Phosphorylation of Histone H2A Inhibits Transcription on Chromatin Templates†§

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The regulation of gene expression via the histone code has, for the most part, revealed that histone modifications cause the recruitment of adaptor proteins that indirectly regulate the synthesis of RNA. Using purified factors to assemble and modify the chromatin and to transcribe the DNA, we investigated whether modifications of histones may directly impact the RNA polymerase II transcription process. We screened proteins known to modify histones for effects on transcription, and we found that the mitogen- and stress-induced kinase, MSK1, inhibited RNA synthesis. Inhibition of transcription by MSK1 was most sensitive when the template was in chromatin, as naked DNA templates were resistant to the effects of MSK1. We found that MSK1 phosphorylated histone H2A on serine 1, and mutation of serine 1 to alanine blocked the inhibition of transcription by MSK1. Furthermore, we found that acetylation of histone H3 by the p300 and CREB-binding protein (CBP) acetyltransferase, PCAF, suppressed the kinase-dependent inhibition of transcription. These results suggest that acetylation of histones may stimulate transcription by suppressing an inhibitory phosphorylation by a kinase as MSK1.

In eukaryotic cells, genomic DNA is ensheathed in chromatin. The core component is the histone octamer bound to DNA to comprise the nucleosome. Nucleosomes are in turn spaced and arrayed by nuclear machinery to effectively open or close the DNA to facilitate or repress gene expression. It has become clear that histone proteins become tagged to signal the activation or repression of genes, and the covalent tags have become known as the histone code (1, 2). Modifications, which include acetylation, methylation, phosphorylation, and ubiquitination, result in binding by other chromatin effector proteins to add (or remove) other tags or modify the chromatin, or the tag may be bound by a transcription factor that catalyzes the initiation of RNA synthesis. As an example, phosphorylation of H3 at serine 10 stimulates acetylation of lysine 14 of H3, which in turn recruits TFII D (3).

Because of the complex nature of the histone code, in which protein complexes are recruited to a gene and modify the chromatin to recruit another complex, it has been primarily studied in the living cell. The histone code, and its linkage to transcription, has been studied in cell-free systems, but in each case crude nuclear extracts were used as sources for protein factors (3–10). As an example, activator-dependent p300-mediated acetylation of histones in reconstituted DNA templates resulted in a powerful stimulation of transcription (6).

Phosphorylation of histones can also be an important regulatory signal. Histone H3 phosphorylation is known to play important roles in both transcription and chromosome condensation during mitosis (11–14). Histone H2B phosphorylation condenses the chromatin and is involved in apoptosis (15, 16). Phosphorylation of H2A and H4 occur, but the downstream effects of phosphorylation of these subunits are unknown. In this study, the mitogen- and stress-induced kinase, MSK1, a mitogen-activated protein kinase that phosphorylates histone H3 in vitro (17, 18), was tested for function in transcription reactions using purified chromatin components and purified RNA polymerase II transcription factors. The MSK proteins have been shown to stimulate transcription in cells via phosphorylation of H3 serine 10, and it has recently been found that this activity primarily resides in the MSK2 protein (18). In the current study, we found, to our surprise, that the MSK1 protein negatively regulated transcription via a phosphorylation of H2A, and we found that the acetylation state of the nucleosomes affected this phosphorylation reaction.

EXPERIMENTAL PROCEDURES

Plasmid Templates—G-less cassette construct G5E4 contained GAL4 response elements, the adenosiviral E4 promoter, and a sequence containing no guanines in the coding strand from the transcription start site to 384 base pairs downstream. All transcription reactions include an internal basal control template, pML-200, which encoded a 210-base-pair G-less cassette.

