ROLE OF HISTONE MODIFICATIONS IN MARKING AND ACTIVATING GENES THROUGH MITOSIS

Ester Valls, Sara Sánchez-Molina and Marian A. Martínez-Balbás#

Instituto de Biología Molecular de Barcelona. CID. Consejo Superior de Investigaciones Científicas (CSIC). Parc Cientific de Barcelona (PCB). Josep Samitier 1-5. 08028 Barcelona. Spain

#Corresponding author
Marian A. Martínez-Balbás
Instituto de Biología Molecular de Barcelona. CID-CSIC
Parc Cientific de Barcelona (PCB).
Josep Samitier 1-5.
08028 Barcelona.
SPAIN
E.Mail:mmbbmc@ibmb.csic.es
Tel.: 34-93-403-49-61
Fax: 34-93- 403-49-79

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SUMMARY

The global inhibition of transcription at the mitotic phase of the cell cycle occurs together with the general displacement of transcription factors from the mitotic chromatin. Nevertheless, the DNase and MnO4K hypersensitive sites are maintained on potentially active promoters during mitosis, helping to mark active genes at this stage of the cell cycle. Our study focuses on the role of histone acetylation and H3 (K4) methylation in the maintenance of the competency of these active genes during mitosis. To this end, we have analyzed histone modifications across the promoters and coding regions of constitutively active, inducible and inactive genes in mitotic arrested cells. Our results shown that basal histone modifications are maintained during mitosis at promoters and coding regions of the active and inducible RNAPII transcribed genes. In addition we have demonstrated that, together with H3 acetylation and H3 (K4) methylation, H4 (K12) acetylation at the coding regions contributes to the formation of a stable mark on active genes at this stage of the cell cycle. Finally, analysis of cyclin B1 gene activation during mitosis revealed that the former occurs with a strong increase of H3 (K4) trimethylation, but not H3 or H4 acetylation, suggesting that histone methyltransferases are active during this stage. These data demonstrate a critical role of histone acetylation and H3 (K4) methylation during mitosis in marking and activating genes during the mitotic stage of the cell cycle.

INTRODUCTION

The packaging of the eukaryotic genome into chromatin has a profound effect on gene function. To counterbalance the repressive nature of chromatin, cells utilize two types of protein complexes, which covalently modify the nucleosome core histones or catalyze nucleosome mobility in an ATP-dependent fashion (1, 2). Covalent modifications of histone proteins, such as acetylation, phosphorylation, methylation and ubiquitination of the N-terminal tails of histones, have been hypothesized to constitute a histone code that controls the pattern of gene expression (3, 4). In particular, histone acetylation correlates with gene activation, by counteracting the repressive nucleosomal environment (5). Acetylation occurs on conserved lysine residues of the N-terminal tails of the core histones, and induces chromatin opening by perturbing higher order chromatin folding. More recently H3-K4 methylation has also been also described as an active gene-specific modification mark, helping to recruit the preinitiation complex on promoters during transcriptional activation (6, 7, 8) in *Saccharomyces cerevisae* as well as in mammalian cells.

In recent years it has become apparent that some histone modifications are involved in preserving the expression patterns through the cell division, contributing to the establishment of cellular memory. Histone methylation is believed to play an important role in maintaining the heterochromatin silenced (4, 9). More recently it has been shown, in mammalian cells, that di- and trimethylated H3-K4 peaks correspond to the 5´coding region, suggesting the implication of these modifications in the establishment and/or in the maintenance of potentially active states (8). In contrast to histone methylation, histone acetylation is not believe to play an important role in cellular memory, since it is a dynamic modification. However, the finding that bromodomains can bind preferentially to acetylated lysines (10), leaves open the possibility that histone acetylation could serve as an active mark during mitosis (4) as has already been proposed (11, 12). Nonetheless, the potential role of histone acetylation in maintaining the active configuration during the mitotic stage of the cell cycle is not completely understood. Within this context we have analyzed the contribution of acetylation and H3-K4 methylation to marking and activating genes during mitosis.

EXPERIMENTAL PROCEDURES

Cell culture and synchronization. Mitotic index determination

HeLa S3 cells were grown in Dulbecco´s modified Eagle´s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10 % calf serum (CS, Invitrogen). Cells were arrested in mitosis by treatment with 50ng/ml nocodazole for 8 h. Mitotic cells were collected by selective detachment by manual shaking of the tissue culture flasks. The mitotic index was monitored by FACS analysis as follows: cells were harvested following trypsinization, washed twice in PBS and fixed in 70% ethanol for 2 h. Fixed and permeabilized cells were then stained with the fluorescent dye DAPI for 10 min and analyzed by flow cytometry with a Becton Dickinson FACS scan according to the manufacturer's instructions. The mitotic index was found to be 95-100%.

Indirect immunofluorescence and immunoblotting

Cells on cover slips were fixed in 4 % p-formaldehyde in PBS for 30 min at room temperature and permeabilized with methanol for 10 min. After blocking with 3% BSA in PBS, 0.1% Tween 20 for 1 h at room temperature, cover slips were incubated with a 1:200 anti acetyl H3 (K9 and 14) antibody (Upstate Biotechnology), 1:200 anti poly-acetyl H4 antibody (Upstate Biotechnology), 1:100 anti phospho-H3 (S10) (Upstate Biotechnology), 1:200 anti dimethyl H3 (K4) antibody (Upstate Biotechnology), 1:200 anti trimethyl H3 (K4) antibody (Abcam), and 1:100 anti acetyl H4 (K12) antibody (Upstate Biotechnology) in PBS, 3% BSA for 2h, followed by incubation for 1 h with Cy3-conjugated goat anti-rabbit IgG, used at 1:250 dilution in PBS, 3% BSA. Following each antibody incubation, cover slips were extensively washed with PBS, 0.05% Tween 20 four times for 10 min each at room temperature. The nuclei were stained with DAPI.

Cell extract preparation. Histone extraction.

Total cell extracts were prepared in IPH buffer (13) by keeping the cells on ice for 20 min or the indicated time, followed by centrifugation at 13,000 rpm for 10 min at 4º C. All buffers contained protease inhibitors (Boehringer). Histones were extracted by ON treatment of the whole cells with 0.25 M HCl at 4º C under rotation. After centrifugation at 12,000 g for 10 min at 4º C, histones were precipitated by cold acetone and resuspended in SDS-protein loading buffer. All buffers contained protease inhibitors (Boehringer), TSA (300 nM), and 25 mM NaF, 5 mM Na orthovanadate, 1 mM Na pyrophosphatase, and 5 uM microcystein (14). Immunoblotting was performed using standard procedures and visualized using an ECL kit (Amersham).

HAT, HDAC and HMT assays

In vitro HAT, HDAC and HMT assays was performed as previously described (6, 15, 16).

Chromatin immunoprecipitation analysis

HeLa cells were treated with 1% formaldehyde at room temperature for 15 min. The reaction was stopped by the addition of glycine to a final concentration 125 mM. Cells were washed once in ice-cold PBS, once with buffer I (0.25% triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM Hepes pH 6'5) and with buffer II (200mM NaCl, 1mM EDTA, 0.5 mM EGTA, 10 mM Hepes pH 6'5) at 4ºC for 10 min. Subsequently, the pellet was resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8, 1 mg/ml protease inhibitors, 1 mM PMSF) and sonicated on ice until cross-linked chromatin was sheared to an average DNA fragment length of 0.2-0.5 kbp. After centrifugation (10-45 min 14 Krpm), soluble cross-linked chromatin was diluted 1:10 in immunoprecipitation (IP) buffer (1% triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris pH 8 and protease inhibitors), divided into aliquots and stored a -80ºC. Chromatin preparations were pre-cleared by incubation with Protein A-Sepharose solution: 30 mg pre-immune serum (35mg/ml), 2 mg herring sperm DNA (1mg/ml), 50 ml Protein A-Sepharose

CL4B (Amersham-Pharmacia) 10% in TE buffer (50 mg/500 ml TE) in IP buffer for 2 h at 4ºC under rotation. The protein A-Sepharose was removed by centrifugation; the pre-cleared chromatin was immunoprecipitated by incubation with the antibody O/N at 4ºC. The immunoprecipitates were washed with buffers TSE I (0.1% SDS, 1% Triton X-100, 2Mm EDTA, 20 mM Tris-HCl pH 8, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2Mm EDTA, 20 mM Tris-HCl pH 8, 500 mM NaCl), buffer III (0'25 M LiCl, 1% NP40, 1% Deoxycholate, 1Mm EDTA, 10 mM Tris-HC l pH 8), and three times with Tris-EDTA (TE) buffer. Washed pellets were eluted with 300 ul of a solution containing 1% SDS, 0.1 M NaHCO3. Eluted pellets were de-cross-linked O/N at 65ºC, and were purified on 50 ul TE buffer in a DNA purification kit (Amersham-Pharmacia). DNA was analyzed by PCR with primer pairs to promoter and coding regions of different genes. Antibodies used were rabbit polyclonal antibody against acetyl H3 (k9, k14), poly-acetyl H4 (K5, K8, K12, K16), phospho H3 (S10), dimethylated H3 (K4), acetyl H4 (K12) (Upstate Biotechnology), trimethylated H3 (K4) (Abcam).

