (10). Recovered DNA was sequenced on an Illumina Genome Analyzer platform and the resulting reads were aligned to the *Drosophila* genome. We, thus, created two genome-wide maps containing 8.7 (NHS) and 9.5 (HS) million aligned reads (see 'Materials and Methods' section).

dMi-2 peaks were called, and we compared the high-resolution NHS and HS dMi-2 ChIP-Seq binding profiles (see 'Materials and Methods' section). The vast majority of dMi-2 binding sites was identified in both samples: of 9378 dMi-2 binding sites determined in NHS cells, 9361 were also detected in HS cells (99.8% overlap). Taken together, these results suggest that HS does not result in a dramatic redistribution of global dMi-2 chromatin association.

Next, we sought to identify chromatin regions displaying a significant HS-dependent increase in dMi-2 binding. We found seven regions, ranging in length from 1 to 9 kb that exhibited a 3.3- to 10.6-fold increase in dMi-2 binding in HS-treated cells (Figure 2A).

Regions 1 and 2 displayed the strongest increase in dMi-2 binding (10.6- and 9.6-fold, respectively). Both map within the 87A locus which contains two *hsp70* genes (Figure 2B). (Note that because the *Drosophila* genome contains six *hsp70* gene copies, reads corresponding to regions that are common to several *hsp70* genes were not aligned in this initial analysis. Therefore, only reads mapping to unique *hsp70* gene sequences are visible.) Region 3 localized to the 87C locus which harbours three *hsp70* genes. These ChIP-Seq results confirm our immunofluorescence analysis of polytene chromosomes [Figure 1B; (10)]. They also show that dMi-2 is not only recruited to *hsp70* loci but to several additional regions.

dMi-2 associates with seven HS genes

We next analysed which genes are contained in regions 4–7 (Figure 2B). Regions 4 and 5 are located within 8 kb of each other. They each contain two *hsp* genes: region 4 harbours *hsp23* and *hsp27* (note that the 3′ portion of *hsp27* has been filtered out due to the presence of repetitive sequences), region 5 encompassed *hsp22* and *hsp26*. All four of these HS genes display an increase in dMi-2 association upon activation. *CG4461*, a non-HS gene located between *hsp22* and *hsp26* did not show significantly increased dMi-2 binding upon HS. Finally, regions 6 and 7 represented the HS genes *hsp68* and *hsp83*, respectively.

Recently, Guertin *et al.* (23) have used ChIP-Seq to identify heat shock factor (HSF) binding sites in S2 cells. They have demonstrated HS-dependent HSF binding to the promoters of *hsp70, hsp68, hsp27, hsp26, hsp23* and *hsp22*. All of these genes displayed HS-dependent dMi-2 recruitment in our ChIP-Seq analysis (Figure 2B). In addition, HSF is recruited to the promoters of *CG3884* and *CG6770*, and these genes are

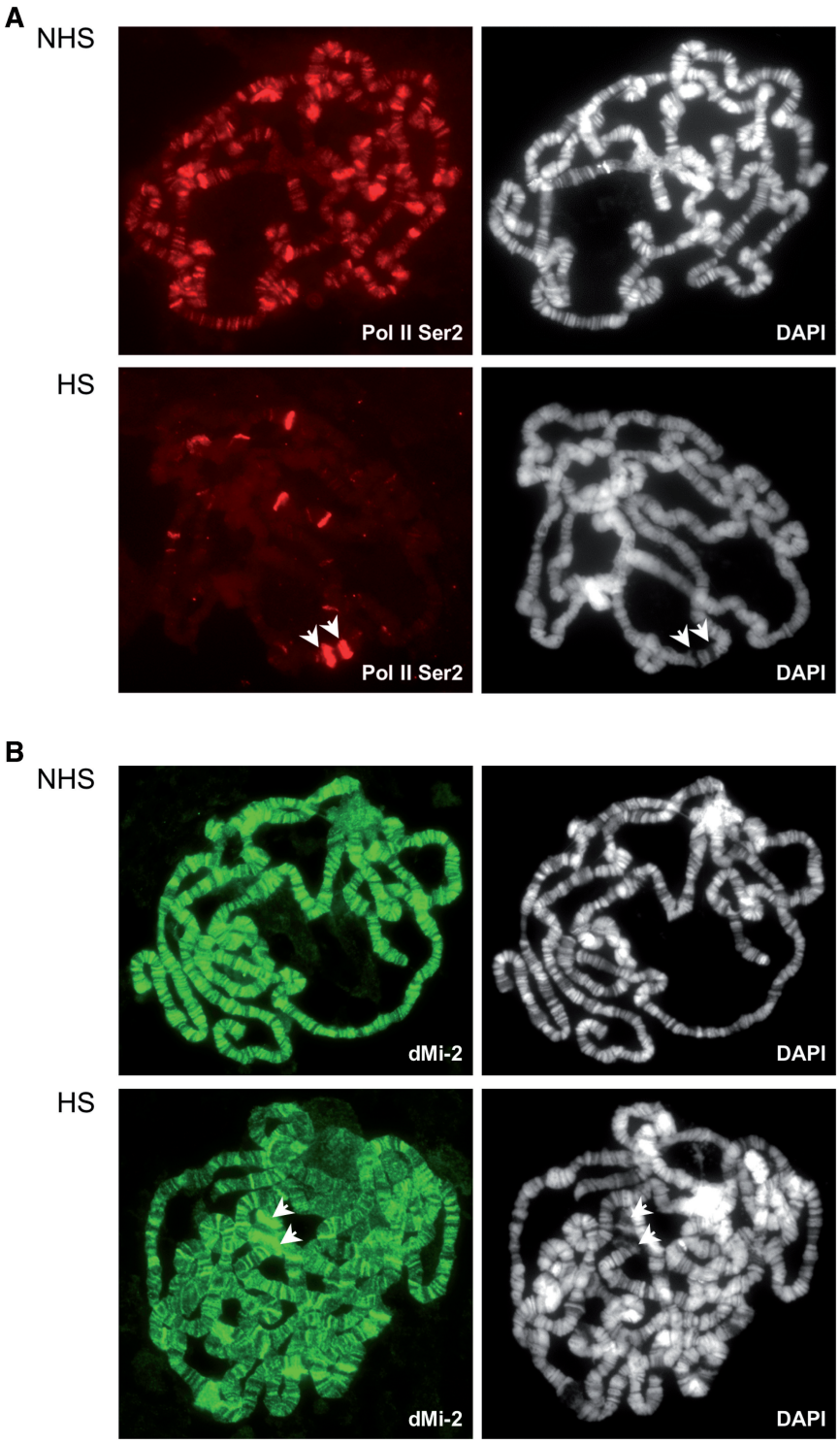
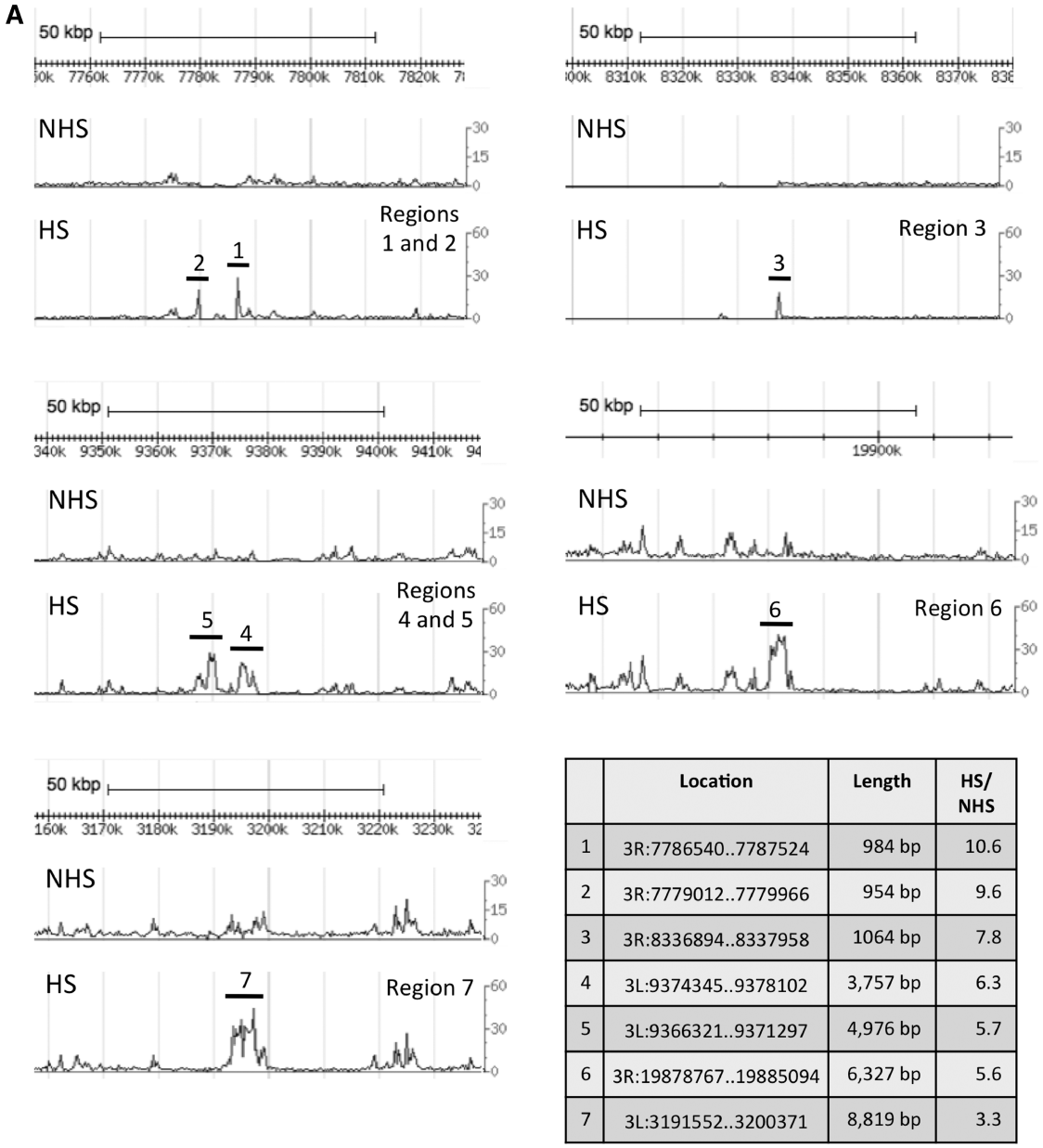