Histone Octamer Preparations—HeLa core histones were purified from the chromatin pellet of whole cell extracts (19) from HeLa cells or butyrate-treated HeLa cells using hydroxyapatite chromatography (20). Full-length histone genes were isolated using full-length IMAGE clones purchased from Invitrogen. The human histone genes were subcloned into pET-21a for expression as untagged proteins in bacteria. IMAGE clones used were: 2988620 (H2A), 2989839 (H2B), 2988279 (H3), and 5113140 (H4). The sequences of the expression constructs were confirmed. Details of the subcloning can be obtained from the authors upon request. Individual histone proteins were purified from inclusion bodies, purified by ion exchange chromatography, mixed with the other

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† The abbreviations used are: TF, transcription factor; MSK, mitogen- and stress-induced kinase; PCAF, p300 and CREB-binding protein associated factor; ACF, ATP-utilizing chromatin assembly and remodeling factor; TAU, Tryptic/acid/urea; nt, nucleotide(s); HAT, histone acetyltransferase.
Histone proteins, slowly renatured, and ~110-kDa histone octamers were isolated by gel filtration, essentially according to published methods (3).

**Protein Purification**—Recombinant chromatin assembly proteins (21), recombinant PCAF (22), and transcription factors (23) were purified by established procedures.

**Chromatin Assembly**—Reconstitution of chromatin using purified histone octamers, DNA templates, and assembly factors was done essentially as described in Ito et al. (21). Briefly, each chromatin assembly reaction (30 μl) contained 0.5 μg of pG5E4, 0.5 μg of pML-200, and 1.4 μg of histone octamers. For recombinant human histones, histone octamer preparations were premixed with 100 nM NAP-1 for 30 min on ice, then incubated with 4 nM Acf-1, 7 nM imitation switch, and DNA templates at 27 °C for 5 h in buffer containing 10 mM HEPES, pH 7.6, 50 mM KCl, 5% glycerol, 1% polyvinyl alcohol, 1% polyethylene glycol, 25 mM MgCl₂, and 4 mM ATP. For histone octamer purified from HeLa cells or butyrate-treated HeLa cells, histones were premixed with 2 μM NAP-1 and then incubated with 10 nM Acf-1, 18 nM imitation switch. When PCAF was tested, histone octamer was first incubated with PCAF and 10 μM acetyl-CoA (Amersham Biosciences) in the assembly buffer for 1 h at 30 °C, followed by the chromatin assembly reaction.

**In Vitro Transcription Assay**—Transcription reactions were performed in a 25-μl volume containing 20 mM Tris-OAc, pH 7.9, 20% glycerol, 1 mM EDTA, 6 mM Mg(OAc)₂, 90 mM KOAc, 0.75 μg of histone (free histone or chromatin), 4 mM ATP, the indicated amount of kinase, and 10 μCi of [γ⁻³²P]ATP (3000 Ci/mmol, Amersham Biosciences). The reactions were incubated at 30 °C for 60 min. After precipitation with 20% trichloroacetic acid and washing with 100% acetone, the pellets were dissolved in the appropriate loading buffer and resolved by SDS-PAGE (17% gel) or Triton/acid/urea (TAU) gel electrophoresis (25). Gels were stained with Coomassie Blue R-250 and destained before drying for autoradiography.

**RESULTS**

**MSK1 Inhibits Transcription from Chromatin Templates**—We tested several enzymes, which are known to modify histones, for effects on *in vitro* transcription assays. We found that one of these, MSK1, exerted a strong inhibitory effect on RNA synthesis. The transcription factors were all purified mammalian proteins: TFIIA, -B, -D, -E, -F, -H, core RNA polymerase II, and cofactor PC4. Histone octamers were initially purified from HeLa cells, and these octamers were assembled into DNA-bound chromatin using ACF and nucleosome assembly protein 1 (NAP1) (21). Micrococcal nuclease assays of these templates revealed that these templates contained a high quality of chromatin (Supplementary Materials Fig. S1), and by other measures developed in this study (see below), the histone octamers were effectively converted to nucleosomes on DNA. We had earlier shown that transcription from chromatin templates requires a topoisomerase activity for elongation (24), but when using the ACF chromatin assembly system, we found that the topoisomerase was not absolutely required as there was a low level of transcription, and the topoisomerase was stimulatory to transcription on these templates (23) and data not shown). In the described experiments in this study, the effects of the tested histone modifications on the transcription reaction were the same regardless of the inclusion of topoi-
somerase (data not shown), and for the sake of maintaining as simple a transcription system as possible we omitted the topoisomerase from the assembly and the transcription reactions used in the figures.