PCR analysis

PCR analysis was performed as described by Breiling and colleagues (17). PCR products measured 220-330 bp in length and were resolved by agarose-gel electrophoresis and revealed by staining with ethidium bromide. Decreasing amounts of input DNA (10, 1, 0.1 and 0.01%) were used to determine the linear range of the PCR reactions for each primer pair and IP. The following primer pairs were used: hsp70 gene designed at the proximal promoter region and the coding region were respectively: 5'-CCC TTC CCA CCG CCA CTC CC-3', 5'-GCA GCT CCT CAG GCT AGC CG-3', 5'-CCC TCT CGC CCT CGT ACA CC-3', 5'-GAG CAT CAA CCC CGA CGA GG-3'. GAPDH gene at promoter region and the coding region were respectively: 5'-GCT CCA ATT CCC CAT CTC AG, lw-CCA GGC TCA GCC AGT CCC AG, up-GGC TGT GGG CAA GGT CAT CC, lw-GGT GGA GTG GGT GTC GC. Primers for the cyclin B1 gene for the promoter and coding regions were respectively: up-CCT TTC CAG GTG GGC GGC GC, lw-GCT GCC GGT CGC AGA GAA TG, up-CTG CCT CTC CAA GCC CAA TGG, lw-GCT GCC CAC GTC CTG CTT G. And, finally, for the IL-2 promoter and coding regions: up-GTT CAG TGT AGT TTT AGG AC, lw-CT CTT CTG ATG ACT CTT TG, up-CCC TAT CAC TCT TTA ATC AC, lw-GCA GTA AAT GCT CCA GTT GT.

ChIP quantification

To quantify ChIP signals, PCR gel bands were measured using QuantityOne software (Bio-Rad), then, values from immunoprecipitated samples were divided by the corresponding 1% input sample (the PCR signals of this input sample were not saturated). Bound/input values were then averaged from multiple (at least three) PCR reactions from independent experiments. All data in the figures correspond to percentages from the 1% input sample.

RESULTS

Global acetylation and H3 (K4) methylation at interphase and mitosis

We first analyzed the levels of histone modification, which define active genes [H3 and H4 acetylation and H3 (K4) di- and trimethylation] in global chromatin at interphase and mitosis. To this end, HeLa S3 cells were synchronized in mitosis by treatment with nocodazole, which inhibits microtubule polymerization in a reverse manner. The mitotic index of the cell population following nocodazole treatment was determined by (1) fluorescence-activated cell sorting (FACS) analysis and measured 90-95% (Figure 1A) and (2) by microscopic examination of condensed chromosomes and nuclear envelope breakdown (data not shown). We then analyzed the acetylation of H3 (at K9 and K14 positions) and H4 poly-acetylation (at K5, 8, 12 and 16 positions) by immunoblotting and indirect immunostaining. Figures 1B and C show that the level of H3 acetylation was maintained during mitosis (Figs. 1B lane 2 and 1C columns 3 and 4) H4 poly-

acetylation, however, decreased after the cells entered mitosis (Figs 1B lane 1 and 1C columns 1 and 2), as was previously shown (18), and recovered by the end of the telophase (18 and data not shown). We subsequently analyzed the H3 (K4) di- and trimethylation levels of global chromatin at the interphase and during mitosis. Figures 1B (lanes 3 and 4) and 1C (columns 5, 6, 7, and 8) show that in contrast to the global decrease of H4 poly-acetylation, H3 (K4) di- and trimethylation levels remained unchanged from the interphase to mitosis. Afterwards, we analyzed the temporal relationship between the levels of di- and trimethylated histone H3 at K4 and chromatin condensation. To this end, we examined histone H3 (K4) methylation states through mitosis. As shown in Figure 1D, high levels of H3 methylation were observed on the chromosomes from the prophase to the telophase. This pattern differed from that of histone hyper-acetylation; in this case, a concomitant decrease occurred as chromatin condensed into metaphase chromosomes and was re-established during late telophase (18 and data not shown). These data suggest that histone methylation plays a different role than that of histone acetylation during mitosis.

As expected, H3 (S10) phosphorylation clearly peaked when the cells entered mitosis, remaining until late telophase (Fig. 1B lane 5; 1C columns 9 and 10).

Unequal subcellular distribution of HAT and HDAC activities during mitosis

Mitotic global histone deacetylation has been postulated to occur by the displacement of HAT and HDAC enzymes from condensing chromosomes as cells undergo mitosis (18). However, if both activities were displaced from chromatin, the final histone acetylation/deacetylation equilibrium would be maintained. To get better insight into this problem, we have analyzed the subcellular distribution of HAT and HDAC activities in asynchronous and mitotic cells. To this end, we extracted them with IPH buffer containing 0.5% Triton X-100 and incubated in ice for different time periods to release loosely bound proteins (soluble fraction). The resulting pellet contained the tightly bound proteins, which included those strongly associated with fragments of the nuclear matrix (pellet). The distribution of HAT and HDAC activities in asynchronous and mitotic cells in soluble and pellet fractions was determined by *in vitro* HAT and HDAC assays, respectively. The level of HAT activity in the lysates changed from asynchrony to mitosis, indeed, during the latter, most of the HAT activity was present in the tightly bound fraction (Fig. 1E). However, this situation was completely different for HDAC activity, which was found mainly in the soluble fraction during mitosis (Fig. 1E). These findings suggest an unequal availability of HAT and HDAC enzymes, potentially explaining the global alteration in acetylation/deacetylation equilibrium, thereby leading to a global histone deacetylation during mitosis

These data are consistent with previous results demonstrating a general loss of histone acetylation when cells undergo mitosis. This led us to analyze whether the promoter regions from active genes also lose acetylation marks during mitosis, and prompted us to investigate the significance underlying the maintenance of general H3 methylation in mitosis.

Histone acetylation and H3 (K4) methylation at active and inducible promoters in mitosis

To investigate the possible role of histone active modifications in the mitotic marking of active or inducible genes in living cells, we performed comparative ChIP analyses. Formaldehyde-cross-linked soluble chromatin was prepared from asynchronous and mitotic HeLa cells, normalized for DNA content, and subjected to immunoprecipitation with antibodies against acetylated H3 (K9, 14), poly-acetylated H4 (K5, 8, 12 and 16), di- and trimethylated H3 (K4) and phosphorylated H3 (S10) (Table 1). Acetylation and methylation levels were determined by quantitative polymerase chain reaction (PCR) analysis at the promoter regions of a constitutively active gene transcribed by RNAP II (glyceraldehydes 3-phosphate

dehydrogenase, GAP-DH) and an inactive RNAPII-transcribed gene (interleukin-2, IL-2).

We first compared the ChIP signals from asynchronous and mitotic cell populations. We found that the H3 acetylated basal signal at the GAP-DH promoter was maintained during mitosis (Fig. 2A). The H4 acetylated basal signal slightly decreased, relative to the asynchronous cells (Fig. 2A), but remained significantly higher than background levels obtained from the inactive gene promoter, IL-2 (Fig. 2B) during mitosis. H3 (K4) methylation was subsequently analyzed at the GAP-DH promoter in mitotic cells, finding that di- and trimethylated H3 (K4) ChIP signals were similar to those from asynchronous cells (Fig. 2A). No methylated H3 (K4) ChIP signals were found at the transcriptionally inactive promoter IL-2 (Fig. 2B). These data suggest that basal H3 and H4 acetylation as well as H3 (K4) methylation marks, are specifically maintained at the GAP-DH promoter during mitosis (although a partial loss of H4 acetylation occurs).

After observing that activation marks are maintained during mitosis in the constitutively active RNAP II-transcribed gene, we sought to analyze the promoter of an inducible gene, the heat shock protein 70, hsp70. Figure 2 C shows that H3 and H4 acetylated ChIP signals in the hsp70 promoter increased following heat shock (HS) stimulation of asynchronous cells, as well as H3 (S10) phosphorylation (Fig. 2C). H3 (K4) methylation, however, did not significantly change after HS (Fig. 2C). These results suggest that both histone acetylation and H3 phosphorylation are involved in the rapid activation of the hsp70 gene, in asynchronous cells. In *Drosophila*, immunostaining studies have revealed that only H3 phosphorylation is involved in hsp70 activation (19). However, mouse hsp70 promoter has been postulated to require only histone acetylation to be activated following HS (20). Our ChIP results suggest that both modifications occur after HS activation in human cells.

Next, we compared the ChIP signals from asynchronous and mitotic cell populations. We found that the H3 acetylated basal signal was maintained during mitosis (Fig. 2D). The H4 acetylated basal signal decreased by 35-40%, relative to the asynchronous cells (Fig. 2D), but remained significantly higher than the background levels obtained from the inactive gene promoter (IL-2) during mitosis (Fig. 2B). Following HS, the mitotic basal levels of acetylated H3 and H4 did not change (data not shown). Finally, H3 (K4) methylation was analyzed at the hsp70 promoter in mitotic cells, finding that di- and trimethylated H3 (K4) ChIP signals were similar to those from asynchronous cells (Fig. 2D). These data confirm the above results: H3 and H4 acetylation marks are maintained during mitosis at the promoter regions of inducible and active genes. In the same manner, H3 (K4) di- and trimethylation levels did not change during mitosis.

As described above, the analysis of the H3, H4 acetylation and H3 (K4) methylation levels of the inactive RNAP II-transcribed promoter IL-2 revealed no modifications, either at synchrony or at mitosis; only H3-S10 phosphorylation was detected after the cells underwent mitosis (Fig. 2B).

