Nucleic Acids Research, 2003, Vol. 31, No. 23 ***6741–6747***
DOI: 10.1093/nar/gkg909

Dynamic methylation of histone H3 at lysine 4 in transcriptional regulation by the androgen receptor

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Received September 18, 2003; Revised and Accepted October 15, 2003

ABSTRACT

The methylation of histone H3 correlates with either gene expression or silencing depending on the residues modified. Methylated lysine 4 (H3-K4) is associated with transcription at active gene loci. Furthermore, it was reported that trimethylated but not dimethylated H3-K4 is exclusively associated with active chromatin in *Saccharomyces cerevisiae*. In the present study, we investigated the H3-K4 methylation at the human prostate specific antigen (PSA) locus following gene activation and repression via androgen receptor (AR). We show that ligand-induced, AR-mediated transcription was accompanied by rapid decreases in di- and trimethylated H3-K4 at the PSA enhancer and promoter. Moreover, the observed decreases in H3-K4 methylation were reversed when AR was inhibited by a specific AR antagonist, bicalutamide. In contrast to the decreases in methylation at the 5′ transcriptional control regions of the PSA gene, H3-K4 methylation in the coding region steadily increased after a lag period of ~4 h. The results suggest a novel role of methylated H3-K4 in transcriptional regulation.

INTRODUCTION

Chromatin, a highly structured and regulated polymer of genomic DNA, histones and non-histone proteins, is the physiological template of all eukaryotic genetic information. Recent advances have firmly established that, beyond its architectural role, chromatin plays a more specific role in the regulation of gene expression. Central to this regulation is the dynamic organization and modification of nucleosomes, which are the basic repeating unit of chromatin and are comprised of 146 bp of DNA wrapped around histone octamers. Structural analysis of the nucleosome has revealed that the N-terminal tails of histones are positioned peripheral to the nucleosomal core, suggesting that they are potentially involved in protein–protein interactions (1). Indeed, histone N-terminal tails are subject to various covalent modifications such as acetylation, phosphorylation, ubiquitination and methylation by specific chromatin-modifying enzymes (2). Such modifications can determine the functional state of chromatin, and therefore affect various downstream processes, possibly by directly altering DNA–histone affinity or by providing recognition motifs for chromatin-associated proteins (3,4).

The role of methylated histone lysines in gene regulation has recently attracted considerable interest. Unlike acetylation or phosphorylation, methylation does not alter the overall charge of the N-terminal tails of histones, implying a mechanism other than regulating nucleosomal compaction via charge neutralization (5). Instead, histone methylation was reported to serve as a recognition motif for chromatin-associated proteins such as HP1, which binds to H3 methylated at lysine 9 to establish heterochromatic regions (6,7). Methylated lysines occur in mono-, di- or trimethylated states that are catalyzed by specific enzymes with varying transcriptional outcomes. For example, human SET7/9 catalyzes monomethylation of histone H3 at lysine 4 (H3-K4), whereas yeast Set1 catalyzes di- and trimethylation of H3-K4, and trimethylated but not dimethylated H3-K4 has been reported to be exclusively associated with active transcription in *Saccharomyces cerevisiae* (8,9). Furthermore, whereas acetylation and phosphorylation have been demonstrated to be reversible, methylation has been suggested to be stable and irreversible, partly due to the fact that no histone demethylase has been discovered to date (10). It is suggested, therefore, that methylation is a permanent modification that serves as a 'molecular memory of transcription' that could be heritably maintained. However, in examples where dynamic transcriptional regulation is essential, reversibility of methylation may be necessary. For example, thyroid hormone receptor (TR), which has the ability to alternately and rapidly repress or activate transcription depending on the absence or presence of cognate ligand, appears to regulate histone methylation of lysine as well as arginine residues concomitant with transcriptional activation of target genes (11).

Nuclear hormone receptors (NR) are members of a large group of structurally related transcription factors that are regulated by lipophilic ligands. The androgen receptor (AR), a member of the nuclear receptor superfamily, activates transcription of specific target genes by binding to androgen responsive elements (AREs) upstream of the transcription start site and by recruiting both coactivators and other components of the general transcriptional machinery (12).

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While transcriptional activation is driven by the binding of 5-α-dihydrotestosterone (DHT) to the AR, antagonists such as bicalutamide repress transcription through the recruitment of corepressors, SMRT and N-CoR, as well as histone deacetylases (HDACs) (13). Although histone arginine methyltransferases such as CARM1 and PRMT1 were reported to facilitate transcriptional activity of NR (14), the role of histone lysine methylation is still unclear.