We tested whether MSK1 can modulate transcription in this purified factor system from these chromatin templates using HeLa-derived histones. We had anticipated that MSK1 would stimulate RNA synthesis because modification of H3 by MSK factors has been reported to be stimulatory to transcription (18). To our surprise, MSK1 inhibited the transcription activated by GAL4-VP16 on chromatin templates (Fig. 1A). Transcription reactions were identical and only differed in the state of the DNA (naked in lanes 1-4 and chromatin in lanes 5-8) and in MSK1 concentration. At high MSK1 concentrations, transcription on naked templates was modestly inhibited, and this was probably because of either an inhibitory modification of a transcription factor, or a nonspecific inhibition because of the MSK1 preparation. By contrast, the inhibition of transcription by MSK1 was more profound and occurred at lower concentrations of kinase in identical reactions except that the template was chromatin. The levels of RNA synthesis were very consistent, and results from three or more repeat experiments, were quantified in Fig. 1D. The most striking effect of MSK1 was observed when quantifying the levels of activated transcription, rather than total transcription. The values represent the ratio of GAL4-VP16-stimulated transcription (390-nt band) to basal transcription (210-nt band), and the ratio was set at 100% in the absence of MSK1 (Fig. 1D). On chromatin DNA templates, compared with the absence of MSK1, GALA-VP16-stimulated transcription was repressed more than 60% in the presence of 16 nM MSK1, a significant difference from the inhibition of transcription on naked DNA templates by MSK1.

We next tested whether the acetylation of histones in chromatin affects the inhibition of transcription by MSK1. Histones purified from butyrate-treated HeLa cells were hyperacetylated (Supplementary Materials Fig. S2), and these were assembled into chromatin and tested in transcription. We found that the hyperacetylated histone octamers enhanced GALA-VP16-stimulated transcription (Fig. 1B, lanes 1 and 5) and significantly reversed the inhibition of transcription by MSK1 (Fig. 1, B and D). This result suggested that the acetylation of histones could negatively affect the inhibition of transcription by MSK1. Because both histone preparations, HeLa and But-HeLa, contained acetylated proteins, and because bacterially expressed recombinant factors lack modifications such as acetylation, we tested the role of acetylation of histones in this reaction using recombinant human histones purified from bacteria. When using chromatin containing recombinant human histones the inhibitory effects of MSK1 occurred at a lower concentration of MSK1 and to a higher level of total inhibition than with chromatin containing histones purified from HeLa cells (Fig. 1, C and D). At the highest concentration of MSK1 tested, the inhibition of transcription by MSK1 was ~80% when the chromatin contained recombinant histones, ~60% with HeLa histones, and less than 40% with either hyperacetylated HeLa histones or naked templates. The reaction conditions were equivalent with regard to basal transcription factors, the DNA templates, assembly factors, and MSK1. The only differences were the state of the histones in the chromatin. These results indicated that MSK1 inhibited transcription with chromatin templates. In addition, pre-existing acetylation of histones on chromatin could block the inhibition of GALA-VP16-stimulated transcription by MSK1.