Histone acetylation and H3 (K4) methylation at active and inducible genes during mitosis

It is possible that the maintenance of acetylation and methylation at active promoters serves to signal the reassembly of transcriptional machinery at the G1 or S phase, in such a case the promoter histone posttranslational modifications could play a pivotal role in marking active genes. We therefore analyzed whether the coding regions are also marked during mitosis by acetylation and methylation. H3 acetylation ChIP signals of the GAP-DH coding region from mitotic cells were reduced by 15-20% compared with those from asynchronous cells during mitosis (Fig 3A). On the other hand, H4 acetylation ChIP signals decreased by 40-50% in mitotic cells relative to asynchronous cells (Fig 3A). We also analyzed the hsp70-coding region using ChIPs. We initially observed that H3 and H4 acetylation, as well as H3 phosphorylation, occur both in the analyzed

hsp70-coding region (Fig.3C) and in the promoter (Fig. 2C) following HS activation. We subsequently compared the ChIP signals from asynchronous and mitotic cell populations. We found that histone acetylation and H3 (K4) trimethylation basal mitotic signals slightly decreased at the hsp70 coding region relative to asynchronous cells (Fig. 3D). On the other hand, H3 (K4) dimethylation levels did not change during mitosis (Fig. 3A, D). As expected, neither histone acetylation nor methylation was detected at the IL-2 coding region (Fig. 3B). These results suggest that coding regions, as well as promoters, of active and potentially active RNAPII transcribed genes maintain the basal acetylation and H3 (K4) methylation levels during the mitotic phase of the cell cycle. These histone posttranslational modifications may play a critical role in marking active gene during mitosis.

H4 K12 acetylation at interphase and mitosis

To identify potentially important sites for cellular memory within chromatin, Gottschling and colleagues conducted a genetic screen in *Saccharomyces cerevisiae* to isolate those mutant alleles of the H3 and H4 genes that set telomeric genes into a silenced state (21). They found that the level of acetylation at lysine 12 within the histone H4 tail functioned as a memory mark for propagating the expression state of a telomeric gene during mitosis. Based on this finding, we determined whether or not H4 (K12) acetylation could affect the maintenance of transcriptional competency of euchromatic genes in mammalian cells. We studied the levels of acetylated H4 (K12) on global chromatin at interphase and mitosis by immunoblotting and indirect immunostaining. Figure 4 (panels A and B) shows that the levels of acetylated H4 (K12) are maintained during mitosis in bulk chromatin. We then analyzed acetyl-H4 (K12) levels at the promoter and coding regions of the above-analyzed genes using ChIP assays; we have included the cyclin B1 gene since it is actively transcribed during mitosis (22). Our results demonstrate that H4 (K12) acetylation is present at the coding regions of active and potentially active genes, either in asynchronous or in mitotic cells (Fig. 4C). However, very weak or nil ChIP signals were detected at the promoter regions of these active genes (Fig. 4C). Moreover, the promoter and coding region of the IL-2 gene were not reactive to anti-acetyl H4 (K12) antibody, neither in asynchronous nor in mitotic cells (Fig. 4C). These results suggest that in histone H4, lysine 12 may serve as a memory mark in the nucleosome for the inheritable transmission of the active or inactive state in euchromatic genes, as has been suggested for telomeric genes (21).

Histone H3 (K4) methylation couples with transcription during mitosis

It has been suggested that histone acetylation cannot occur during mitosis, due to the displacement of HAT enzymes away from mitotic chromatin during the early prophase (18). We therefore decided to analyze whether the activation of mitotically transcribed genes involves ongoing histone acetylation and/or methylation. To this end we analyzed the levels of acetylation at the cyclin B1 promoter during mitosis (when it was actively transcribed) using ChIP assays. The results in Figure 5A reveal no changes in the H3 and H4 acetylation levels following gene activation during mitosis. However, the di- and trimethylated H3 (K4) and phosphorylated H3 (S10) signals clearly increased after gene activation (Fig. 5A). This suggests the existence of an ongoing HMT activity coupled with cyclin B1 transcription during mitosis. H3 (S10) phosphorylation may be a necessary signal for mitotic entry, or it may be involved in cyclin B1 transcription activation, as has been suggested for other genes (19). Once we determined that active histone methylation, at the promoter region, may be involved in gene activation during mitosis, we analyzed whether it also occurs at the coding region of the cyclin B1 gene. Activation of this gene strongly correlates with an increase of trimethylated H3 (K4) (Fig. 5B), but not of acetylated H3 and H4. In fact, the basal levels of acetylated H4 present in interphase decreased by 30-40%

during mitosis (Fig 5B), while acetylated H3 was not detectable in the analyzed region (Fig 5B). These results suggest that HMTs are maintained as active enzymes during mitosis, capable to methylate some mitotically active genes.

Based on this premise, we analyzed the HMT activity in asynchronous and mitotic cells. As shown in Table 2, the global HMT activity associated with mitotic and asynchronous cells was similar, suggesting the possibility that HMTs are maintained as active enzymes during mitosis.

DISCUSSION

Our current study demonstrates that although bulk chromatin is generally deacetylated during mitosis, acetylation and methylation marks of some active genes persist in the parental nucleosomes during mitosis. The general removal of acetyl groups from the modified lysines residues of core histones (23, 24) may be necessary for the compaction of chromatin, which involves neutralization of DNA negative charges (25). Several studies have shown that in the absence of histone H1, the acetylated nucleosome complexes remain in an extended conformation in contrast to the nonacetylated nucleosomes. (26, 27). Furthermore, the consequence or the linker histones binding, required to allow high levels of chromatin compaction, specifically inhibit acetylation of oligonucleosomes (28). On the other hand, the maintenance of some acetylated loci may be important in preventing some active genes from becoming silenced, as has been proposed (11, 12). Acetylation would serve as a signal for the propagation and maintenance of active chromatin states through cell division. How would histone modifications serve as an epigenetic mark during mitosis? According to the histone code hypothesis, different modifications are recognized by specific associated proteins, responsible for reading the code (4, 29). Thus, permanent acetylation and methylation of promoter in active genes would serve to specifically bind certain effector proteins. Candidates for such interactions include (i) the Brd4 protein that contains two bromodomains and remains bound to mitotic chromatin in a bromo-dependent manner (30, 31) and (ii) TFIID complex which specifically interacts with acetylated histone H3 at lysines 9 and 14 (32). H3 (K4) methylation could also provide a specific high-affinity interaction surface for SNF2H binding (33), or for some chromodomain-containing complexes (34). Interestingly, immunostaining analysis of the global H3 and H4 acetylation and H3 (K4) methylation levels in mitotic cells revealed that residual mitotic acetylation and methylation correspond to discreet regions of the genomic chromatin, suggesting that previously active genes, or genes that need to be rapidly reactivated after mitosis, co-exist in the same chromatin domains.

Our results are consistent with Kouskouli and Talianides´s (12) data for the human constitutively active HNF-4, HNF-1 and albumin genes for H3 and H4 acetylation and H3 (K4) methylation. Moreover, the present study expands the data for inactive but inducible (human hsp70), inactive (IL-2) and mitotically active (cyclin B1) genes. In addition we have shown that H4 (K12) acetylation at the coding regions in tandem with H3 acetylation and H3 (K4) methylation, contributes to the formation of a stable mark on active genes during mitosis. Finally, our results show that activation of cyclin B1 gene strongly correlates with an increase of trimethylated H3 (K4) but not H3 and H4 acetylation. Thus, these data provide the first evidence that HMTs may be maintained as active enzymes during this stage of the cell cycle. Therefore, the regulation of HMT enzymes during mitosis may differ from that of HAT/HDAC enzymes (18, 35, 36). This issue is currently under investigation.

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REFERENCES

1. Konberg, R.D., and Lorch, Y. (1999*) Curr. Opin. Genet. Dev.* **9**, 148-151

2. Martens, J.A., and Winston, F. (2003) *Curr. Opin. Gent. Dev.* **13,** 136-142

3. Strahl, B.D., and Allis, C.D. (2000) *Nature* **403,** 41-45

4. Turner, B.M. (2002) *Cell* **111,** 285-291

5. Th´ng, J.P. (2001) *Biochem. Cell. Biol.* **79,** 305-311

6. Santos-Rosa, H., Schneider, R., Bannister, A.J., Sheriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., and Kouzarides, T. (2002) *Nature* **419,** 407-411

7. Fischle, W., Wang, Y., and Allis, C.D. (2003) *Curr Opin Cell Biol* **15,** 172-183

8. Schneider, R., Bannister, A.J., Myers, F.A., Thorne, A.W., Crane-Robinson, C., and Kouzarides, T. (2004) *Nat. Cell. Biol.* **6,** 73-77

9. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001) *Nature* **410,** 116-120

10. Zheng, L., and Zhou, M. (2002) *FEBS Lett.* **513**, 124-128

11. Jeppesen, P. (1997) *BioEssays* **19,** 67-74

12. Kouskouti, A., and Talianidis, I. (2005) *The EMBO J.* **24,** 347-357

13. Bannister, A.J., and Kouzarides, T. (1996) *Nature* **384,** 641-643

14. Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J., and Reed, S.I. (1994) *Cell* **76,** 1013-1023

15. Martínez-Balbás, M.A., Bannister, A., Martín, K., Haus-Seuffert, P., Meisterernst, M., and Kouzarides, T. (1998) *The EMBO J.* **17**, 2886-2893

16. Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. (1998). *Nature* **391**, 597-601

17. Breiling, A., Turner, B.M., Bianchi, M.E., and Orlando, V. (2001) *Nature* **412**, 651-655

18. Kruhlak, M.J., Hendzel, M.J., Fischle, W., Bertos, N.R., Hameed, S., Yang, X.J., Verdin, E., and Bazett-Jones, D.P. (2001) *J. Biol. Chem.* **276,** 38307-38319

19. Nowak, S.J., and Corces, V.G. (2004) *Trends Gent.* **20,** 214-220

20. Thomson, S., Hollis, A., Hazzalin, C.A., and Mahadevan, L.C. (2004) *Mol. Cell* **15**, 585-594

21. Smith, C.M., Haimberger, Z.W., Johnson, C.O., Wolf, A.J., Gafken, P.R., Zhang, Z., Parthun, M.R., Gottschling, D.E. (2002) *Proc. Natl. Acad. Sci. U S A* **99,** 16454-16461