In this study, we report that changes in methylated H3-K4 status occur at various loci within the human prostate specific antigen (PSA) gene during early stages of transcriptional regulation by the AR. Decreases in both di- and trimethylated H3-K4 accompanied AR binding at the enhancer and promoter and were completely reversed by the addition of an AR antagonist, bicalutamide. Conversely, substantial increases in di- and trimethylated H3-K4 were observed in the coding region of the PSA gene as a function of gene expression. Together these results suggest distinct functions conferred by histone methylation at the transcriptional control regions versus coding regions of active genes.

MATERIALS AND METHODS

Cell culture and reagents

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS (Gemini Bioproducts, Woodland, CA). DHT was purchased from Sigma-Aldrich (St Louis, MO). Bicalutamide was obtained from ICI Pharmaceuticals (UK).

Chromatin immunoprecipitation (ChIP) assays

LNCaP cells (5×10^6 cells/150 mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% charcoal/dextran-stripped FBS (Gemini Bioproducts, Woodland, CA) for 3 days. Cells were treated with DHT and/or bicalutamide for various times as indicated, cross-linked by adding formaldehyde (1%) directly to the culture medium, and incubated at room temperature for 10 min. The cells were washed twice with ice-cold PBS and harvested by scraping and centrifugation at 3000 *g* for 5 min. The cell pellets were resuspended in 0.5 ml lysis buffer [1% SDS, 10 nM EDTA, 50 nM Tris–HCl pH 8.0, with 1× complete protease inhibitor cocktail (Roche, Indianapolis, IN)] and incubated for 20 min on ice. The cell lysates were sonicated at setting 4 on a Branson Sonifier Cell Disruptor 185 for 10 s. The sonication was repeated five times (with 1 min incubations on ice in between sonications) and insoluble materials were removed by centrifugation at 15 500 *g* for 10 min. For each immunoprecipitation, 100 µl of supernatant containing soluble chromatin was diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.0, 167 mM NaCl, 1× protease inhibitor cocktail). After preclearing with 75 µl of protein G–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C for 1 h, the supernatant was immunoprecipitated by incubating at 4°C overnight with 25 µl anti-MLL, 25 µl anti-AR (N20, Santa Cruz Biotechnology, Santa Cruz, CA), 5 µl anti-dimethyl H3-K4, 5 µl anti-AcH3 (Upstate Biotechnology, Lake Placid, NY) or 5 µl anti-trimethyl H3-K4 (Abcam, Cambridge, UK). Immune complexes were obtained by incubating with 50 µl of protein G–Sepharose at 4°C for 1 h. Immunoprecipitates were sequentially washed for 5 min each in low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% IGEPAL CA 630, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH 8.0) and TE buffer (twice). Washed beads were extracted with 250 µl of elution buffer (1% SDS, 100 mM $NaHCO_3$) twice, the elution was combined, and the protein–DNA cross-linking was reversed by incubation at 65°C overnight. Each sample was treated with 20 µg of proteinase K (Gibco BRL, Grand Island, NY) in proteinase K buffer (50 mM Tris–HCl pH 6.5, 10 mM EDTA) at 45°C for 1 h. DNA was purified by phenol/chloroform extraction, precipitated by ethanol with glycogen, and resuspended in 100 µl of H_2O. One percent of total soluble chromatin was processed in parallel without immunoprecipitation, and values obtained from this DNA were used as denominators to calculate immunoprecipitated DNA as percentage of input.