Inhibition by MSK1 Is Specific for Transcription Stimulated by GALA-VP16—Both GALA-VP16-activated and basal transcription decreased as MSK1 was added to the chromatin-containing reaction but the stimulated transcription was disproportionately affected (Fig. 1). Because the longer transcript was more sensitive to the effects of MSK1, either the elongation rate or the activation process was negatively affected by the MSK1 kinase. To determine the role of GALA-VP16 in the inhibition of transcription, the inhibitory effects of MSK1 were tested in the absence of GALA-VP16 using the chromatin containing recombinant histones. Under these basal transcription conditions, MSK1 modestly decreased the levels of RNA product, but the effects on the 390-nt transcript were balanced by the effects on the 210-nt internal control RNA. By contrast, the levels of transcription of the GALA-VP16-stimulated RNA were disproportionately inhibited by the MSK1 (Fig. 2, A and B). Visual inspection of the gel reveals that the presence of the GALA-VP16 activator strongly stimulated transcription of the 390-nt RNA (lane 5), but in the presence of 16 nM MSK1 the level of synthesis of the 390-nt RNA was equal to the basal levels (compare lane 8 to lane 4). These results indicated that inhibition of transcription by MSK1 directly affected the transcriptional activation process.

Substrate Specificity of MSK1 Kinase—MSK1 and MSK2 have been shown to be histone H3 kinases, but in living cells, MSK2 was the major H3 kinase (17, 18). To confirm that the H3 subunit was phosphorylated by MSK1 and to test whether this modification was responsible for the inhibition of activated

**Fig. 2.** MSK1 activity inhibits transcription stimulated by GALA-VP16. A, chromatin templates assembled with recombinant human histones were tested in transcription reactions without (lanes 1–4) or with (lanes 5–8) GALA-VP16. MSK1 was included as indicated. The exposure time of the autoradiograph in lanes 1–4 was longer than for lanes 5–8. B, quantitative analysis of the effects of MSK1 on GALA-VP16-stimulated transcription. The results are based on three repeat experiments, and the relative transcription levels were calculated as in Fig. 1D.
transcription, kinase assays were performed using histone octamers purified from HeLa cells or from recombinant proteins expressed in bacteria. Consistent with prior observations, free histone octamers from both preparations were primarily modified on H3, with lower levels of phosphorylation detected on each of the other histones (Fig. 3A, lanes 1 and 2). Because the modifications of histones were poorly resolved by standard SDS-PAGE, we confirmed the identities of phosphorylated histones using a TAU gel (Fig. 3B, lanes 1 and 5). The identities of the phosphorylated species were readily assigned in the case of the recombinant histones, because the composition of these histones is uncomplicated (lanes 1 and 3). The interpretation of the HeLa-derived phosphorylated histones was more complex because multiple histone variants and modifications were present.

Association of histones with DNA has been observed to affect the specificity of their modifications (26). We tested whether the phosphorylation of histones by MSK1 was affected by association with DNA in chromatin. Surprisingly, phosphorylation of H3 by MSK1 was completely repressed in chromatin. Instead, the principal phosphorylated product was H2A when using recombinant human histones, and in chromatin from HeLa histones the main phosphorylated products were H4 and to a significantly lesser extent H2A (Fig. 3A, lanes 3 and 5). The sequences of the amino termini of H4 and H2A amino termini have similar effects. We do not understand why phosphorylation of H4 was only observed in the HeLa-derived nucleosomes, but we suggest that the multiple histone variants and multiple modifications found in a cell (Fig. 3B and Supplementary Materials Fig. S2) cause this effect. That H2A was the principal phosphorylated histone of MSK1 when using the chromatin from recombinant histones was confirmed by a TAU gel (Fig. 3B, lane 2). The nearly complete loss of phosphorylation of histone H3 suggested that there were few free histone octamers remaining in the chromatin preparations. Taken together, these results suggested that MSK1 repressed transcriptional activation on chromatin through phosphorylation of H2A. The quantitative difference between the effects on transcription of MSK1 on recombinant human histones and HeLa histones may reflect differences in phosphorylation of H2A.