22. Sciortino, S., Gurtner, A., Manni, I., Fontemaggi, G., Dey, A., Sacchi, A., Ozato, K., and Piaggio, G. (2001*) The EMBO Rep* **11,** 1018-1023

23. Jeppesen, P., Mitchell, A., Turner, B., and Perry, P. (1992) *Cromosoma* **101,** 322-332

24. Turner, B.M., and Fellows, G. (1989) *Eur. J. Biochem.* **179,** 131-139

25. Schwarz, P.M., and Hansen, J.C. (1994) *J. Biol. Chem.* **269,** 16284-16289

26. García-Ramirez, M., Rocchini, C., and Ausio, J. (1995) *J. Biol. Chem.* **270,** 17923-17928

27. Tse, C., Sera, T., Wolffe, A.P., and Hansen, J.C. (1998) *Mol. Cell. Biol.* **18,** 4629-4638

28. Herrera, J.E., West, K.L., Schiltz, R.L., Nakatani, Y., and Bustin, M. (2000) *Mol. Cell. Biol.* **20,** 523-529

29. Jenuwein, T., and Allis, C.D. (2001) *Science* **293,** 1074-1080

30. Dey, A., Chistsaz, F., Abbasi, A., Mistelli, T., and Ozato, K. (2003) *PNAS* **100,** 8758-8763

31. Kanno T., Kanno, Y., Siegel, R.M., Jang, M.K., Lenardo, M.J., and Ozato, K. (2004) *Mol. Cell* **13,** 33-43

32. Christova, R., and Oelgeschlager, T. (2002) *Nat. Cell. Biol.* **4,** 79-82

33. Santos-Rosa, H., Schneider, R., Bernstein, B.E., Karabetsou, N., Morillon, A., Weise, C., Schreiber, S.L., Mellor, J., and Kouzarides, T. (2003) *Mol. Cell.* **12,** 1325-1332

34. de la Cruz, X., Llois, S., Sánchez-Molina, S., and Martínez-Balbás, M.A. (2005) *BioEssays* **27,** 164-175

35. Cimini, D., Mattiuzzo, M., Torosantucci, L., and Degrassi, F. (2003) *Mol. Biol.of the Cell* **14,** 3821-3833

36. Kim, J.-M., Liu, H., Tazaki, M., Nagata, M., and Aoki, F. (2004) *J. Cell. Biol*. **162,** 37-46

The abbreviations used are: HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase, GFP, green fluorescent protein; Ac-H3, acetylated histone H3 at lysines 9 and 14; Ac-H4, poly-acetylated histone H4; H3-K4-dimet, dimethylated histone H3 at lysine 4; H3-K4-trimet, trimethylated histone H3 at lysine 4; H3-S10-P, phosphorylated histone H3 at serine 10; Ac-H4-K12, acetylated histone H4 at lysine 12; ChIP, chromatin immunoprecipitation; BSA, bovine serum albumin; DAPI, 6'-diamidino-2-phenylindole, PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

FIGURE LEGENDS

Figure 1
Global histone acetylation and methylation in asynchronous and mitotic cells

(A) FACS analysis of untreated (asynchronous) and nocodazole treated (mitotic) HeLa S3 cells. 99% of mitotic cells were obtained after the treatment.
(B) Western blot analysis of asynchronous and mitotic HeLa S3 cells. Histones from asynchronous and mitotic cultures were purified and analyzed by Western blot using antibodies that specifically recognized acetylated histone H3 at lysines 9 and 14 (Ac-H3), acetylated histone H4 at lysines 5, 8, 12 and 16 (Ac-H4), dimethylated H3 at lysine 4 (H3-K4-Dimet), trimethylated H3 at lysine 4 (H3-K-Trimet) and phosphorylated H3 at serine 10 (H3-S10-P).
(C) Immunocytological analysis of asynchronous and mitotic cells. p-formaldehyde-fixed cells were stained with the antibodies indicated in (B) (red). Cells were co-stained with DAPI to reveal the DNA (blue).
(D) Relationship between chromatin condensation and H3 (K4) methylation levels. Immunocytological analysis of HeLa cells at various phases of mitosis (as indicated). Cells were stained with antibodies specific for either di-or trimethylated of histone H3 (K4) in red. Cells were co-stained with DAPI to reveal the DNA (blue).
(E) Unequal subcellular distribution of HAT and HDAC activities during mitosis. Equal numbers of asynchronous and mitotic HeLa cells were extracted with IPH buffer containing 0.5% Triton X-100 on ice for 5, 10, or 15 min. The HAT and HDAC activities present in the resulting soluble and pellet fractions were determined by *in vitro* HAT and HDAC assays. Pellet/Soluble ratios for each time and each activity (HAT and HDAC) are shown in the figure.

Figure 2
Acetylation and methylation of nucleosomes at active promoters in asynchronous and mitotic cells
Comparative ChIP analysis were performed in parallel with the same numbers of asynchronous (A) and mitotic (M) cells using the antibodies described in Table 1. The immunoprecipitates were analyzed by quantitative PCR using specific primers for GAP-DH promoter (A), IL-2 promoter (B) and hsp70 (D). (C) Comparative ChIP analysis performed in parallel with the same numbers of asynchronous heat shocked (+) and not heat shocked cells (-) and analyzed by quantitative PCR using specific primers for the hsp70 promoter. PCR products were resolved in agarose gels and quantified with QuantityOne software (Bio-Rad). The values on the y-axis represent the amount of immunoprecipitated DNA as a percentage of the 1% input sample. Relative histone modification levels in mitotic (M) and asynchronous (A) cells were calculated from three independent experiments. A schematic representation of the GAP-DH, IL-2 and hsp70 promoters and coding regions, as well as the position of the two primer pairs used for ChIP is shown at the top of each panel. (+) corresponds to HeLa cells heat shocked at 43 ºC for 30 min.

Figure 3
Acetylation and methylation of nucleosomes at coding regions in asynchronous and mitotic cells
ChIP analysis were performed as indicated in the legend of Figure 2, and the immunoprecipitates were analyzed by quantitative PCR using specific primers for the GAP-DH coding region (A), IL-2 coding region (B) and hsp70 coding region (D). (C) corresponds to comparative ChIP analysis performed in parallel with the same numbers of asynchronous heat shocked cells at 43 ºC for 30 min (+) and not heat shocked cells (-) and analyzed by quantitative PCR using specific primers for the hsp70 coding region.

Figure 4
H3-K12 acetylation in asynchronous and mitotic cells
(A) Western blot analysis of histones from asynchronous (A) and mitotic (M) HeLa S3 cells using antibodies that specifically recognize acetylated histone H4 at lysine 12 (Ac-H4-K12).
(B) Immunocytological analysis of asynchronous and mitotic cells with the antibody that specifically recognizes acetylated histone H4 (K12) (red). Cells were stained with DAPI to reveal the DNA (blue).

(C) ChIPs analysis were performed as indicated in the legend of Figure 2 using antibodies that specifically recognize acetylated histone H4 (K12) (Ac-H4-K12). The immunoprecipitates were analyzed by quantitative PCR using specific primers for the promoters and coding regions of the indicated genes: GAP-DH, IL-2, hsp70 and cyclin B1.

Figure 5

Acetylation and methylation of nucleosomes from the mitotically active gene cyclin B1

(A) (B) ChIPs analysis were performed as indicated in the legend of Figure 2, and the immunoprecipitates were analyzed by quantitative PCR using specific primers for the cyclin B1 promoter (A) and coding region (B). A schematic representation of the cyclin B1 promoter and coding region as well as the position of the two primer pairs used for ChIP, is shown at the top of panel A.

Table 1

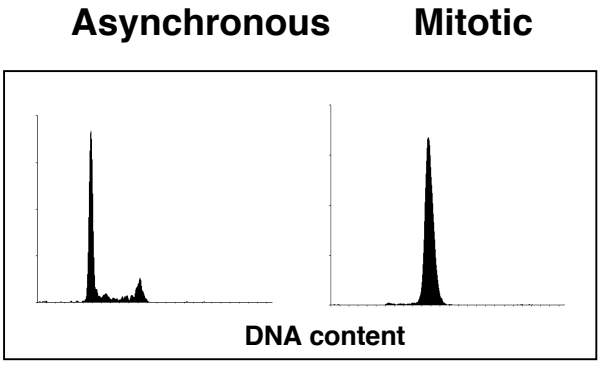
Antibody	Antibody recognizes modifications	Source or reference	Intephase	Mitosis
Anti-acetyl-H3	Lysines 9 and 14 acetylated of H3	Upstate Biotechnology 06-599	+	+
Anti-acetyl-H4	Lysines 5,8,12,16 acetylated of H4	Upstate Biotechnology 06-866	+	+/-
Anti-phospho-H3	Serine 10 phosphorylated of H3	Upstate Biotechnology 06-570	-	+
Anti-dimethyl-H3-Lys4	Lysine 4 dimethylated of H3	Upstate Biotechnology 07-030	+	+
Anti-trimethyl-H3-Lys4	Lysine 4 trimethylated of H3	Abcam ab8580	+	+