Real-time PCR of ChIP DNA

Immunoprecipitated DNA was analyzed using quantitative real-time PCR as described previously (15). Briefly, triplicate PCRs for each sample were mixed with AmpliTaq Gold PCR master mix (Applied Biosystems, Branchburg, NJ), forward and reverse primers and probe to be analyzed by Bio-Rad iCycler optical system. The primers and probes were: enhancer forward, 5′-GCCTGGATCTGAGAGAGATATC-ATC-3′; reverse, 5′-ACACCTTTTTTTTTCTGGATTGTTG-3′; promoter forward, 5′-CCTAGATGAAGTCTCCATGAG-CTACA-3′; reverse, 5′-GGGAGGGAGAGCTAGCACTTG-3′; 3′ forward, 5′-TCATCATGAATCGCACTGTTAGC-3′; reverse, 5′-GCCCAAGTGCCTTGGTATACC-3′; E-P forward, 5′-CAGTGGCCATGAGTTTTGTTTG-3′; reverse, 5′-AACCAATCCAACTGCATTATACACA-3′; exon 3 forward, 5′-CACACCCGCTCTACGATATGAG-3′; reverse, 5′-GAGCTCGGCAGGCTCTGA-3′; enhancer probe, 5′-6-FAM-TGCAAGGATGCCTGCTTTACAAACATCC-BHQ-1-3′; promoter probe, 5′-6-FAM-CAATTACTAGATCACC-CTGGATGCACCAGG-BHQ-1-3′; 3′ probe, 5′-6-FAM-TGAATCATCTGGCACGGCCCAA-BHQ-1-3′; E-P probe, 5′-6-FAM-CCCAACGCAACTTAACCTAACAAG-BHQ-1-3′; and exon 3 probe, 5′-6-FAM-CTCCAGCCACGACCT-CATGCTGCT-BHQ-1-3′ (Biosearch Technologies, Novato, CA). This assay provides a precise quantitation of target DNA and is based on the principle of fluorophore release from a self-quenching probe; the instrument measures the number of cycles (C_t) required for fluorescence to exceed a set threshold. A standard curve of known target DNA is constructed in parallel from which the relative amount of target DNA in the sample is calculated. Values are presented as percentage input, which is analyzed at the same time. The precise quantitative nature of this analysis is superior to analyses commonly used by others that rely on semi-quantitative end-point assessments of PCR bands on agarose gels.

Histone isolation and immunoblotting

Total histones were isolated as described (16) with the following modifications. LNCaP cells were washed once with ice-cold PBS, harvested in 500 µl PBS containing 10 mM sodium butyrate and centrifuged at 700 *g* for 1 min. The pellet was resuspended in 1 ml lysis buffer (1% Triton X-100, 8.6% sucrose, 10 mM Tris–HCl pH 6.5, 10 mM sodium butyrate, 50 mM sodium disulfite, 10 mM $MgCl_2$) and centrifuged at 1000 *g* for 1 min. The lysis was repeated three times until a white nuclear pellet was attained. After a final wash with 10 mM Tris–HCl pH 7.4, 13 mM EDTA, the pellet was resuspended in ice-cold distilled water. H_2SO_4 was added to a final concentration of 0.4 N and incubated on ice for 1 h. After centrifugation at 10 000 *g* for 5 min, the supernatant containing total histones was precipitated with 10× vol of acetone at –20°C overnight. The total histone pellet was recovered by centrifugation at 1000 *g* for 5 min and resuspended in distilled water. Total protein concentration of the isolated histones was assayed using a Bio-Rad Protein Assay Kit (Hercules, CA) according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices Emax Microplate Reader (Sunnyvale, CA). Equal amounts of total histones were analyzed by SDS–PAGE, transferred to Hybond-P membrane (Amersham Pharmacia Biotech), and probed with the indicated antibodies. Anti-unmodified H3 (Upstate Biotechnology) were used as controls. HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Detection was performed using the enhanced chemiluminescence western blotting system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Images are representative of three independent immunoblots and were analyzed by Fluor-S Max MultiImager Quantification System (Bio-Rad).

Real-time RT–PCR of RNA

PSA mRNA was analyzed as described previously using quantitative RT–PCR (15).

RESULTS AND DISCUSSION

Di- and trimethylation of H3-K4 decrease during transcriptional activation by AR

In *S.cerevisiae*, the precise methylation status of histone H3-K4 was reported to determine transcriptional activity. The trimethylated but not dimethylated form of H3-K4 is preferentially associated with active transcription, and thus it was hypothesized that the dimethylated state may function as a transcriptionally permissive signal whereas the trimethylated state exclusively denotes active transcription (9). We have sought to address whether the transcriptional status conferred by H3-K4 methylation is conserved in human by investigating a nuclear hormone receptor model where transcription can be rapidly activated or repressed. To this end, we examined H3-K4 methylation in AR-mediated PSA gene transcription by utilizing a human prostate cancer cell line, LNCaP, which expresses both PSA and AR. PSA gene transcription is tightly regulated by AR. Upon ligand induction, ARs bind to AREs located in the proximal promoter and upstream enhancer of the PSA gene, and transcription is initiated following the subsequent recruitment of coactivators and RNA polymerase II (RNAP II) to both control regions (17).