Figure 1. Redistribution of RNA polymerase II and dMi-2 on polytene chromosomes after heat shock. Immunofluorescence staining of polytene chromosomes with (**A**) RNA polymerase II (pol IIser2) antibody and DAPI and (**B**) dMi-2 antibody and DAPI. (Upper panels) no heat shock (NHS); (lower panels) heat shock (HS) conditions. Position of *hsp70* loci 87A and 87C are indicated by arrows.

transcriptionally upregulated approximately 10-fold after a 20 min HS (23). However, we did not detect a significant HS-dependent increase in dMi-2 binding to these genes (Supplementary Figure S1). This argues that while dMi-2 associates with many active HS genes, HSF binding and HS-dependent transcriptional activation is not sufficient for dMi-2 recruitment. We conclude that, in addition to being recruited to *hsp70* genes, dMi-2 binding to six additional HS genes is significantly enhanced upon HS.



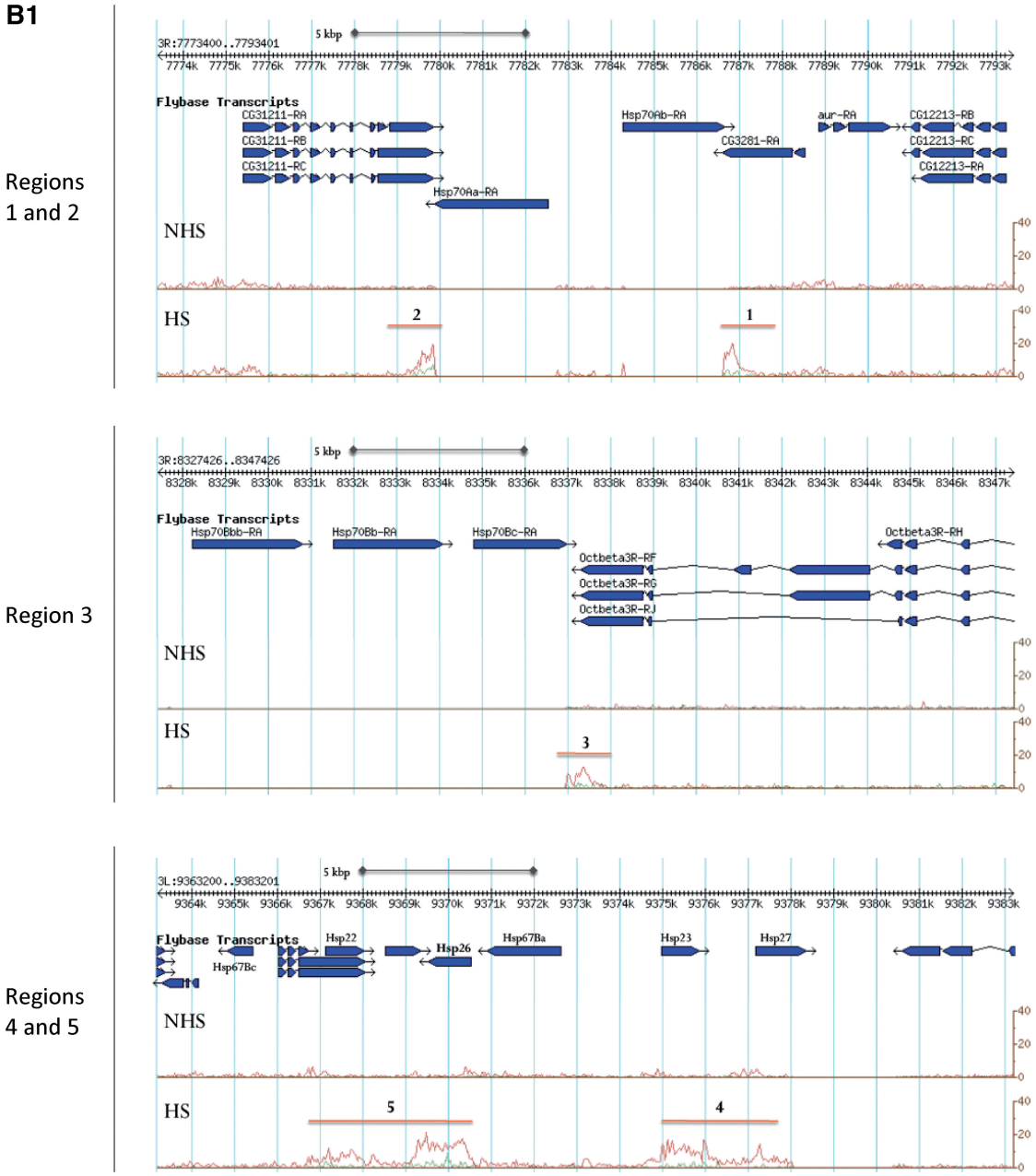
	Location	Length	HS/ NHS
1	3R:7786540..7787524	984 bp	10.6
2	3R:7779012..7779966	954 bp	9.6
3	3R:8336894..8337958	1064 bp	7.8
4	3L:9374345..9378102	3,757 bp	6.3
5	3L:9366321..9371297	4,976 bp	5.7
6	3R:19878767..19885094	6,327 bp	5.6
7	3L:3191552..3200371	8,819 bp	3.3

Figure 2. Seven chromosomal regions display increased dMi-2 binding after heat shock. (**A**) ChIP-Seq identified seven regions which displayed increased dMi-2 binding after heat shock. Chromosomal locations, lengths and fold-enrichment (HS/NHS) are given in the table. Genome browser images of dMi-2 ChIP-Seq tracks from control (NHS) and heat-shocked (HS) cells are shown. Reads are displayed as coverage per base pair (*Y* axis). (**B**) All seven regions are close to or overlap with known heat shock genes. Genome browser images of dMi-2 (red) and IgG (green) ChIP-Seq tracks from control (NHS) and heat-shocked (HS) cells are shown. Reads are displayed as coverage per base pair (*Y* axis). (**C**) Validation of ChIP-Seq data by ChIP-qPCR. Regions of *hsp70*, *hsp26* and *CG3884* that were analysed by dMi-2 and IgG ChIP-qPCR are indicated below the ChIP-Seq profile which is reproduced for comparison. Error bars denote standard deviations from three (*hsp70*) or two (*hsp26, CG3884*) biological replicates.

dMi-2 binds to the bodies of transcribed HS genes

During HS, dMi-2 association with several tested sequences between the transcriptional start (TSS) and polyadenylation site of *hsp70* is enhanced (10). In contrast, no increased binding to the *hsp70* promoter has been detected (10). To assess dMi-2 binding over other HS genes, we analysed the high-resolution ChIP-Seq profile. For each HS gene analysed, it was evident that dMi-2 binding spanned the gene body (Figure 2B). The activated HS genes analysed displayed a weak increase in read density when IgG was used instead of dMi-2 antibody (Figure 2B). We attribute this to a preferential release of small DNA fragments from transcriptionally active regions (24).

We validated the ChIP-Seq results by ChIP-qPCR for the *hsp70, hsp26* and *CG3884* genes (Figure 2C). In agreement with the ChIP-Seq results, HS-dependent dMi-2 binding to the transcribed portions, but not the promoter regions, of *hsp70* and *hsp26* was evident. Importantly, no such HS-dependent increase was



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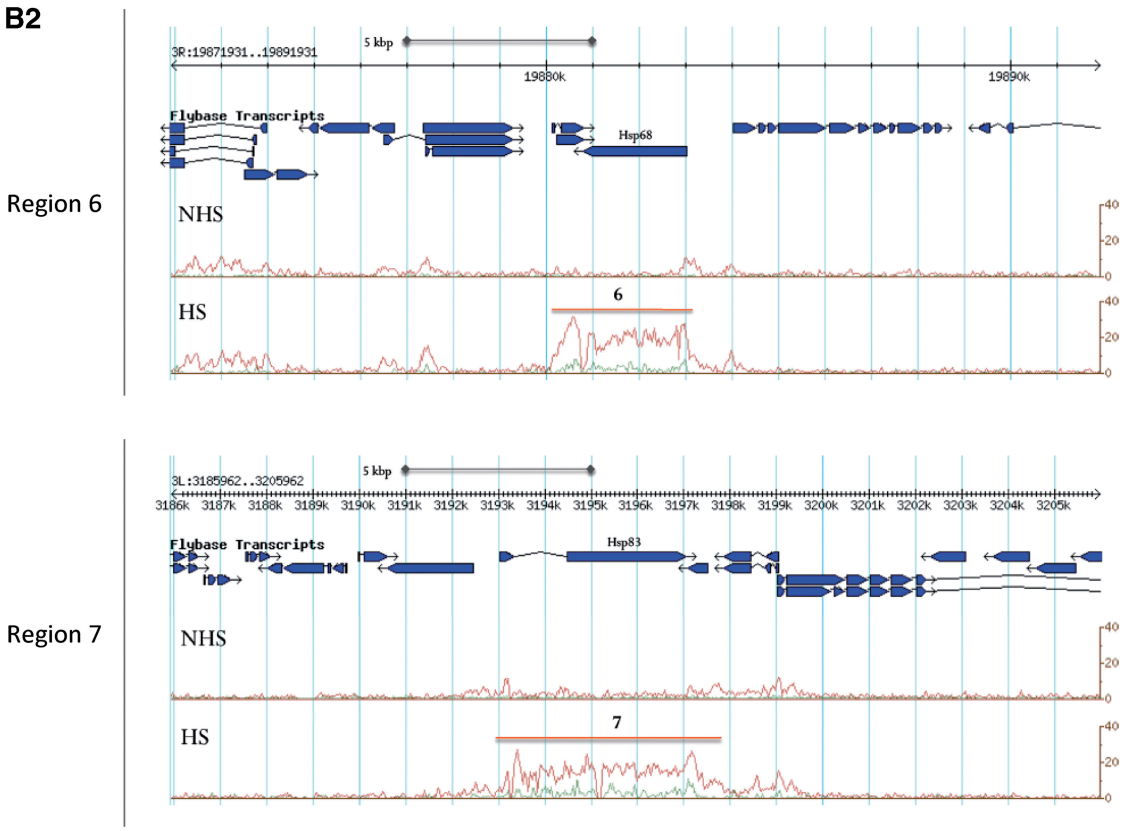
detected when IgG instead of dMi-2 antibody was used demonstrating that the increased dMi-2 ChIP signals reflect dMi-2 binding to chromatin rather than transcriptional activity. Moreover, again in agreement with the ChIP-Seq results, we did not detect dMi-2 recruitment to the *CG3884* gene.