Table 2

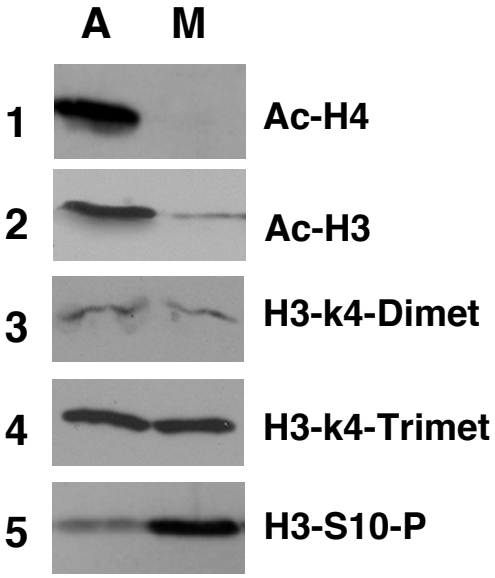
Total cell extract	Ratio of interphase to mitotic mean HMT activity n=3
10 ug	0.94
20 ug	0.90
40 ug	1.07

Figure 1

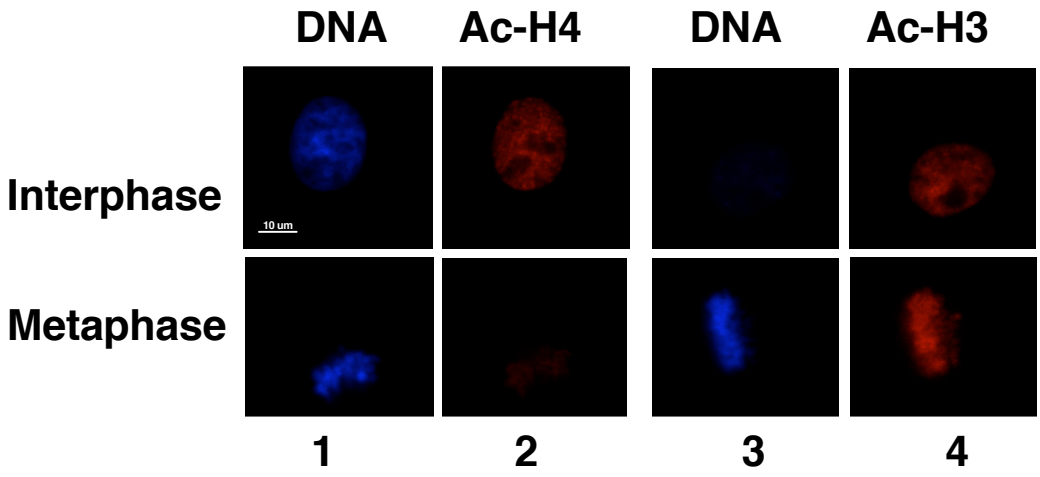
A


Asynchronous
Mitotic
DNA content

B


A
M
1
Ac-H4
2
Ac-H3
3
H3-k4-Dimet
4
H3-k4-Trimet
5
H3-S10-P

C


DNA
Ac-H4
DNA
Ac-H3
Interphase
Metaphase
10 um
1
2
3
4

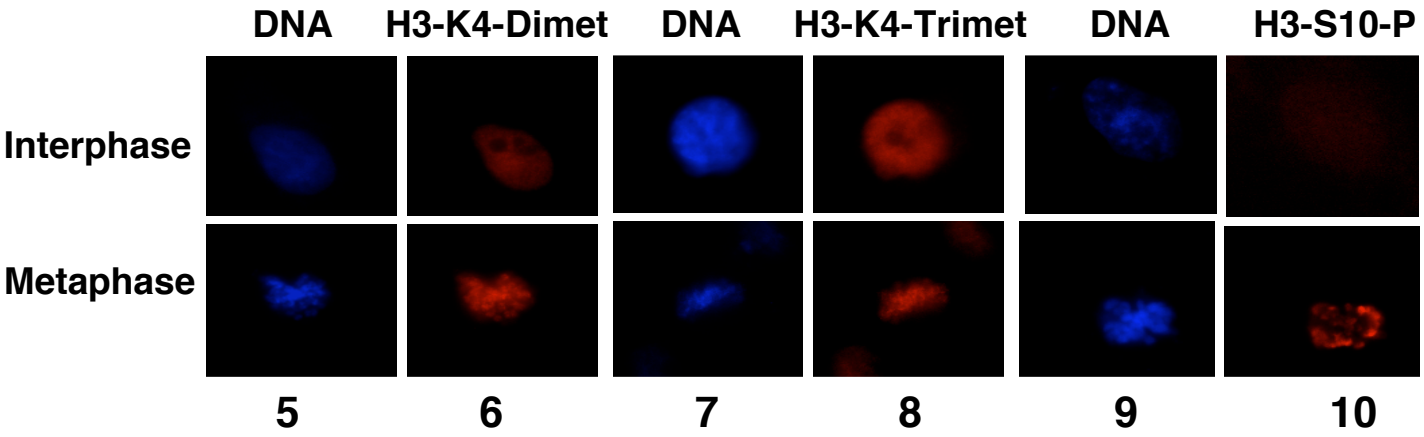
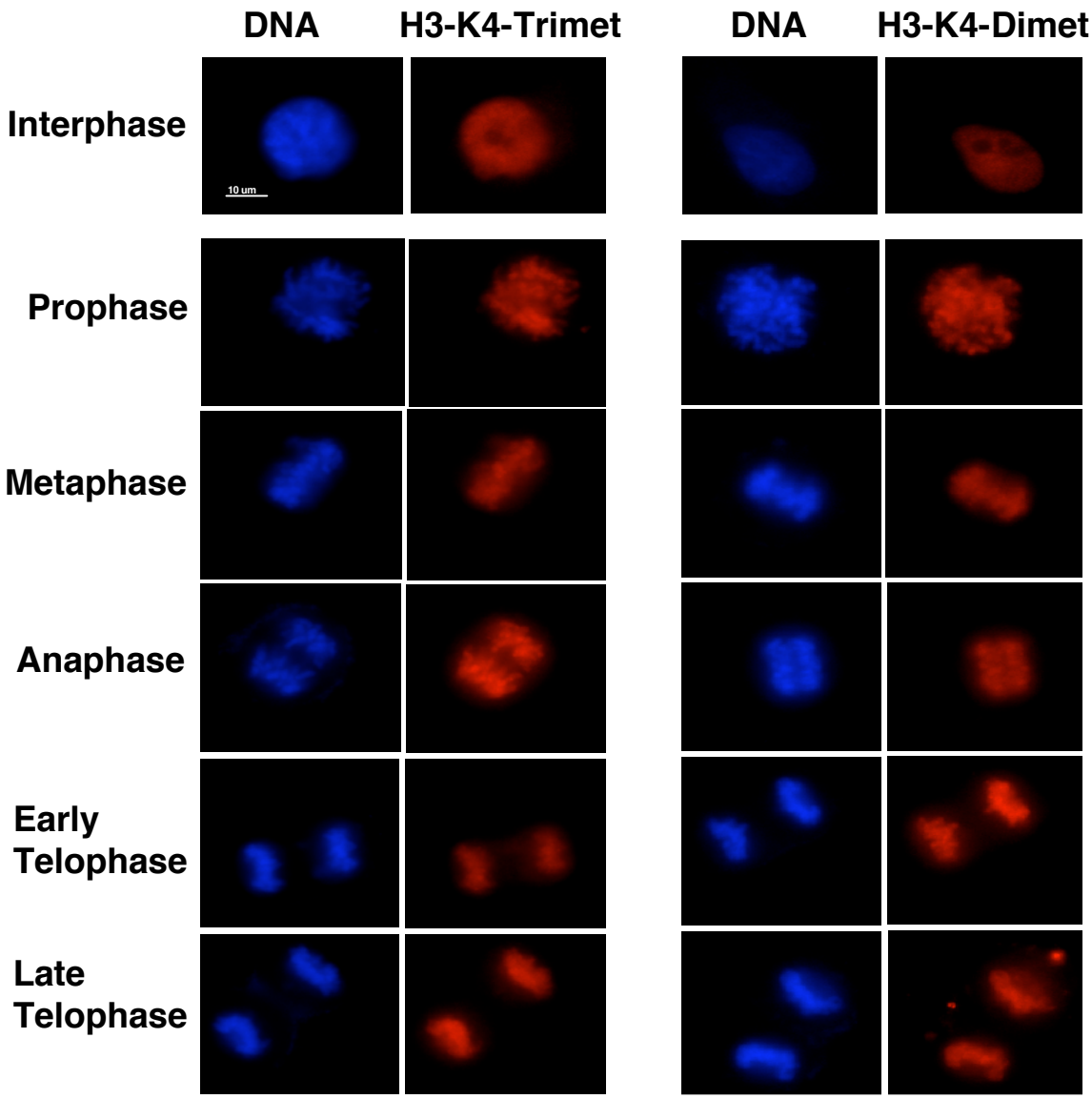

DNA
H3-K4-Dimet
DNA
H3-K4-Trimet
DNA
H3-S10-P
Interphase
Metaphase
5
6
7
8
9
10

Figure 1

D


DNA
H3-K4-Trimet
DNA
H3-K4-Dimet
Interphase
Prophase
Metaphase
Anaphase
Early Telophase
Late Telophase
10 um