In order to study AR-mediated effects at target gene loci, we quantitated specific histone modifications as well as AR occupancy using ChIP analysis and specific real-time PCR primers that span various loci in the PSA gene (Fig. 1A). Specifically, we examined the proximal promoter, the distal enhancer (~4.1 kb upstream of the transcription start site), and exon 3 (~3.2 kb downstream of the transcription start site). As controls, we also designed primers at a region between the enhancer and promoter (E-P) and a region 3′ to the poly-A addition site (3′). AR occupancy followed a previously described (15) pattern at the enhancer and promoter with the quantitative difference possibly due to the presence of multiple AREs at the enhancer (Fig. 1C). AR occupancy was essentially absent in the three loci without AREs. Acetylation of H3 (K9 and K14) increased at the enhancer, promoter and exon 3 but not at either E-P or 3′ (Fig. 1D). As with AR occupancy, acetylation also displayed a quantitative difference between the enhancer and promoter, with a significantly higher level at the enhancer. This may be due to the multiple bound-ARs recruiting a proportional number of histone acetyltransferases (HATs) at the enhancer. In support, a recent study demonstrated that p160 coactivators and CBP/p300 HATs are preferentially recruited to the enhancer (17). Also, biochemical and genetic studies have revealed that the enhancer is required for maximal PSA expression and thus, together, establish the enhancer as a crucial control region (18). Interestingly, acetylation at the coding region was higher than either at the enhancer or promoter, indicating that acetylation is not limited to the transcriptional control regions and may play a significant role in elongation. *In vitro*, nucleosomes present a considerable obstacle to the elongating RNAP II causing transcription inhibition, which can be relieved by acetylation (19,20). Furthermore, RNAP II association is reduced by hypoacetylation in the coding regions in yeast (21). Thus, acetylation may be important in transcription elongation as well as initiation.

Strikingly, both di- and trimethylated H3-K4 decreased with ligand induction at the enhancer and promoter, but not at the other three loci (Fig. 1E and F). The observation that both di- and trimethylated H3-K4 are decreased cannot be explained by the possibility that further methylation of dimethylated lysine occurred to form trimethylated lysine, or that trimethylated lysine was demethylated to form dimethylated lysine. To address the concern that trimethyl H3-K4 antibody (Abcam) may cross-react with methylated H3-K9, we analyzed for the presence of methylated H3-K9 with the specific antibody (Upstate) in ChIP assays and found that methylated H3-K9 was essentially absent at the PSA gene (data not shown). Thus, the decrease in trimethylated H3-K4 upon transcription activation is not due to the decrease in methylated H3-K9 detected by cross-reactive antibody.

To further investigate the apparent decreases in di- and trimethylated H3-K4 as a function of AR occupancy, we monitored both methylation states at various time points after DHT addition. Interestingly, the decreases in di- and trimethylated H3-K4 were rapid (within 15 min) and sustained (up to 24 h, data not shown) at both the PSA enhancer and promoter (Fig. 2). The data suggest that histone replacement and dilution of histone methylation by replication are unlikely