To better characterize the distribution of dMi-2 over active HS genes, we considered promoter regions (defined as 500-bp upstream of TSSs), the 5′ and 3′ halves of the transcribed regions and a 500-bp region downstream of the annotated polyadenylation site separately and determined the relative enrichment of dMi-2 binding upon HS (Figure 3A). As expected, we detected no significant enrichment of dMi-2 binding at genes such as GAPDH1, that are not activated by heat shock, irrespective of which region was analysed (enrichment factors below 1.0). In contrast, strong enrichment was detected within both halves of the transcribed regions of HS genes. In four out of five HS genes, dMi-2 binding was more strongly enriched within the 3′ half than the 5′ half of the gene (*hsp22*: 4- versus 5-fold, *hsp23:* 12- versus 16-fold, *hsp26:* 4- versus 9-fold, *hsp27:* 4- versus 4-fold, *hsp68:* 7- versus 12-fold). There was no strong recruitment to the promoter regions of the six HS genes analysed (enrichment factors ranged from 1.2- to 1.7-fold). Surprisingly, however, our analysis revealed strong



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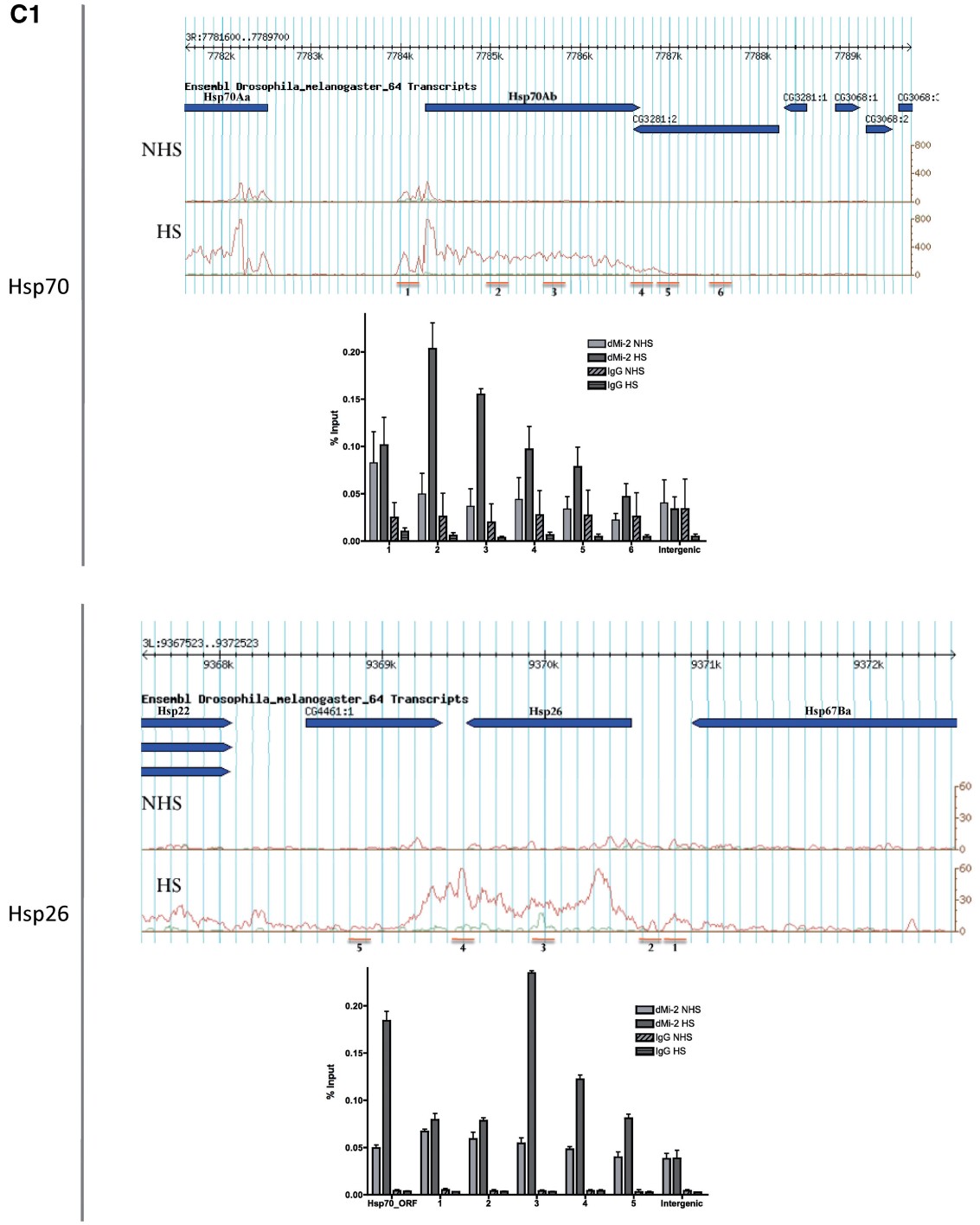
enrichment in the 500-bp region downstream of the annotated polyadenylation sites (*hsp22:* 6-fold, *hsp23:* 11-fold, *hsp26:* 6-fold; *hsp68:* 11-fold). We confirmed the positions of annotated polyadenylation sites by comparing them to modENCODE RNA-seq data obtained from S2 cells (modENCODE RNA-Seq 983 untreated S2-DSRC, data not shown). Enrichment factors for the regions downstream of the polyadenylation sites were lower than those for the 3′ halves and similar or higher than those for the 5′ halves of HS genes.