**Acetylation of Histones Modulated the Effects of the MSK1 Kinase**

The data in Fig. 1 suggested that acetylation of histones decreased the inhibitory effect of MSK1 activity on transcription. We tested candidate HAT proteins for modulating the effects of MSK1 by inclusion in the chromatin assembly reaction and then assaying in transcription. Acetylation of recombinant histones by the 93-kDa PCAF polypeptide stimulated transcription (Fig. 4A, lanes 1 and 5), which is consistent with the known stimulatory effect of acetylation on transcription in general. Of interest, this stimulatory effect of PCAF on transcription, independent of MSK1, was not observed with histone octamers purified from HeLa cells (data not shown), suggesting that the effects of the HAT activity were most effective if there were no baseline acetylations of the histones. As was predicted from the results of Fig. 1, pretreatment of histones with PCAF decreased the inhibitory effect on transcriptional activation by MSK1 (Fig. 4A). The results were highly reproducible, and significantly shifted the curve plotting the inhibition by MSK1 (Fig. 4B). The concentration of MSK1 at which the reaction was inhibited by 50% shifted from 5.5 to 8.5 nM. The magnitude of the effect of PCAF on the inhibition of transcription by MSK1 was small when comparing these results with those obtained using HeLa histones purified from butyrate-treated cells. This result was expected because the recombinant PCAF subunit would only affect a small percentage of the possible acetylations and with low efficiency. By comparison, the HAT domain of CBP did not affect the inhibition of transcription by MSK1 (data not shown). A protein kinase assay revealed that PCAF decreased the level of phosphorylation of H2A by MSK1 (Fig. 4C). Consistent with prior results (22), PCAF acetylated primarily histone H3 (Fig. 4D), suggesting that the effect of PCAF on the phosphorylation activity was not via a single polypeptide, but rather that there is a trans effect of acetylation of one histone subunit and phosphorylation of another.

**Serine 1 of H2A Is the Target of the MSK1 Kinase**

H2A serine 1 was mutated to alanine, and histone octamers containing H2A-S1A were assembled into chromatin similarly as were the recombinant histones with wild-type sequence (Supplementary Materials Fig. S1). Both, wild-type and mutant, nucleosomes repressed transcription, relative to naked DNA (Fig. 5A). Whereas MSK1 inhibited transcription from chromatin...
containing wild-type recombinant histones, the synthesis of the 390-nt RNA from chromatin containing H2A-S1A was comparatively resistant to MSK1 (Fig. 5A, lanes 7 and 8). Independent of MSK1, transcription from either, wild-type or mutant, chromatin was repressed, but the mutant H2A-S1A consistently resulted in a slightly higher level of transcription. We endeavored to make the level of transcription from the H2A-S1A mutant histones equivalent to the level of transcription from the wild-type chromatin by including a higher amount of these mutant histone octamers in the chromatin assembly reaction. When the concentration of histone octamers used in assembling the chromatin was increased by 50% for the mutant H2A-S1A, the level of transcriptional repression was more pronounced, but the transcription was still resistant to the effects of MSK1 (Fig. 5A, lanes 8–11). The modest inhibition in the accumulation of the 390-nt transcript in reactions containing the H2A-S1A was offset by a similarly modest inhibition of the accumulation of the 210-nt basal control transcript. Quantified results of three repeat experiments revealed a significant shift in the inhibition of transcription by MSK1 (Fig. 5B). Analysis of the phosphorylation products by MSK1 revealed that free histone octamers had equally intense labeling of H3, when comparing the wild-type and mutant histones. By contrast, the level of H2A labeling was greatly decreased (Fig. 5C), supporting that serine 1 is the principal site of phosphorylation on H2A in free histones by MSK1. When the histones were bound to DNA in chromatin, the MSK1 kinase only labeled the H2A on the wild-type nucleosome (Fig. 5C). These data reveal that H2A serine 1 phosphorylation directly represses transcription on chromatin.