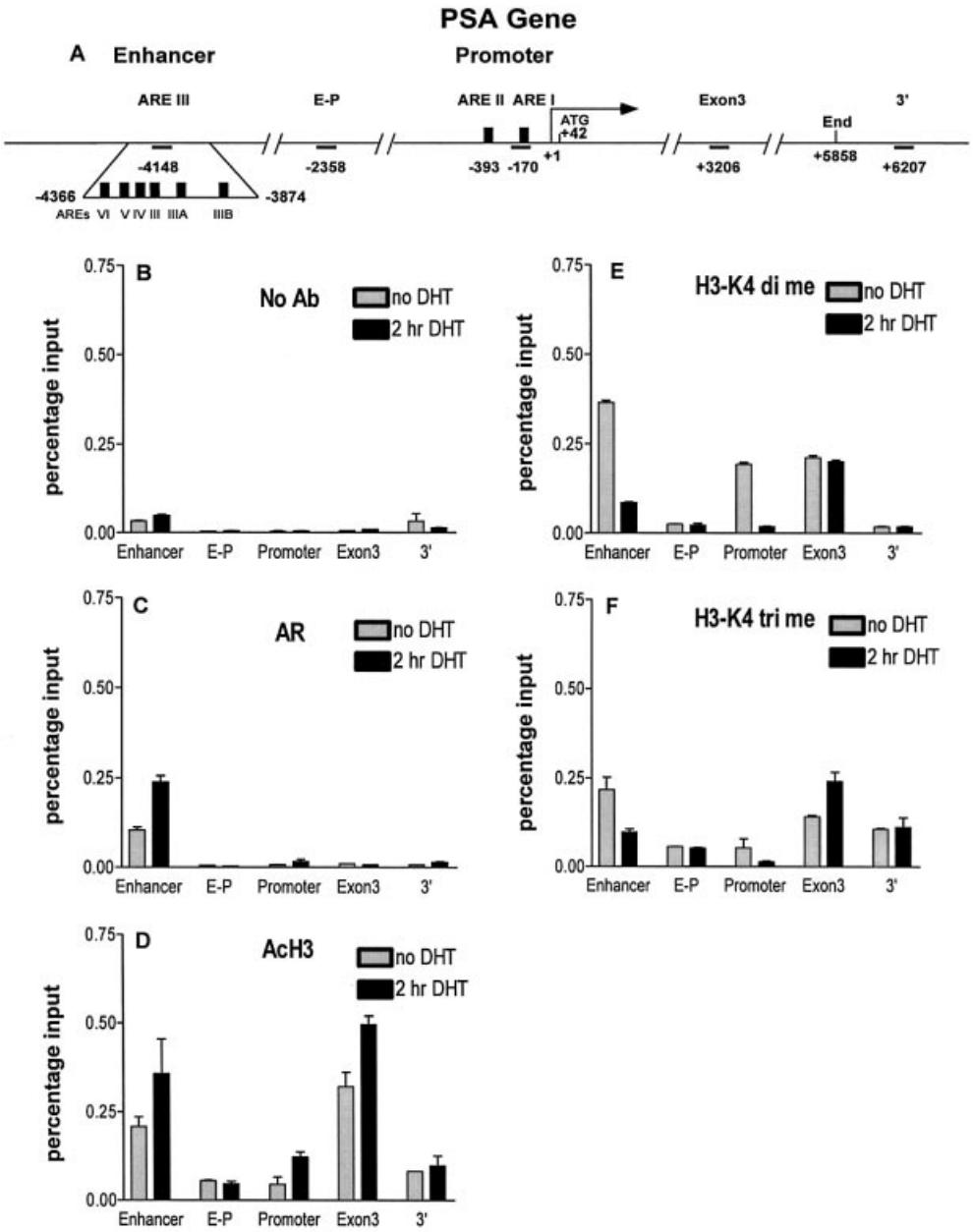

Figure 1. ChIP analysis of AR occupancy and histone H3 modifications at the PSA gene. (**A**) Schematic representation of the PSA gene indicating five target loci (horizontal bars). Vertical bars represent approximate locations of AREs. E-P is a region between the enhancer and promoter without any known function. LNCaP cells were incubated in 5% charcoal-stripped FBS media with either vehicle (EtOH) or DHT (10 nM) for 2 h and analyzed as described in Materials and Methods as follows: (**B**) no antibody control, (**C**) AR occupancy, (**D**) acetylated H3 at lysines 9 and 14, (**E**) dimethylated H3 at lysine 4 and (**F**) trimethylated H3 at lysine 4. Values are presented as percentage input and error bars represent the standard error of the mean of triplicate determinations using quantitative real-time PCR. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. Results shown are representative of five independent experiments.

due to the rapid kinetics. To address whether the observed decreases in di- and trimethylated H3-K4 occurred locally at specific loci, we isolated total histones after either 15 min or 4 h of incubation with native ligand and immunoblotted for changes in global H3-K4 methylation. No significant changes in acetylated H3, di- and trimethylated H3-K4 and unmodified H3 were observed after short or prolonged treatment with DHT (Fig. 3). The data suggest that the apparent decreases in di- and trimethylated H3-K4, as well as increase in acetylated H3, are not global effects conferred by DHT treatment but rather are locally regulated events at specific DHT-dependent loci.

Inhibition of AR activity by AR antagonist reverse di- and trimethylation alterations of H3-K4

We assessed whether AR regulates the apparent decreases in di- and trimethylated H3-K4 and, importantly, whether both modifications are reversible. To this end, we quantitated H3-K4 methylation at the enhancer after treating LNCaP cells briefly (30 min) with DHT and then inhibiting AR activity by