E

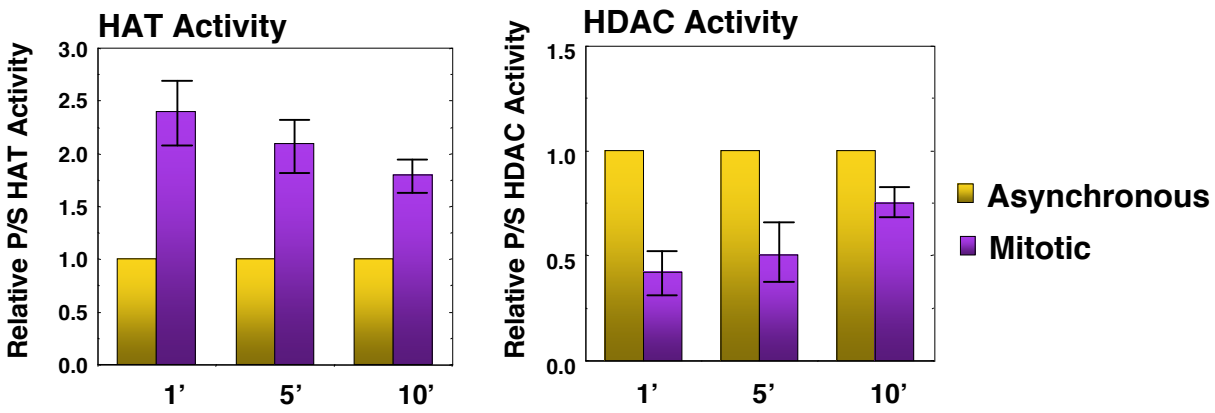
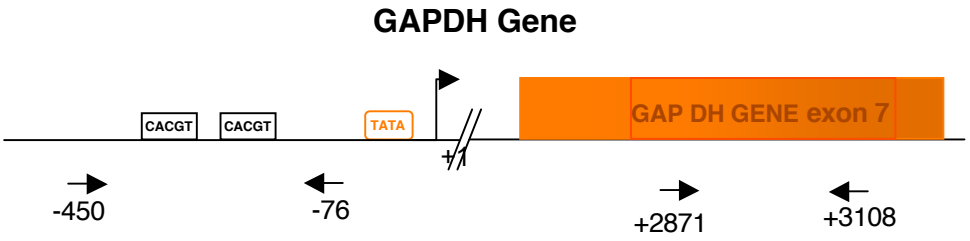
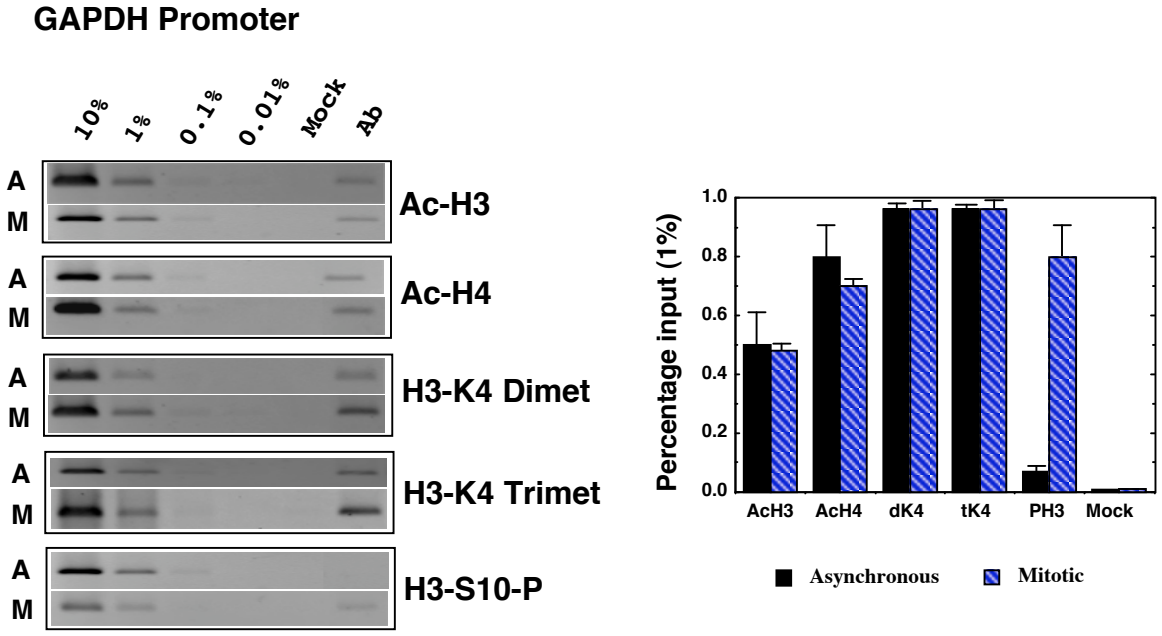

HAT Activity
Relative P/S HAT Activity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1'
5'
10'
HDAC Activity
Relative P/S HDAC Activity
1.5
1.0
0.5
0.0
1'
5'
10'
Asynchronous
Mitotic

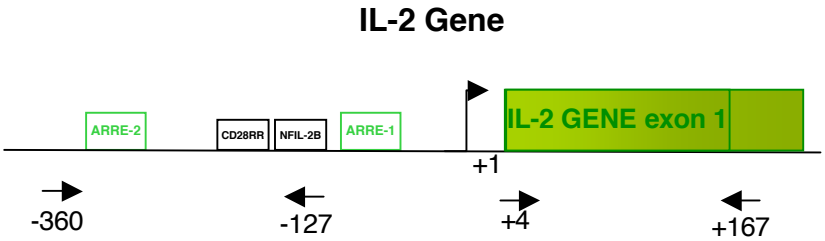
Figure 2

A


GAPDH Gene
CACGT
CACGT
TATA
+1
GAP DH GENE exon 7
-450
-76
+2871
+3108


GAPDH Promoter
10%
1%
0.1%
0.01%
Mock
Ab
A
M
Ac-H3
Ac-H4
H3-K4 Dimet
H3-K4 Trimet
H3-S10-P
Percentage input (1%)
1.0
0.8
0.6
0.4
0.2
0.0
AcH3
AcH4
dK4
tK4
PH3
Mock
Asynchronous
Mitotic

B


IL-2 Gene
ARRE-2
CD28RR
NFIL-2B
ARRE-1
IL-2 GENE exon 1
+1
-360
-127
+4
+167

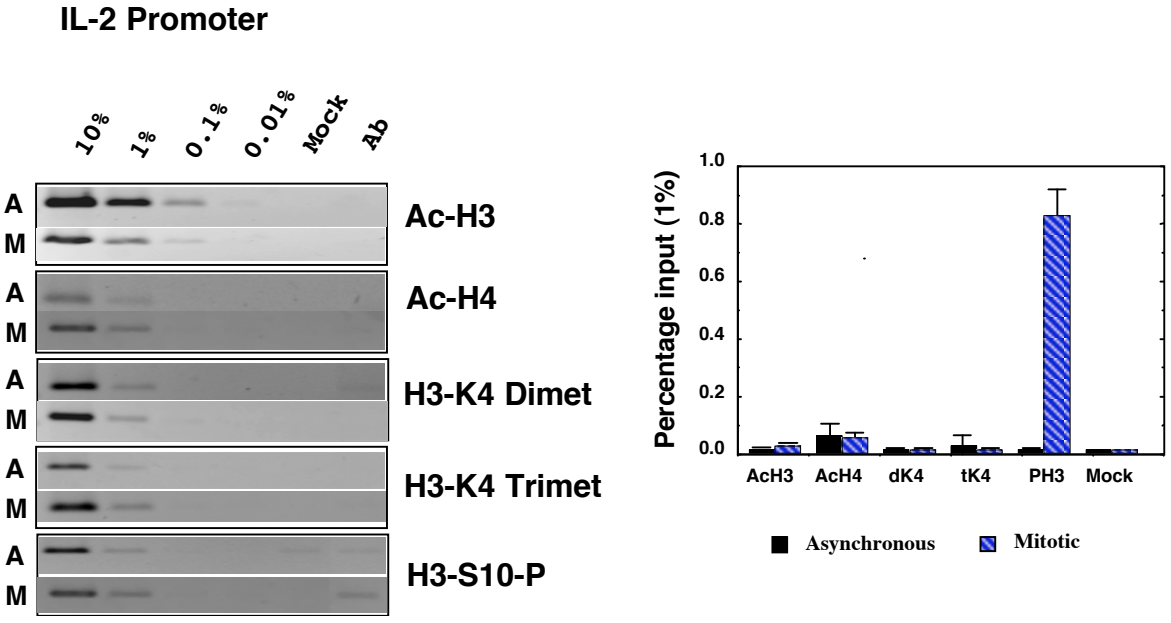
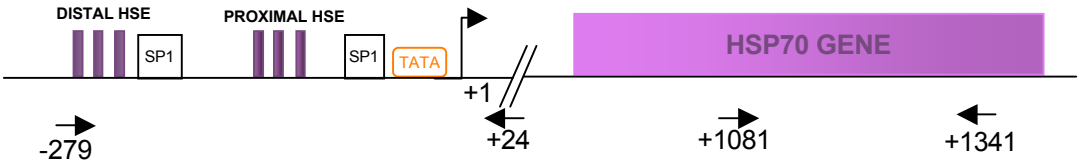
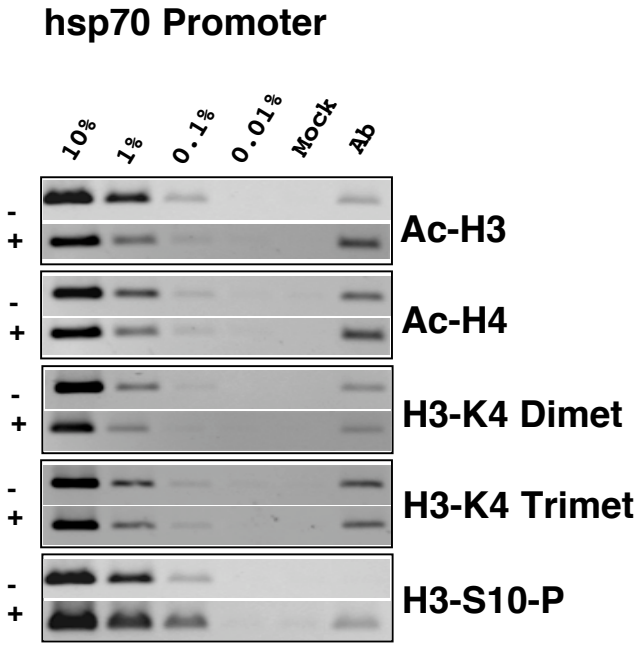
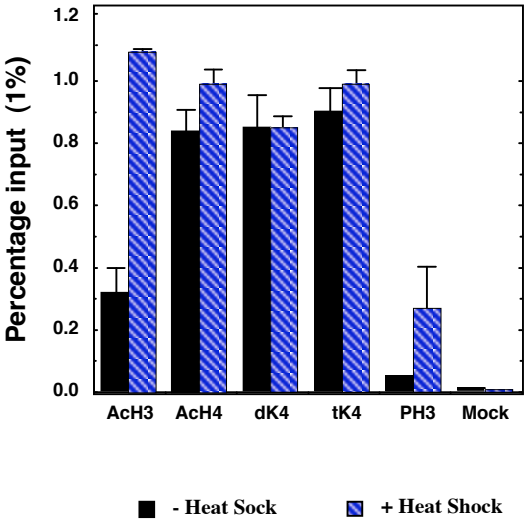

