Histone H1b Phosphorylation Is Dependent upon Ongoing Transcription and Replication in Normal and ras-transformed Mouse Fibroblasts

(Received for publication, January 10, 1997, and in revised form, February 5, 1997)

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We have previously shown that mouse phosphorylated histone H1b (pH1b) was localized near nuclear sites that contained splicing factors. This observation suggested to us that pH1b was associated with transcribing chromatin. Here we investigated the relationship between phosphorylation of H1b and transcription. We demonstrate that treatment of normal or ras-transformed mouse fibroblasts with the transcription inhibitor actinomycin D for 70 min results in a dramatic decrease in the level of pH1b. Similar results were observed when transcription was inhibited by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). When DRB was removed, the level of pH1b was restored after 2 h. Treatment of the cells with aphidicolin, a potent inhibitor of replication, resulted in a marked decrease in the level of pH1b after 30 min. This is the first report showing a dependence of histone modification upon ongoing transcription and replication. Inhibition of transcription or replication may hinder accessibility of H1b to the H1 kinase, supporting the idea that pH1b is associated with transcribing chromatin. Phosphorylation of H1b may be required to maintain a more decondensed chromatin structure to facilitate transcription and replication processes.

Due to their role in chromatin compaction, H1 histones are considered as general repressors of transcription (1). However, H1 is associated with transcriptionally active chromatin (2). Recent studies show that we need to revise our thinking about H1 histones as general repressors to coparticipants with transcription factors in regulating transcription. H1 shares structural features found in transcription factors and can function as a positive or negative gene-specific regulator of transcription (3).

Mouse H1 consists of the subtypes H1a, H1b, H1c, H1d, H1e, and H1f that differ in amino acid sequence (4). There are changes in the pattern of synthesis of different H1 subtypes and a differential loss of subtypes during the formation of nondividing cells. In addition the subtypes differ in the degree and type of phosphorylation during the cell cycle (5). The observation that each H1 subtype has unique properties led to the proposal that they differ from each other functionally (5).

H1 has a tripartite structure consisting of a central globular core and lysine-rich N- and C-terminal domains. These domains interact with linker DNA to stabilize the compaction of chromatin, and they contain the sites of phosphorylation, many of which have cdc2/cdk2 consensus sequence sites. Phosphorylation of H1 may alter its interaction with linker DNA and, in turn, modulate chromatin structure (6). H1 phosphorylation is highest in the M phase of the cell cycle when chromatin is highly condensed; however, chromatin condensation has been shown to occur in the absence of H1 phosphorylation (7). There are a number of examples suggesting that H1 phosphorylation is more likely involved in chromatin decondensation rather than condensation (6). It was proposed that H1 phosphorylation could act as a first step mechanism for inducing chromatin decondensation, which would allow access of factors for gene activation or replication as well as for chromosome condensation (6).

Mouse fibroblast 10T½ cells transformed by ras, fes, mos, myc,raf, or combinations of ras, myc, and mutant p53 have an increased level of pH1b in comparison with the parental cells (8, 9). We have suggested that the increase in pH1b levels is a result of the persistent activation of the MAP2 kinase signal transduction pathway leading to an increase in CDK2/cyclin E-associated H1 kinase activity. In agreement with our results it has recently been demonstrated that fibroblasts lacking the tumor suppressor retinoblastoma susceptibility gene product also exhibit an increased level of phosphorylated H1 and relaxed chromatin structure and that deregulation of CDK2 may be directly involved (10).

Indirect immunofluorescence studies using an anti-pH1b antibody revealed a punctate pattern of nuclear staining in parental and oncogene-transformed mouse fibroblasts (8). This pattern colocalized with the pattern observed when cells were stained with B1C8, an antibody raised against a human nuclear matrix protein that colocalizes with SC-35 domains and co-immunoprecipitates exon-containing RNA from in vitro splicing reactions (11, 12). Recent studies have shown that there is a high degree of spatial association of specific actively transcribed genes with SC-35 domains, whereas the nontranscribed genes do not show preferential association (13). We have therefore suggested that pH1b is complexed to transcriptionally active chromatin, which is localized near sites of RNA splicing (8).