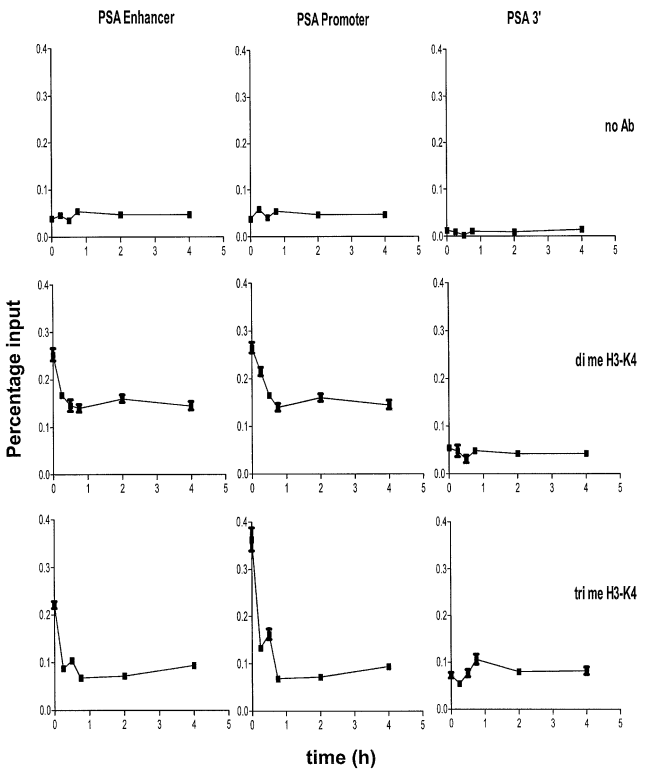

Figure 2. Kinetic analysis of di- and trimethylated H3-K4 at the PSA enhancer, promoter and a 3′ region. LNCaP cells were incubated with either vehicle (representing time 0) or DHT (10 nM) for the indicated times and analyzed using quantitative PCR for no antibody control (top row), dimethylated H3-K4 (middle row) and trimethylated H3-K4 (bottom row) at the PSA enhancer (left column), promoter (middle column) and 3′ region (right column). Values are presented as percentage input and error bars represent the standard error of the mean of triplicate determinations using quantitative real-time PCR. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. Results shown are representative of three independent experiments.

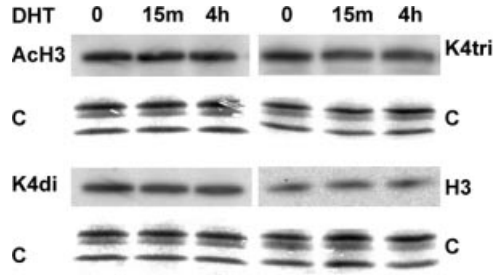

Figure 3. Immunoblot of total histones isolated from LNCaP cells after DHT treatment. Total histones were isolated from LNCaP cells (5×10^6 cells/150 mm dish) after incubation with either vehicle (representing time 0) or DHT (10 nM) for 15 min and 4 h. Total histones were immunoblotted for acetylated H3, dimethylated H3-K4, trimethylated H3-K4 and unmodified H3. C, corresponding Coomassie-stained core histones represent the loading and are shown below each immunoblot. Results shown are representative of three independent experiments.

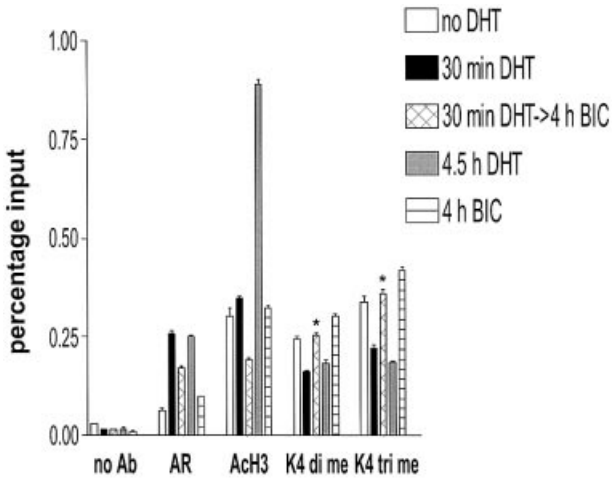

Figure 4. Bicalutamide antagonizes the effect of DHT at the PSA enhancer. LNCaP cells were treated with DHT (10 nM) for 30 min, washed with PBS and treated with bicalutamide (10 μM) for 4 h. ChIP DNA were analyzed as described in Materials and Methods for no antibody control and antibodies against AR, acetylated H3, dimethylated H3-K4 and trimethylated H3-K4. Controls include vehicle, 30 min, 4.5 h DHT and 4 h bicalutamide treatments. Values are presented as percentage input and error bars represent the standard error of the mean of triplicate determinations using quantitative real-time PCR. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. *Two-tailed *P*-values (*t*-test) of comparisons between the 30 min DHT treatment and bicalutamide reversal values are 0.0012 for dimethylated H3-K4 and 0.0084 for trimethylated H3-K4. Results shown are representative of three independent experiments.