Visual inspection of the ChIP-Seq profiles revealed that dMi-2 binding was continuous along the body of the HS genes. However, three HS genes displayed a strong decrease in dMi-2 binding near or at the poyadenylation site followed by an dMi-2 peak beyond the polyadenylation signal [Figure 2B*; hsp70Bc* (Region 3), *hsp22* (Region 5), *hsp68* (Region 6)]. In the case of *hsp68*, this downstream dMi-2 peak encompassed the gene *CG6000*, which is transcribed in the opposite orientation to *hsp68*. To test if transcription of *CG6000* is activated upon heat shock, we performed RT-qPCR (Figure 3B). Direct comparison of *hsp68* and *CG6000* transcription revealed that whereas *hsp68* transcription is activated more than 200-fold upon heat shock, *CG6000* transcript levels are not significantly changed. This suggests that dMi-2 recruitment to the region downstream of the *hsp68* polyadenylation site is a consequence of increased *hsp68* transcription rather than an increase in *CG6000* transcription.

In summary, we conclude that dMi-2 binding extends beyond the polyadenylation site of activated HS genes into the region where transcription termination takes place.

Association of dMi-2 with the decondensed 87A locus

Upon HS, polytene chromatin at the two loci (87A and 87C), which together contain six copies of the *hsp70* gene, undergoes dramatic structural changes that culminate in the formation of decondensed transcriptional puffs (Figure 1A). The 87A locus contains the *hsp70Aa* and *hsp70Ab* genes, and is flanked by two insulator sequences, scs and scs′ (Figure 4). These insulators prevent the spreading of chromatin decondensation into neighbouring regions. In addition, the rapid loss of nucleosomes that occurs within the first 2 min after HS is confined to the region between scs and scs′ (11). We sought to analyse dMi-2 binding to the entire 87A locus to determine how locus-wide chromatin alterations influence dMi-2 binding. During the initial alignment procedure, ChIP-Seq reads mapping to identical regions of the six *hsp70* genes were automatically discarded as repetitive sequences. To allow analysis, we assumed that all six *hsp70* genes would bind dMi-2 to the same extent and distributed all reads equally among the six gene copies. This revealed that increased dMi-2 binding was limited to the bodies of the divergently transcribed *hsp70Aa* and *hsp70Ab* genes. No increase of dMi-2 binding to *CG31211* and *CG3281*, two transcribed genes, which lie within the 87A locus but are not



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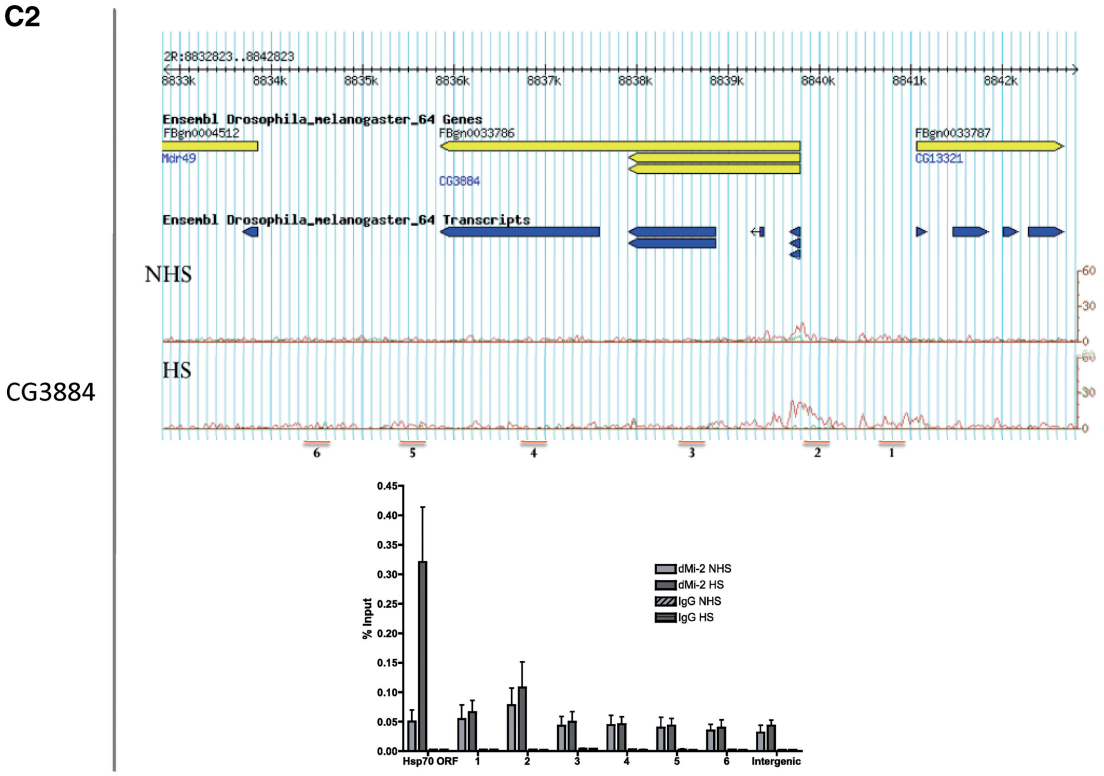
upregulated upon HS was detected. Also, in agreement with previous results, dMi-2 was not recruited to the promoter regions of *hsp70Aa* and *hsp70Ab* (10). As was the case for other activated HS genes, the region of increased dMi-2 binding extended several hundred base pairs beyond the polyadenylation sequence. Taken together, these results suggest that, even within a region that exhibits general chromatin decondensation and nucleosome depletion, dMi-2 recruitment is confined to the transcribed part of activated HS genes.

Strong transcription is not sufficient for dMi-2 recruitment

We considered the possibility that dMi-2 accumulation at active HS genes was mostly driven by their high level of RNAP II transcription. We asked if dMi-2 would associate with the bodies of other highly transcribed genes. We, therefore, studied dMi-2 interaction with two other classes of genes—constitutively active genes encoding ribosomal proteins and induced metallothionein genes. We compared



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dMi-2 binding to the transcribed regions of 34 genes encoding protein subunits of the 40S ribosome (RpS gene family) with adjacent regions of equal length (5′ and 3′). There was no significant enrichment of dMi-2 binding to the body of the highly transcribed RpS genes compared to their surrounding regions (Supplementary Figure S2). To rule out the possibility that dMi-2 would associate with RpS genes under HS conditions, we also performed this analysis using ChIP-Seq data obtained from HS-treated cells (Supplementary Figure S2). However, these data also did not reveal increased dMi-2 binding to RpS genes. We conclude that dMi-2 is not enriched at RpS genes, despite the fact that these genes are strongly transcribed.