To investigate the relationship of H1 phosphorylation and transcriptionally active chromatin, we treated parental and
ras-transformed 10T½ mouse fibroblasts with the transcription inhibitors actinomycin D and DRB. We monitored the level of pH1b by Western blotting with anti-pH1b, and we found that the inhibition of transcription in parental or ras-transformed mouse fibroblasts resulted in a decrease in the level of pH1b. To our surprise, we found that treatment of parental and ras-transformed cells with the replication inhibitor aphidicolin resulted in a marked decline in pH1b levels.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The CIRAS-2 cell line was derived by transfection of 10T½ cells with the T-24 Ha-ras oncogene (14). 10T½ and CIRAS-2 cell lines were maintained at 37 °C under a humidified atmosphere containing 7% CO₂ in medium supplemented with penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml). Cells grown in α-minimal essential medium plus 10% fetal bovine serum (Intergen, Purchase, NY). Cells were plated in 15 ml of the above medium at 5 × 10⁵ cells/150-mm diameter plastic tissue culture dish. Three days later the medium was replaced, and the cells were subjected to the various treatments described below. After treatment, the medium was removed, and the monolayer was washed twice with phosphate-buffered saline, pH 7.3, and trypsinized. The cells were pelleted, and the pellet was stored at −80 °C.

Manipulation of Cell Lines with Transcription and Replication Inhibitors—Mouse fibroblast 10T½ cells were subjected to the following treatments: (a) 10 μg/ml actinomycin D for 30 and 70 min, (b) 150 μM DRB for 30 min, 70 min, and 2 h, (c) 10 μM aphidicolin for 30 min, or (d) 150 μM DRB and 10 μM aphidicolin for 70 min. Then the medium was removed and replaced with fresh medium without DRB, and the cells were allowed to recover for 2 h. CIRAS-2 cells were subjected to the same treatments as described above except the 150 μM DRB treatment was done for 2- and 3-h time periods.

Isolation of Histone H1—Cells (5 × 10⁵) were seeded in 150-mm plastic tissue culture dishes and grown in 10% fetal bovine serum in α-minimal essential medium and harvested 72 h later by trypsinization. Approximately 4 × 10⁶ to 1 × 10⁶ cells were used for histone preparation. Cells were homogenized in 5 ml of nuclear preparation buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.5 mM MgCl₂, 0.65% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). Nuclei were recovered by centrifugation at 1500 × g for 10 min. All centrifugations were carried out at 4 °C. Nuclei were resuspended in 3 ml of RSB buffer (10 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 10 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride). Nuclei were extracted with 0.4 M H₃SO₄ and then with 5% perchloric acid to isolate H1 (15). The perchloric acid precipitate contained the nucleosomal histones. The supernatant was dialyzed with 0.1 M acetic acid and then distilled H₂O. The samples were lyophilized and resuspended in distilled H₂O.

Electrophoresis and Western Blotting—Proteins were analyzed by AE (acetic acid, 6.7 M urea, 0.375% (w/v) Triton X-100)-15% polyacrylamide gels and SDS-15% polyacrylamide gels. The proteins were recovered by centrifugation at 1500 × g for 10 min. All centrifugations were carried out at 4 °C. Nuclei were resuspended in 3 ml of RSB buffer (10 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 10 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride). Nuclei were extracted with 0.4 M H₃SO₄ and then with 5% perchloric acid to isolate H1 (15). The perchloric acid precipitate contained the nucleosomal histones. The supernatant was dialyzed with 0.1 M acetic acid and then distilled H₂O. The samples were lyophilized and resuspended in distilled H₂O.

Electrophoresis and Western Blotting—Proteins were analyzed by Western blotting (16). The membrane containing H1 was immunochemically stained with the anti-phH1b IgG and goat anti-rabbit antibody linked to horseradish peroxidase (Sigma) using the ECL detection system (Amersham Corp.). Equal amounts of protein were loaded on an SDS-15% polyacrylamide gel, and the gel was stained with Coomassie Blue. The relative amount of pH1b was determined by scanning the autoradiogram obtained from Western blots with anti-phH1b. A 3250F densitometer (protein and DNA Imagewage Systems, Huntington Station, NY) was used for scanning and densitometric analysis was performed using the ImageMaster 1-D software (Pharmacia Biotech Inc.). The relative level of pH1b in each sample was determined as described previously (8). Each experiment was performed in triplicate, and the values obtained for pH1b levels represent the average of three separate experiments.