replacing DHT with a specific AR antagonist, bicalutamide. The bicalutamide treatment alone increased AR occupancy by 2-fold but decreased DHT-mediated AR occupancy (Fig. 4), which is consistent with recent evidence that the antagonist functions by binding to AR to induce receptor binding to AREs and recruitment of corepressors such as SMRT (22). Strikingly, the AR-mediated decreases in di- and trimethylated H3-K4 were completely reversed to transcriptionally inactive levels by the bicalutamide treatment. The data indicate that the apparent decreases in di- and trimethylated H3-K4 are regulated in the AR-mediated transcription and are reversible. Furthermore, since the bicalutamide treatment alone can induce AR occupancy but does not decrease the H3-K4 methylation levels, it seems unlikely that either AR or corepressors bind to the methylation signals to mask epitopes in the ChIP assay. However, it is entirely possible that methylated H3-K4 serves as a recognition motif for chromatin-associated proteins that positively regulate transcription such as HATs and coactivators. In this view, AR-mediated transcription or AR itself recruits positive regulators to the transcriptional control regions (i.e. the enhancer and promoter) and bind to di- and/or trimethylated H3-K4 signals. The binding of bulky proteins, or protein complexes, could result in masking of epitopes in ChIP analysis, thereby causing apparent decreases in di- and trimethylated H3-K4.

It has been suggested that one of the possible mechanisms of histone methylation turnover is the proteolytic cleavage of histone tails, specifically between lysines 4 and 9 of histone H3 (23). The proteolysis of H3 tails addresses the active elimination of methylation prior to the histone replacement during DNA replication and, notably, without the elimination of acetylation at lysines 9 and 14. However, this mechanism may not account for our data, which illustrate a dynamic regulation of H3-K4 methylation. An alternate mechanism,

consistent with the active removal of methylation, is the replication-independent exchange of histone H3 with a variant H3.3 (24). In *Tetrahymena*, it was suggested that H3.3 replaces H3 during transcription since H3.3 is only present in the macronucleus where transcription is active (25). Although we cannot rule out this possibility, our data seem to indicate that with the decrease in methylation there is a continued accumulation of acetylation, which may be inconsistent with the mechanism of replacing modified H3 with unmodified H3.3. Still, it may be possible that acetylation and methylation occur on discrete nucleosomes, and only the histones that have been methylated are replaced with the variant.

The active and controlled reversibility of H3-K4 methylation is consistent with the most direct mechanism, which is the enzymatic action of a demethylase. Histone demethylase activity was first reported in 1973 (26), but the enzyme responsible for the activity has never been identified. More recently, it was hypothesized that Elp3, the yeast HAT and elongation factor, is a candidate for the histone demethylase based on its sequence similarity to enzymes that use *S*-adenosyl-L-methionine (SAM) in oxidative reactions (27).

Distinct H3-K4 methylation pattern in the coding region versus transcriptional control regions of the PSA gene

It was reported that dimethylated H3-K4 is present in both the promoter and coding regions of genes in yeast, but with a bias towards the coding regions. Moreover, trimethylated H3-K4 occurs in the 5′ portion of coding regions, which may indicate that trimethylation denotes an early phase of elongation (9,28). Our data suggest that, in human, transcriptional control regions such as the PSA enhancer and promoter are enriched in both di- and trimethylated H3-K4, which can be dynamically regulated. Also, there was an indication that trimethylated H3-K4 in the coding region is regulated (Fig. 1F). To investigate further the role of H3-K4 methylation in the PSA coding region, we quantitated H3-K4 methylation levels at PSA exon 3 over various time periods after induction by DHT. Remarkably, dramatic and significant increases in both di- and trimethylated H3-K4 were observed after a 4 h lag period (Fig. 5A). The qualitative, quantitative and temporal differences in H3-K4 methylation at the coding region versus transcriptional control regions indicate that di- and trimethylated H3-K4 serve distinct roles at those loci. It may be possible that H3-K4 methylation at the transcriptional control regions plays a role in transcription initiation, whereas at the coding region H3-K4 methylation is involved in more downstream processes such as transcription elongation or RNA processing. In yeast, it was shown that Set1, an H3-K4 methylase, is recruited by the RNAP II elongation machinery to the 5′ portion of active coding regions, and the pattern of trimethylated H3-K4 strongly correlates with Set1 occupancy (28). We examined whether trimethylated H3-K4 at the coding region correlates with MLL (the human homolog of Set1) occupancy, and observed that MLL did not locate to either exon 3 or the other loci (data not shown). Together, the data suggest that trimethylated H3-K4 is not limited to the 5′ portion of coding regions, and the accumulation of trimethylated H3-K4 is not due to the recruitment of MLL at exon 3.