IL-2 Promoter
10%
1%
0.1%
0.01%
Mock
Ab
A
M
Ac-H3
Ac-H4
H3-K4 Dimet
H3-K4 Trimet
H3-S10-P
Percentage input (1%)
1.0
0.8
0.6
0.4
0.2
0.0
AcH3
AcH4
dK4
tK4
PH3
Mock
Asynchronous
Mitotic

Figure 2

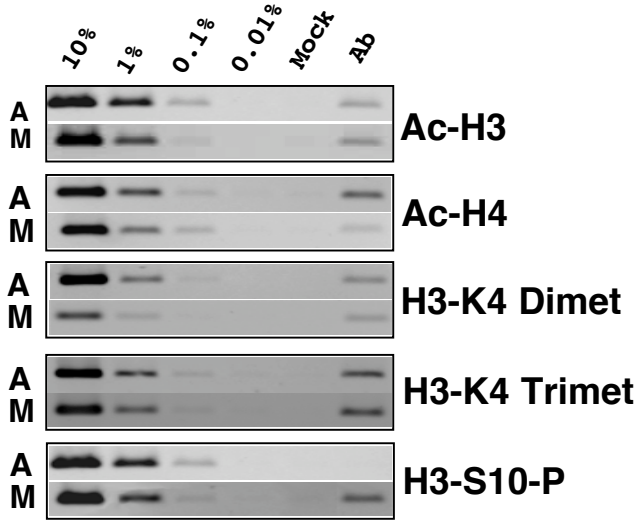
C


DISTAL HSE
SP1
PROXIMAL HSE
SP1
TATA
+1
HSP70 GENE
-279
+24
+1081
+1341


hsp70 Promoter
10%
1%
0.1%
0.01%
Mock
Ab
- +
Ac-H3
Ac-H4
H3-K4 Dimet
H3-K4 Trimet
H3-S10-P


Percentage input (1%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
AcH3
AcH4
dK4
tK4
PH3
Mock
- Heat Sock
+ Heat Shock

D


hsp70 Promoter
10%
1%
0.1%
0.01%
Mock
Ab
A M
Ac-H3
Ac-H4
H3-K4 Dimet
H3-K4 Trimet
H3-S10-P

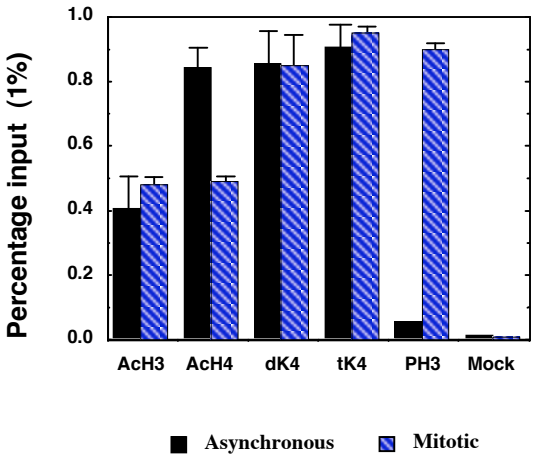
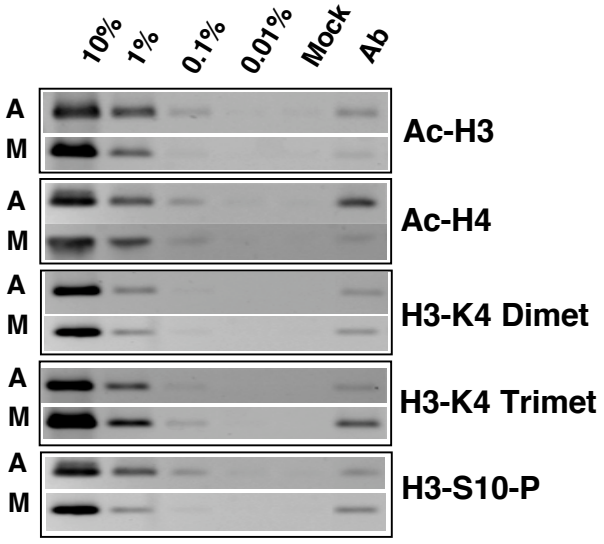
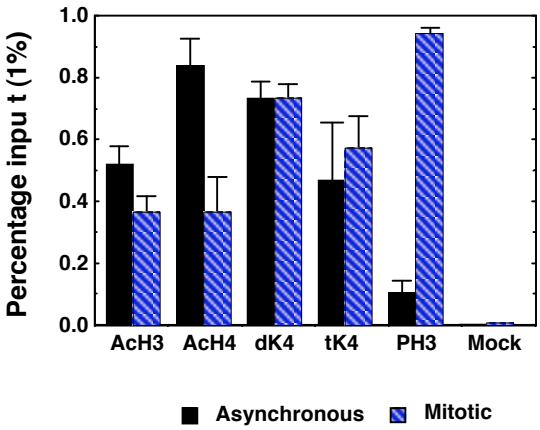

Percentage input (1%)
1.0
0.8
0.6
0.4
0.2
0.0
AcH3
AcH4
dK4
tK4
PH3
Mock
Asynchronous
Mitotic

Figure 3

A

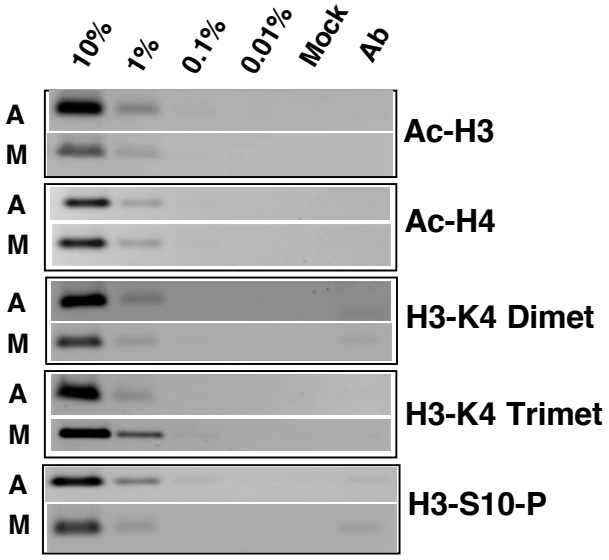
GAPDH Coding Region





B

IL-2 Coding Region



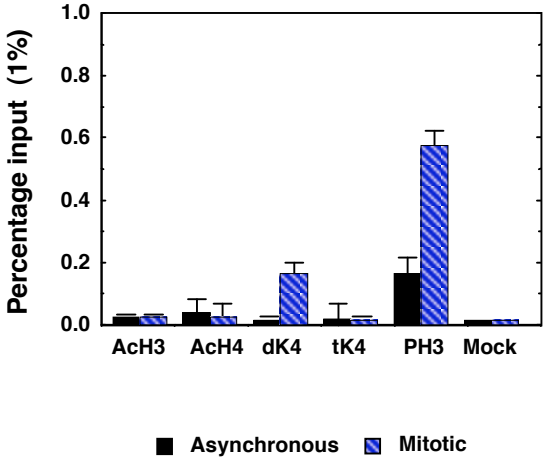
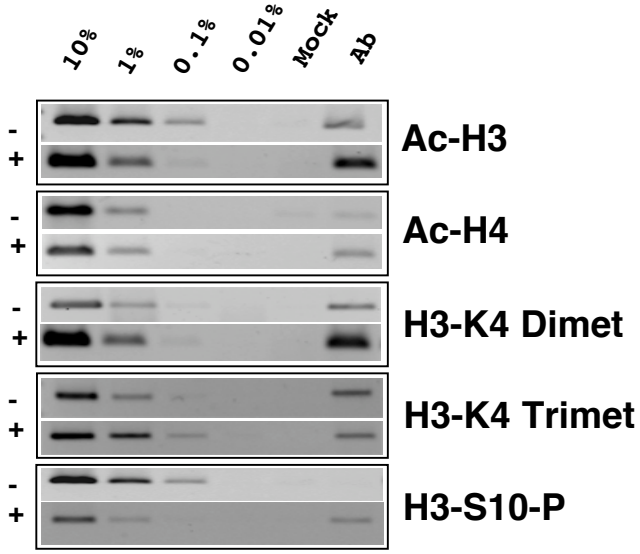
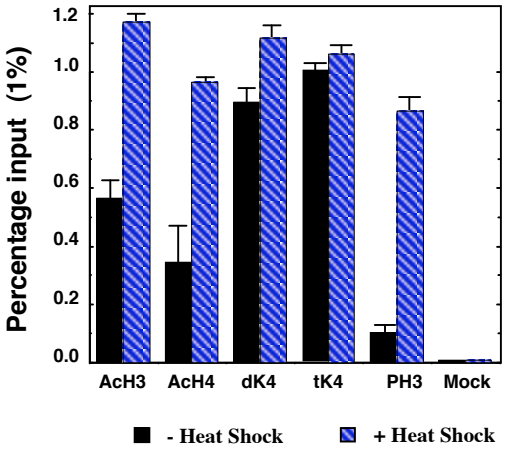