Like HS genes, the metallothionein genes are strongly induced under stress conditions. Addition of Cd^{+} resulted in a 25-fold activation of MtnA transcription (Figure 5A, upper panel). We used ChIP followed by qPCR to assess dMi-2 binding to the transcribed part of the MtnA gene under non-induced and induced conditions. dMi-2 binding to the MtnA gene was not significantly altered following its activation (Figure 5B, lower panel). Taken together, these results indicate that neither strong constitutive transcription nor the strong induction of an inactive gene is sufficient to recruit dMi-2. Recruitment of dMi-2 to HS genes requires, therefore, additional signals.

We finally tested if strong transcription driven by a HS gene promoter would be sufficient for dMi-2 recruitment. We made use of a cell line containing a stably integrated expression vector. This vector drives expression of an *Escherichia coli* Dam methylase fusion protein under control of the *hsp70* HS gene promoter. Transcription of this cDNA was increased 200-fold following exposure of cells to HS (Figure 5B). However, no recruitment of dMi-2 to promoter or transcribed region was detected by ChIP-qPCR. We conclude that strong transcription driven by the *hsp70* promoter is not a sufficient signal for dMi-2 recruitment. Clearly, other features of the endogenous *hsp70* loci that are absent from the reporter gene studied must be required to attract dMi-2 upon HS.

DISCUSSION

We have previously shown that the ATP-dependent nucleosome remodeler dMi-2 is recruited to the *hsp70* heat shock gene following its activation by temperature shift. In the current study, we use a genome-wide ChIP-Seq approach to identify six additional HS genes, which likewise display a heat shock-dependent increase in dMi-2 binding.

Remarkably, in all cases, dMi-2 binding was found to extend across the body of the gene. Specifically, we did not detect significant dMi-2 recruitment to the promoter regions of heat shock genes, which harbour binding sites for the HSF and GAGA factor. Instead, dMi-2 is recruited to a region starting at the TSS and extending several hundred base pairs beyond the polyadenylation site.

Transcription by RNAP II continues past the polyadenylation site until transcription is terminated at one of multiple positions downstream. Termination sites for the *hsp26* gene have been mapped by $KMnO_4$

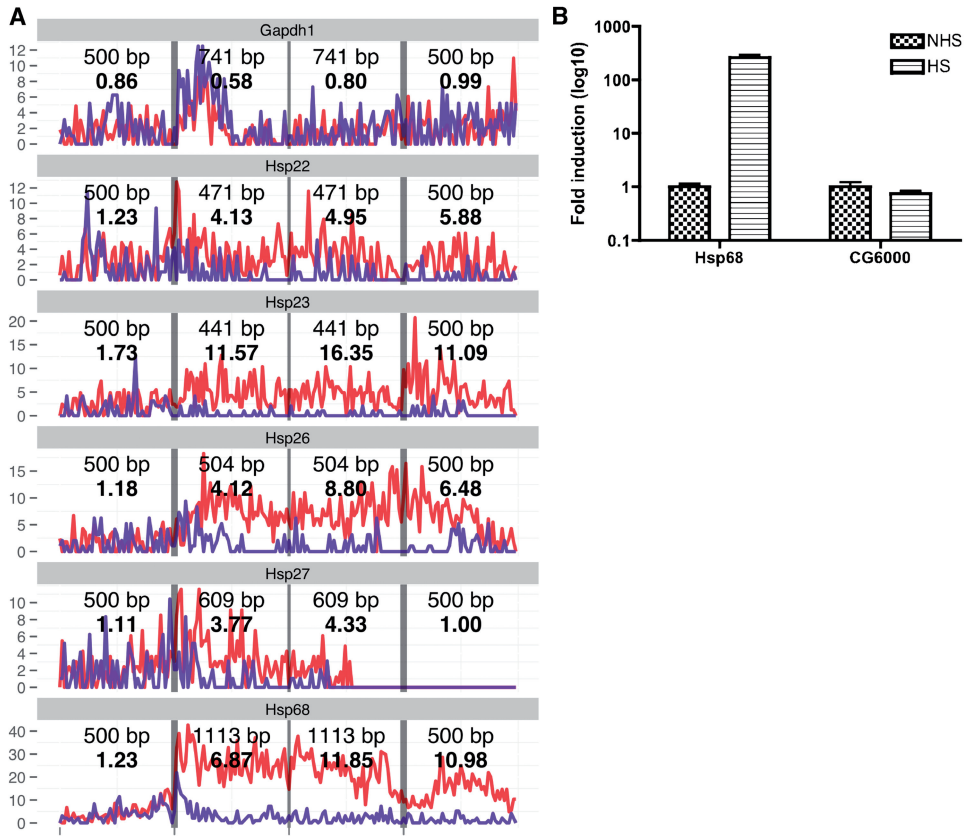

Figure 3. Distribution of dMi-2 over the bodies of GAPDH and six heat shock genes. (**A**) dMi-2 binding levels under control (blue lines) and heat shock (red lines) conditions. Genes are divided up into 500-bp upstream of the transcriptional start site, the 5′ and 3′ halves of the transcribed part and 500-bp downstream of the polyadenylation sequence. Numbers in bold fold enrichment of dMi-2 binding to indicated regions under HS versus NHS conditions. Reads were shifted 95-bp downstream to the approximate binding site (estimated from fragment lengths via MACS) and binned into 50 bins per subregion. *Y* axis displays bin read count normalized to one million reads. For details see text. (**B**) RT-qPCR analysis of *hsp68* and *CG6000* expression in control (NHS) and heat-shocked (HS) cells. Values are expressed relative to the value in control cells.

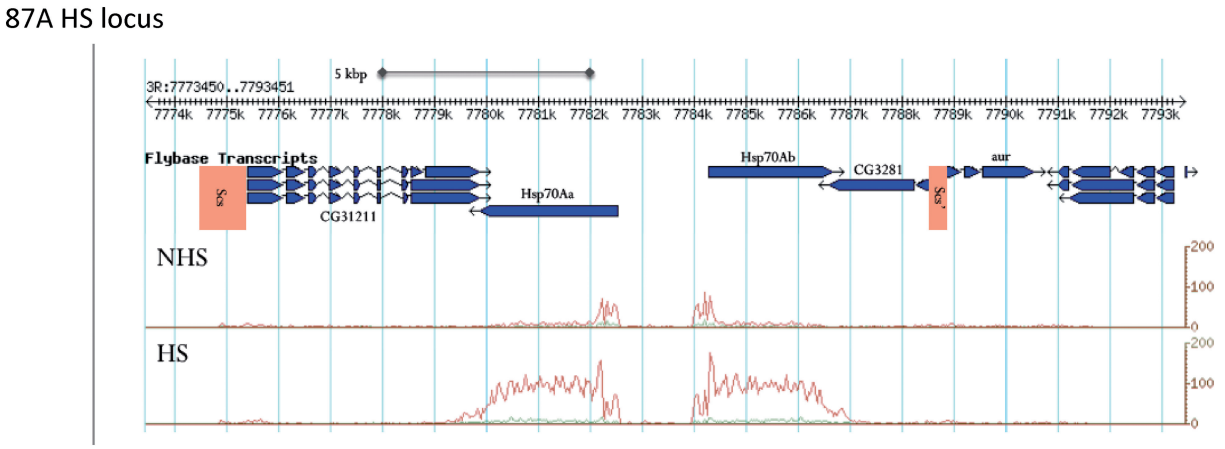

Figure 4. Heat shock-dependent dMi-2 association is restricted to the transcribed part of the *hsp70* locus 87A. Genome browser view of dMi-2 (red) and IgG (green) ChIP-Seq tracks from control (NHS) and heat-shocked (HS) cells across the *hsp70* locus 87A are shown. The positions of the silencers *scs* and *scs′* which flank the locus are indicated. Reads are displayed as coverage per base pair (*Y* axis).