**DISCUSSION**

It was anticipated that phosphorylation of histones would stimulate transcription, because it has been shown that MSK factors phosphorylate histone H3 and activate transcription (17). The H3 kinase activity was most robust with the MSK2 kinase, rather than with MSK1 (18). To our surprise, we found that MSK1 more efficiently catalyzed the phosphorylation of H2A at serine 1, and this modification rendered the substrate less efficient as a template for transcription. An intriguing aspect of these results was that GAL4-VP16-stimulated transcription was disproportionately affected when compared with basal transcription. Thus, whatever feature is activated by GAL4-VP16 in the transcription process, it is reversed by the phosphorylation of H2A. The data in this study do not, however, reveal whether MSK1 in living cells in fact participates in the silencing of genes by the phosphorylation of H2A, just that phosphorylation of H2A inhibits transcription. In the cell, another kinase could execute this function. Indeed, other kinases, including RSK2 and Aurora-B, can modify H2A and inhibit transcription in vitro similarly (data not shown).

This effect of MSK1 on transcription was entirely dependent upon the form of the template. Templates containing nucleosomes were very sensitive to transcription inhibition by MSK1 kinase, whereas naked DNA templates were resistant to inhibition by the MSK1 activity. It is formally possible that MSK1 phosphorylates a basal transcription factor when in the context of histones, but the simpler explanation is that the histones themselves were the target of MSK1 that mediated the transcription inhibition. Because acetylation of the histones or mutation of serine 1 of H2A rendered the template resistant to the effects of MSK1, it is most likely that the effect of MSK1 on transcription was mediated by the phosphorylation of histones. When the histones were recombinant, then H2A was solely responsible for the effect. The results were more complex when the histones were derived from HeLa cells because H4 was also phosphorylated. Whether the H4 phosphorylation was important for the inhibition of RNA synthesis could not be tested because mutations could not be made from the HeLa-derived H4. We suggest that the nearly identical amino termini of H2A and H4 cause the similar inhibition of transcription when...
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FIG. 5. Serine 1 of H2A is required for inhibition of transcription by MSK1. A, transcription on templates containing H2A-S1A was not inhibited by MSK1. Templates for transcription reactions included naked DNA (lanes 1 and 2), chromatin reconstituted using wild-type human histones (lanes 3–5), chromatin reconstituted using H2A-S1A (lanes 6–8), or histone octamers with H2A-S1A at a 50% excess relative to the samples in lanes 6–8 (lanes 9–11). MSK1 kinase assay using the various histones were assayed by TAU gel: wild-type human histones associated with DNA in chromatin (lane 1), histones containing H2A-S1A in chromatin (lane 2), wild-type human histone octamers in the absence of DNA (lane 3), and histones containing H2A-S1A as free octamers (lane 4). The positions of unmodified histones are indicated. Lanes 1–4, lanes 6, lanes 8, lanes 10, lanes 12. B, three experiments as in panel A were quantified for the effect of MSK1 on GAL4-VP16-stimulated transcription. C, MSK1 kinase assay using the various histones were assayed by TAU gel: wild-type human histones associated with DNA in chromatin (lane 1), histones containing H2A-S1A in chromatin (lane 2), wild-type human histone octamers in the absence of DNA (lane 3), and histones containing H2A-S1A as free octamers (lane 4). The positions of unmodified histones are indicated. Lanes 1 and 2 were exposed to film for a longer period of time than lanes 3 and 4.

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phosphorylated by MSK1. However, we cannot explain why H4 was not phosphorylated in the recombinant histones except to posit that some other modification in the cellular histones renders the H4 phosphorylation by MSK1 favorable.

Acetylation of histones, particularly hyperacetylated chromatin rendered the template significantly resistant in transcription assays to the effect of MSK1 (Fig. 1B). The acetylation of H3 by recombinant PCAF partially inhibited the phosphorylation of H2A by MSK1 (Fig. 4). These results are consistent with an early finding by the Allfrey lab (27) in which hyperacetylation of H2A phosphorylation of H2A caused a decrease in the activation of transcription by GAL4-VP16. Second, acetylation of histones inhibited H2A phosphorylation. Third, when using recombinant histones, which completely lack acetylated modifications, the impact of acetylation by PCAF was revealed in the absence of the inhibitory kinase. Both basal and activated transcription were boosted by treatment of the chromatin with PCAF. Taken together, these results reveal modifications of histones that directly alter the template and its response to a transcriptional activator.

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