Figure 3

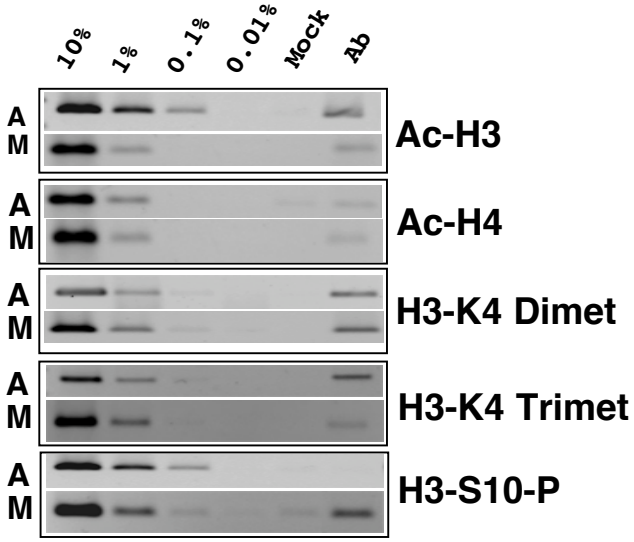
C

hsp70 Coding Region





D



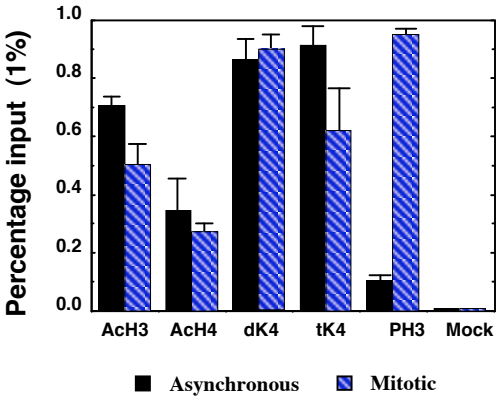


Figure 4

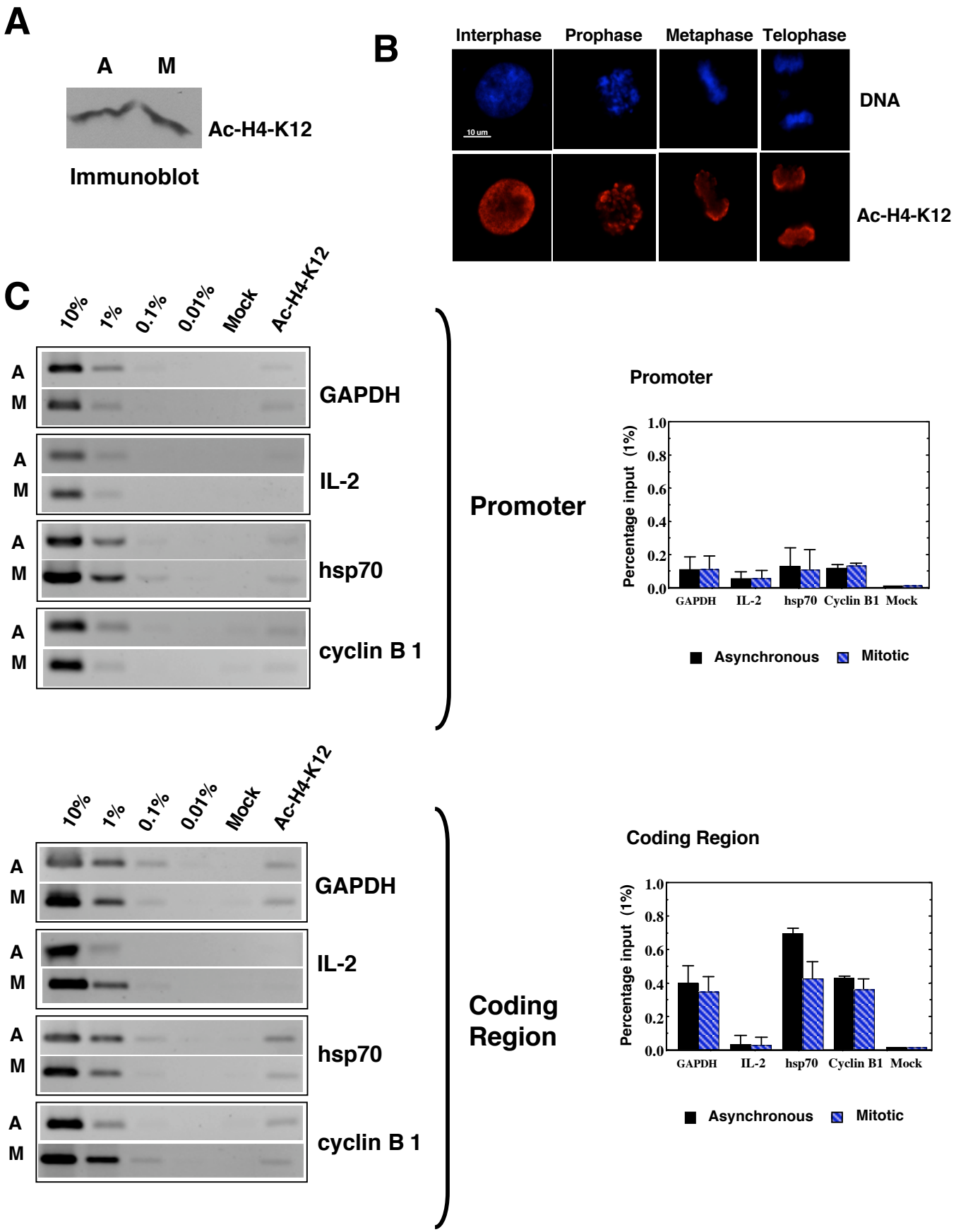
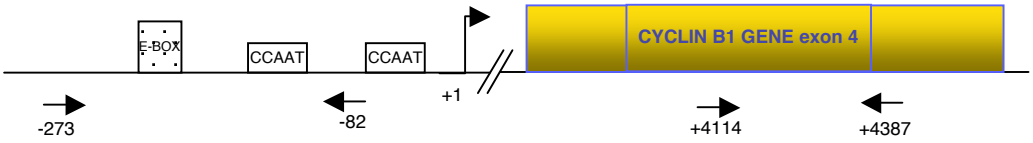
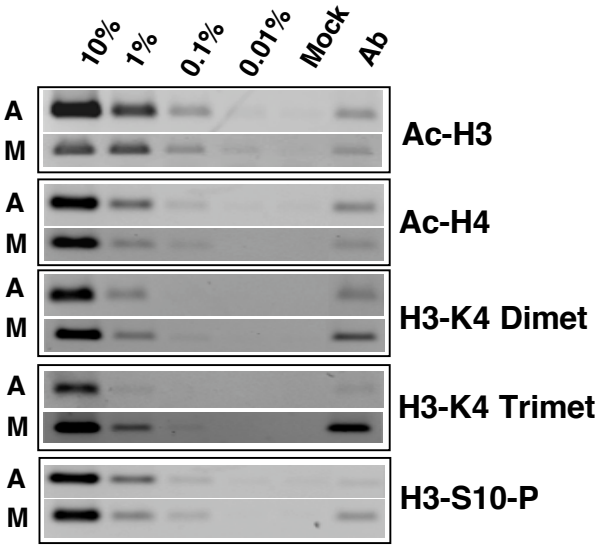

A
A
M
Ac-H4-K12
Immunoblot
B
Interphase
Prophase
Metaphase
Telophase
10 um
DNA
Ac-H4-K12
C
10%
1%
0.1%
0.01%
Mock
Ac-H4-K12
A
M
GAPDH
IL-2
hsp70
cyclin B 1
Promoter
Promoter
Percentage input (1%)
1.0
0.8
0.6
0.4
0.2
0.0
GAPDH
IL-2
hsp70
Cyclin B1
Mock
Asynchronous
Mitotic
Coding Region
Coding Region
Percentage input (1%)
1.0
0.8
0.6
0.4
0.2
0.0
GAPDH
IL-2
hsp70
Cyclin B1
Mock
Asynchronous
Mitotic

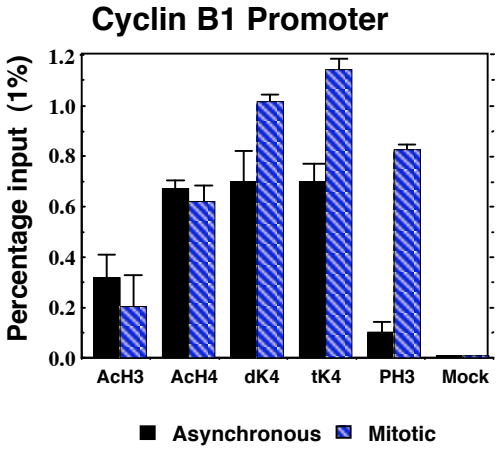
Figure 5

A



Cyclin B1 Promoter





B

Cyclin B1 Coding Region