hypersensitive site mapping (25). The furthest detectable hypersensitive site was located at a distance of 526 bp from the polyadenylation site. Increased dMi-2 binding to the active *hsp26* gene can be detected ~300-bp downstream of the polyadenylation site (Figure 2B). Thus, the dMi-2 bound region lies within the region that is transcribed by RNAP II.

dMi-2 binds nascent *hsp70* and *hsp83* transcripts *in vivo* (10). We hypothesize that this interaction of dMi-2 with nascent transcripts governs the distribution of dMi-2 over

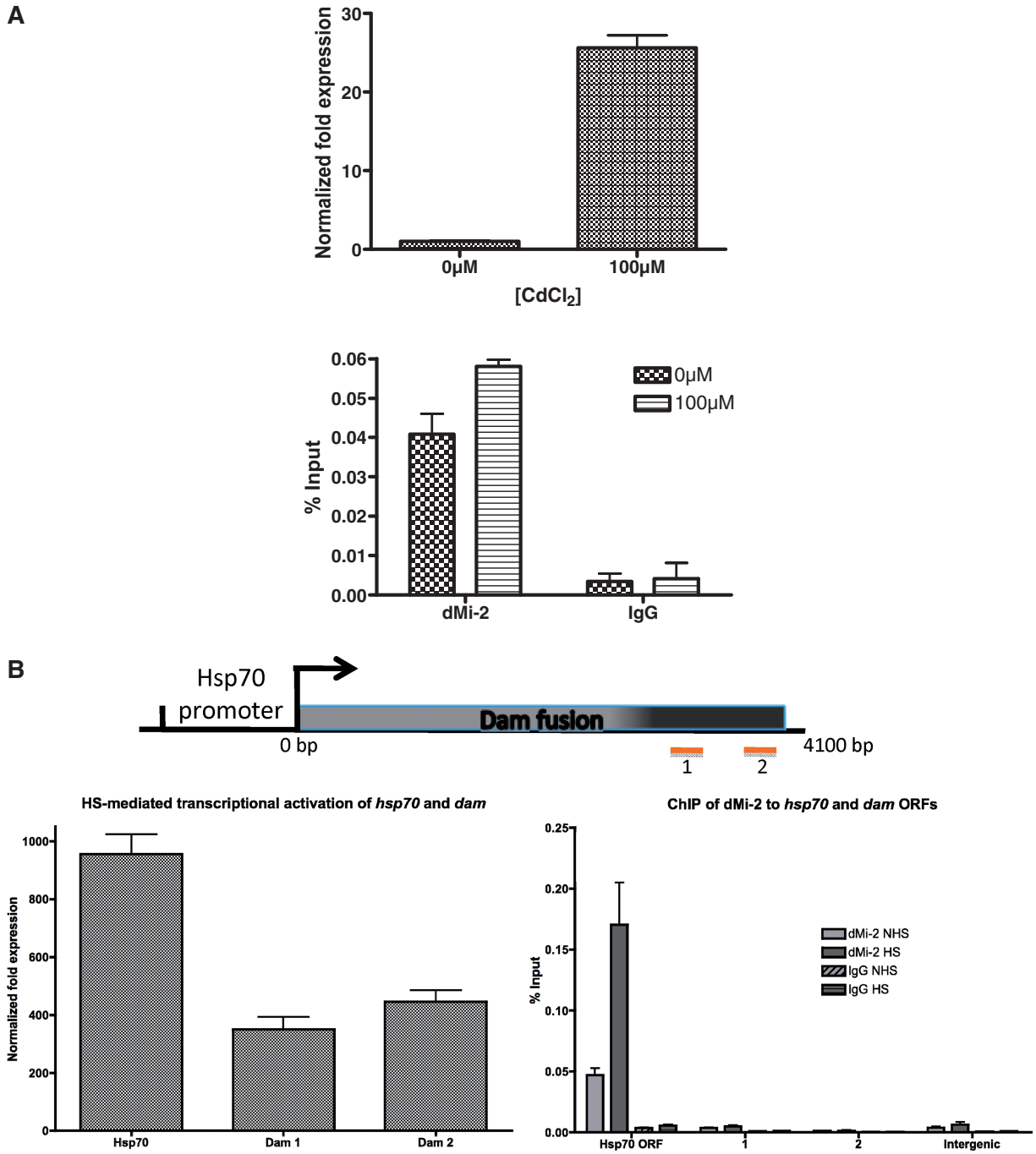

Figure 5. dMi-2 does not associate with other stress-induced genes. (**A**) *Upper panel*: RT-qPCR analysis of *MtnA* expression in control cells (0 μM) and cells treated with $CdCl_2$ (100 μM). Values are expressed relative to the value in control cells. (Lower panel) ChIP analyses of dMi-2 binding to the *MtnA* gene in control cells (0 μM) and cells treated with $CdCl_2$ (100 μM). IgG was used as a negative control. Error bars denote standard deviations from triplicate samples. (**B**) (Upper panel) schematic representation of Dam fusion cDNA under control of hsp70 promoter. Regions analysed by RT-qPCR and ChIP-qPCR are indicated. (Left) HS-induced transcriptional activation of endogenous *hsp70* genes and *hsp70-Dam fusion* reporter gene were determined by RT-qPCR and are displayed as fold expression (expression level under NHS conditions was set to 1). (Right) dMi-2 and IgG ChIP-qPCR on reporter gene under HS and NHS conditions. (**C**) Model. dMi-2 distribution over active heat shock genes is closely correlated with transcription.

active heat shock genes. This hypothesis predicts that dMi-2 levels should be lower within the 5′ halves of HS genes, where RNA transcripts are still short, higher within the 3′ halves of HS genes, where transcripts reach their maximum length, and decline again past the polyadenylation signal, where the message has been cleaved off and only short transcripts are produced prior to their termination. Indeed, the relative enrichment of dMi-2 binding that we have observed across the six heat shock genes analysed supports this hypothesis. In all cases,