RESULTS AND DISCUSSION

Actinomycin D at 10 μg/ml strongly inhibits RNA polymerase I and RNA polymerase II transcription (17). 10T½ and CIRAS-2 cells were treated with 10 μg/ml actinomycin D for 30 and 70 min. H1 isolated from these cells was electrophoretically resolved on 15% SDS-polyacrylamide gels, transferred to membranes, and immunochemically stained with anti-phH1b. Fig. 1 (A and B) shows the position of migration of H1b, H1c, and H1d, H1e, and H1f subtypes on an SDS-15% polyacrylamide gel. The relative level of pH1b in treated cells was compared with that of untreated cells and was determined as described previously (8). Incubation of 10T½ cells with 10 μg/ml actinomycin D resulted in a decrease in the level of pH1b equivalent to 0.12 ± 0.01 (n = 3) of the control after 30 min, whereas after 70 min the pH1b signal was undetectable. In the CIRAS-2 cells there was no decrease in the level of pH1b after 30 min; however, after 70 min the level was reduced to 0.20 ± 0.01 (n = 3). Transcriptionally active genes are often located near sites of RNA splicing (13). Our previous observation of pH1b being co-localized with RNA splicing domains suggested to us that pH1b may be bound to transcriptionally active chromatin. The present data showing that levels of pH1b are dependent upon ongoing transcription adds further support for this association. The only other histone modification known to be dependent upon ongoing transcription is ubiquitination of histone H2B (18).

The adenosine analog DRB selectively inhibits transcription by RNA polymerase II (19). 10T½ cells were treated with 150 μM DRB for 70 min and 2 h (Fig. 2). After 70 min the level of pH1b was reduced to 0.13 ± 0.01 (n = 3) of the control. The signal was undetectable after a 2-h treatment. CIRAS-2 cells were treated for 2- and 3-h time periods. Fig. 2 shows that after 2 h the level of pH1b was reduced to a level 0.53 ± 0.04 (n = 3) of that of the control, and this level remained the same even after a 3-h treatment.

DRB is a reversible inhibitor of RNA polymerase II. We wanted to determine whether the level of pH1b was restored in the DRB-treated cells when DRB was removed. We incubated 10T½ cells in the presence of 150 μM DRB for 70 min and then removed the DRB containing medium and replaced it with fresh medium that did not contain DRB. The cells were allowed to recover for 2 h. Fig. 3 shows that after a 2-h period of time the level of pH1b had been restored.

Aphidicolin is a potent inhibitor of DNA polymerase α and inhibits DNA replication (20, 21). 10T½ and CIRAS-2 cells were incubated for 30 and 70 min in the presence of 10 μM aphidicolin. Fig. 4 shows that inhibition of replication by aphidicolin resulted in a dramatic reduction in the level of pH1b after 30 min in both the 10T½ parental and the CIRAS-2-transformed cell lines. In 10T½ cells the level of pH1b was reduced to a level 0.08 ± 0.01 (n = 3) of that of the control and...
in CIRAS-2 cells to a level 0.23 ± 0.02 (n = 3) of that of the control after 30 min. Inhibition of replication results in a dramatic decrease in the level of pH1b.

The effect of the phosphorylation of H1 on chromatin structure and chromatin replication has been investigated. Ajiro et al. have studied the phosphorylation sites of human histones H1A and H1B during the HeLa S-3 cell cycle (22). They observed two different S phase phosphorylations, one that precedes and one that follows the onset of replication. This led to the suggestion that the phosphorylation of H1 at a particular site on the C-terminal may produce a chromatin conformation that permits DNA replication (22). Further evidence that supports the involvement of H1 phosphorylation in the regulation of chromatin structure during replication comes from studies done with salt-treated SV40 minichromosomes reconstituted with differentially phosphorylated forms of H1. The minichromosomes reconstituted with S phase H1 had a higher replication efficiency than those reconstituted with G0 or M phase H1 (23). It was suggested that the chromatin structure induced by the phosphorylation of H1 affects the efficiency of replication.