To address whether there is a correlation between H3-K4 methylation and PSA mRNA expression, we quantitated both

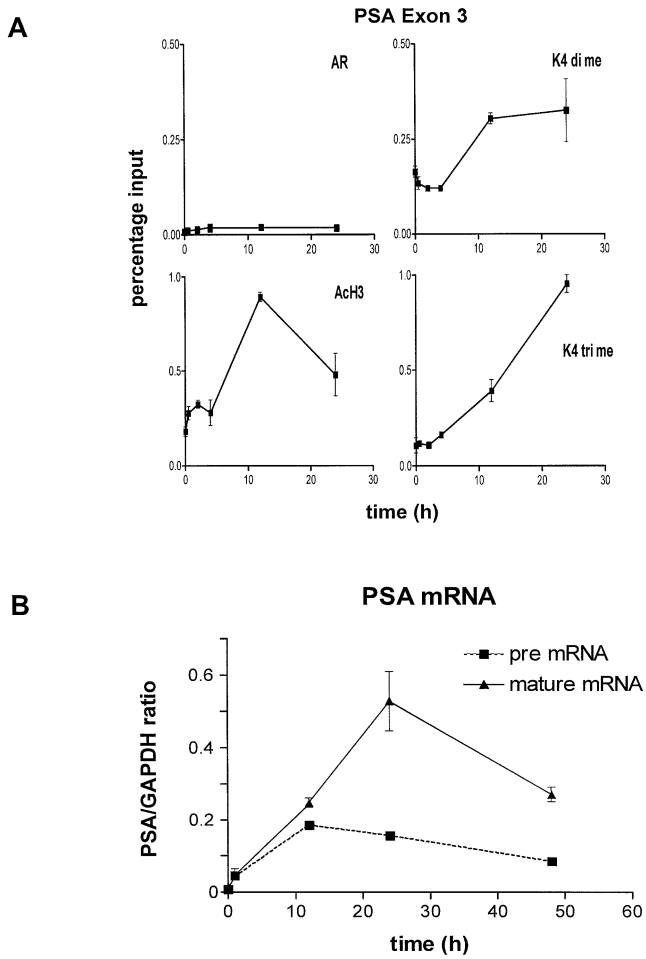

Figure 5. Exon 3 H3-K4 methylation and PSA mRNA expression after prolonged exposure to DHT. (**A**) LNCaP cells were treated with DHT (10 nM) over a 24 h period and analyzed after ChIP of the exon 3 locus using antibodies against AR, acetylated H3, dimethylated H3-K4 or trimethylated H3-K4, as indicated. (**B**) LNCaP cells were treated with DHT over a 48 h period, and pre- and mature mRNA were quantitated by real-time RT–PCR as described previously (15), with forward primers designed in intron 3 and exon 3, respectively, and the reverse primer and probe specific for exon 4. Values are presented as percentage input and error bars represent the standard error of the mean of triplicate determinations using quantitative real-time PCR. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. Results shown are representative of three independent experiments.

pre- and mature mRNA levels by quantitative real time RT–PCR. The pre-mRNA level reached a plateau at 12 h after ligand induction, whereas the mature mRNA level reached a plateau at 24 h after induction by DHT (Fig. 5B). The increased PSA mRNA production up to 24 h was similar to the kinetics previously reported by others (29). Although the potential positive correlation needs to be investigated further, there seemed to be qualitative and quantitative similarities between the pre-mRNA level and H3-K4 dimethylation and between the mature mRNA level and H3-K4 trimethylation. This raises an interesting possibility that di- and trimethylated H3-K4 serve discrete functions, with trimethylated H3-K4 being associated with more downstream events, such as RNA processing, than dimethylated H3-K4.

Our data clearly demonstrate that the H3-K4 methylation observed in human is different to that in yeast. Thus, the process of marking the coding region with histone methylation signal may be an important mechanism that humans evolved to allow efficient gene expression after a prolonged environmental stimulus. This is also the first demonstration that trimethylated H3-K4 is associated with inactive transcription. Although the exact mechanism of gene regulation conferred by the various covalent modifications is still unclear, it is becoming increasingly evident that understanding the effects of specific modifications such as methylation of histone H3 will be critical to the understanding of the steroid hormone regulation of gene expression.

ACKNOWLEDGEMENTS

We thank Michael Stallcup and Lucio Comai for critically reading the manuscript. Grant support was from NIH/NCI (R01CA84890) (to G.A.C.), United States Department of Defense (DAMD17-00-1-0102) (to G.A.C.) and the National Health and Medical Research Council of Australia (ID187641) (to W.D.T.).

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