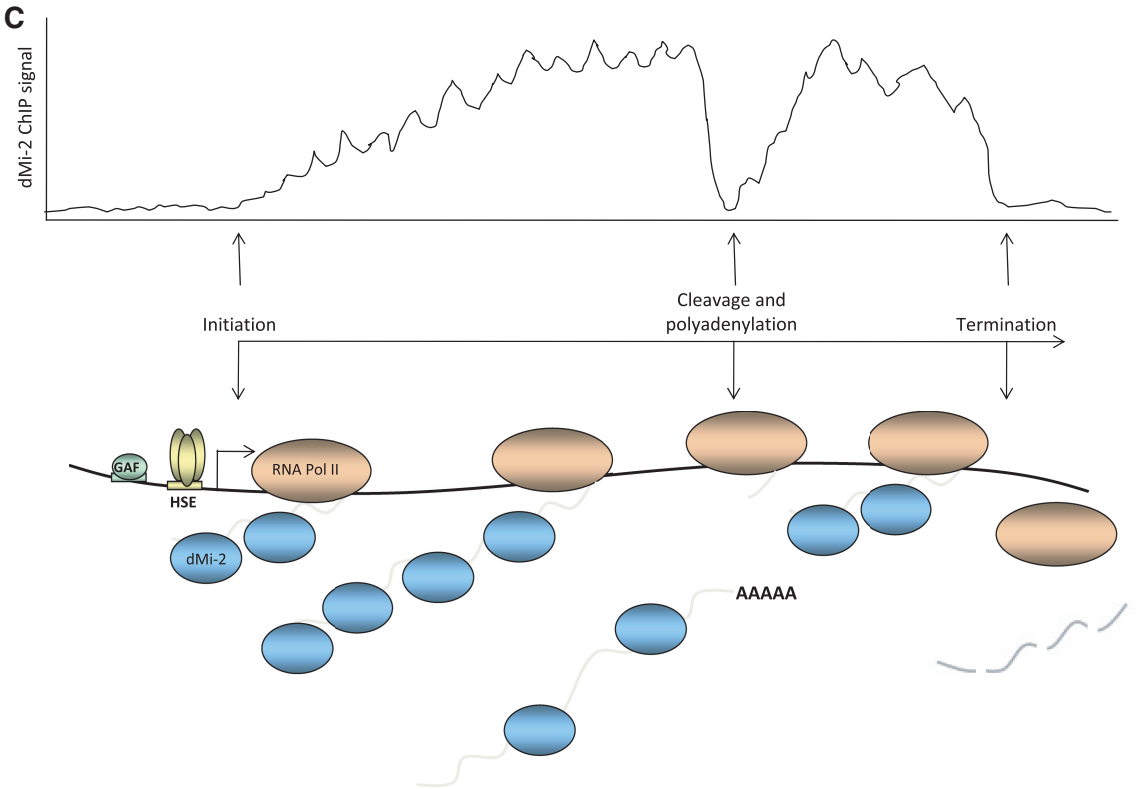

Figure 5. Continued.

dMi-2 enrichment is higher in the 3′ half compared to the 5′ half of genes and declined again in the region beyond the polyadenylation site.

Several HS genes exhibit a 'dip' in dMi-2 binding around the polyadenylation site. This is also consistent with the hypothesis that dMi-2 binding is mediated by an interaction with nascent RNA. We propose that the decline in dMi-2 binding near the polyadenylation site is a consequence of RNA cleavage there. The downstream dMi-2 peak might reflect dMi-2 interacting with the RNA produced by terminating RNA polymerase II (Figure 5).

While the interaction of dMi-2 with nascent RNA appears to contribute to its association with chromatin, it is not sufficient for recruiting dMi-2 to active gene loci. This view is supported by several findings. First, two genes that have been shown to be activated more than 10-fold upon heat shock in a HSF-dependent manner do not display a significant increase in dMi-2 binding (23). Secondly, genes that are strongly transcribed in a constitutive fashion, such as the genes encoding ribosomal protein subunits, do not bind more dMi-2 than neighbouring, untranscribed regions. Thirdly, strong activation of metallothionein A by Cd treatment does not result in increased association of dMi-2 with the promoter or the transcribed part of the gene. Fourthly, dMi-2 is not recruited to a reporter gene under control of the *hsp70* promoter that is upregulated 200-fold following heat shock. Fifthly, inhibition of transcriptional elongation does not affect the recruitment of dMi-2 to several regions within the activated *hsp70* gene (10). These findings suggest that the initial recruitment of dMi-2 to heat shock genes requires additional signals. In case of the *hsp70* gene in Kc cells, one signal appears to be provided by poly-ADP-ribosylation of the locus (10). We have not observed consistent effects of treatment with the PARP inhibitor PJ34 on dMi-2 recruitment in the S2 cells used for this study (data not shown). The relative contribution of poly-ADP ribosylation to dMi-2 recruitment in different biological contexts is therefore unclear. It is also not known, whether poly-ADP-ribosylation does also occur during the activation of other HS genes.

A rapid loss of nucleosomes from the 87A locus after HS has been described (11). Interestingly, nucleosome loss is not restricted to the two *hsp70* genes residing within 87A. Instead, it includes the entire region flanked by the insulator elements scs and scs′. This property of the 87A locus has allowed us to address the question if dMi-2 chromatin association correlates with nucleosome depletion. Interrogation of our ChIP-Seq data revealed that dMi-2 recruitment was restricted to the transcribed part of the *hsp70* genes even within the larger nucleosome-depleted locus. This underscores the importance of transcription for governing dMi-2 chromatin distribution.

Taken together, our results support a two-step recruitment model of dMi-2 (Figure 5C). Initial recruitment does not depend on RNA synthesis. Rather, it is likely to be facilitated by other signals that are specific for HS gene activation, one of which might be poly-ADP-ribosylation in certain contexts (10). Other potential recruitment signals might include binding to PARP itself, which is

located near the 5′-end of the *hsp70* transcription unit and migrates across the gene following heat shock, the interaction with histone variants deposited at *hsp70* or particular histone modifications that are generated during the heat shock response (26,27).

Once dMi-2 is brought to activate HS genes by one or more of these mechanisms, it interacts with nascent RNA and by doing so associates with the transcribed body of the gene. It is tempting to speculate that this association with nascent RNA influences transcription and co-transcriptional processes. Indeed, we have detected quantitative changes in levels and processing of *hsp70* gene transcripts in transgenic flies with compromised dMi-2 activity (10). Our ChIP-Seq study suggests that dMi-2 associates with and regulates an entire suite of heat shock genes and provides the basis for a more systematic analysis of dMi-2's role in the heat-shock response.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1 and 2.

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