It has been determined that the accessibility of H1 to the H1 kinase is directly related to its position in the chromatin fiber and that H1 must be displaced from the chromatin fiber in order for it to be phosphorylated by an H1 kinase (24). Transcription and replication processes may be the initial disruptive events that lead to the displacement of H1b from the chromatin fiber and render it accessible to the H1 kinase. Further H1 phosphorylation may then be required to maintain an open chromatin conformation for replication and transcription. It has been proposed that phosphorylation of H1 may be involved in inducing chromatin decondensation, thereby allowing access to factors for gene expression or replication in addition to chromatin condensation at mitosis (6). We propose that when transcription or replication processes are inhibited, H1b is no longer exposed to the H1 kinase. Dephosphorylation by protein phosphatases would then reduce the steady state levels of pH1b.

The effect of transcription and replication inhibition on pH1b levels in 10T½ and CIRAS-2 cells differs in terms of time to elicit reduced levels of pH1b. Both aphidicolin and actinomycin D or DRB take longer to act in the CIRAS-2 cells. The chromatin of ras-transformed mouse fibroblasts is less condensed than the chromatin of parental cells (8, 25). We proposed that oncogene activation of the MAP kinase signal transduction pathway increases the activity of an H1 kinase, resulting in elevated levels of phosphorylated H1 and decondensation of chromatin (8). This increased kinase activity and less condensed chromatin structure may contribute to the extended times required to observe a reduced level of pH1b when transcription or replication is inhibited. The extended response time to see a reduction in pH1b levels may also reflect a reduced protein phosphatase activity. Protein phosphatase 1 is thought to be responsible for dephosphorylating H1 (26). Protein phosphatase 1 activity is reduced when it is phosphorylated by cyclin-dependent kinases.

**FIG. 2. Effect of inhibition of transcription with DRB on level of H1b phosphorylation.** H1 was extracted from 10T½ cells (A) that were untreated (lane 1) or incubated with 150 μM DRB for 70 min (lane 2) or 2 h (lane 3). H1 was extracted from CIRAS-2 cells (B) that were untreated (lane 1) or treated with 150 μM DRB for 2 (lane 2) or 3 h (lane 3). H1 (2 μg) was electrophoretically resolved on SDS-15% polyacrylamide gels, transferred to membranes, and immunochemically stained with anti-pH1b. A and B show Coomassie Blue-stained gels. C and D show the immunochemically stained membranes. pb is the phosphorylated isoform of H1b.

**FIG. 3. Effect of removal of DRB treatment on level of H1b phosphorylation.** H1 was extracted from 10T½ cells that were untreated (lanes 1), incubated with 150 μM DRB for 70 min (lanes 2), or 70 min and a 2-h recovery (lanes 3) as described under “Materials and Methods.” H1 (2 μg) was electrophoretically resolved on SDS-15% polyacrylamide gels, transferred to membranes, and immunochemically stained with anti-pH1b. A shows the Coomassie Blue-stained gel. B shows the immunochemically stained membrane. pb is the phosphorylated isoform of H1b.

**FIG. 4. Effect of inhibition of replication with aphidicolin on level of H1b phosphorylation.** H1 was extracted from 10T½ (A) and CIRAS-2 (B) cells that were untreated (lanes 1) or incubated with 10 μM aphidicolin for 30 min (lanes 2). H1 (2 μg) was electrophoretically resolved on SDS-15% polyacrylamide gels, transferred to membranes, and immunochemically stained with anti-pH1b. A and B show Coomassie Blue-stained gels. C and D show the immunochemically stained membranes. pb is the phosphorylated isoform of H1b.
Thus, oncogene activation of the MAP kinase signal transduction pathway may result in the activation of cyclin-dependent kinases and deactivation of protein phosphatase 1.

In summary, we have demonstrated that the maintenance of levels of phosphorylated H1b in chromatin is dependent on ongoing transcription or replication. This observation suggests that pH1b is associated with transcriptionally active chromatin. Therefore, we propose that H1b phosphorylation may be required to maintain an open chromatin structure, thus facilitating subsequent rounds of transcription or replication.

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