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Proteasome Activity Modulates Chromatin Modifications and RNA Polymerase II Phosphorylation To Enhance Glucocorticoid Receptor-Mediated Transcription▽†

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The 26S proteasome modulates steroid hormone receptor-dependent gene transcription at least in part by regulating turnover and recycling of receptor/transcriptional DNA complexes, thereby ensuring continued hormone response. For the glucocorticoid receptor (GR), inhibition of proteasome-mediated proteolysis or RNA interference-mediated depletion of specific proteasome subunits results in an increase in gene expression. To facilitate transcription, proteasome inhibition alters at least two features associated with modification of chromatin architecture and gene transcription. First, proteasome inhibition increases trimethyl histone H3K4 levels with a corresponding accumulation of this modification on GR-regulated promoters in vivo. Secondly, global levels of phosphorylated RNA polymerase II (Pol II) increase, together with hormone-dependent association of the phosphorylated Pol II, with the promoter and the body of the activated gene. We propose that apart from modulating receptor turnover, the proteasome directly influences both the transcription machinery and chromatin structure, factors integral to nuclear receptor-regulated gene transcription.

The glucocorticoid receptor (GR), as other members of the steroid hormone receptor superfamily, acts as a hormone-dependent transcription factor that mediates transcriptional and physiological responses to glucocorticoids (32). The hormone-bound GR translocates to the nucleus, where it interacts directly with specific DNA elements, termed glucocorticoid receptor elements (GREs), within promoters to regulate transcription of genes in various target tissues. To activate or repress transcription, the GR recruits a number of coregulator complexes, including the ATP-dependent chromatin remodeling complexes that alter local chromatin architecture to regulate gene transcription (24). Cellular levels of the receptor and receptor coregulator complexes tightly control GR-mediated transcriptional and physiological responses. Hormone binding also results in degradation of GR protein by the 26S proteasome, an activity implicated in the regulation of gene transcription mediated by the receptor (23).

The 26S proteasome consists of a 20S proteolytic core, capped at both ends by the 19S regulatory complex, which recognizes the polyubiquitin-tagged substrates (4). The 19S consists of two subcomplexes, the lid and the base, composed of AAA-type ATPases, one of which is Sug1, also known as thyroid receptor interacting protein (TRIP1) (4). Like many other transcription factors, proteolysis of steroid hormone receptors by the 26S proteasome has been proposed to limit their transcriptional output (35, 42). Additionally, the 26S proteasome is implicated in recycling of transcriptional complexes on chromatin to facilitate multiple rounds of transcription initiation (5).

Recent studies have linked the 26S proteasome with other transcriptional activities independent of proteolysis of specific activators (3, 5, 30). Chromatin immunoprecipitation (chIP) experiments reveal direct interaction between DNA sequences on yeast and mammalian gene promoters and specific proteasome subunits (15, 17, 33, 38). Although in some cases the specific functions of these interactions are not clear, recent studies, particularly of yeast, associate specific proteasome components with distinct chromatin modifications and transcriptional processes (10, 12, 15, 26). For example, efficient elongation by RNA polymerase II (Pol II) requires the 19S regulatory particle, while transcription termination requires an active proteasome (15). It is not clear whether these additional transcriptional activities of the 26S proteasome contribute to steroid hormone receptor-mediated gene regulation.

Inhibiting proteasomal degradation increases transcriptional activity of some, but not all, steroid hormone receptors (7, 8, 20, 31, 52). This implies the significance of the 26S proteasome in sequential events underlying transcription initiation. In the case of the GR, inhibiting proteolysis of the receptor by the proteasome-specific inhibitor MG132 results in an increase in GR-mediated transcriptional activation from the mouse mammary tumor virus (MMTV) promoter (8, 52). Additionally, proteasome inhibition increases GR-mediated transactivation from transient and open or closed chromatin MMTV templates (8). Although transactivation from a chromatin template is normally associated with regions of hypersensitivity on the integrated MMTV promoter, inhibiting proteasome activity does not increase nuclease hypersensitivity at the promoter. We sought to define other mechanisms apart from proteolysis of the receptor that mediate the hormone-dependent increase

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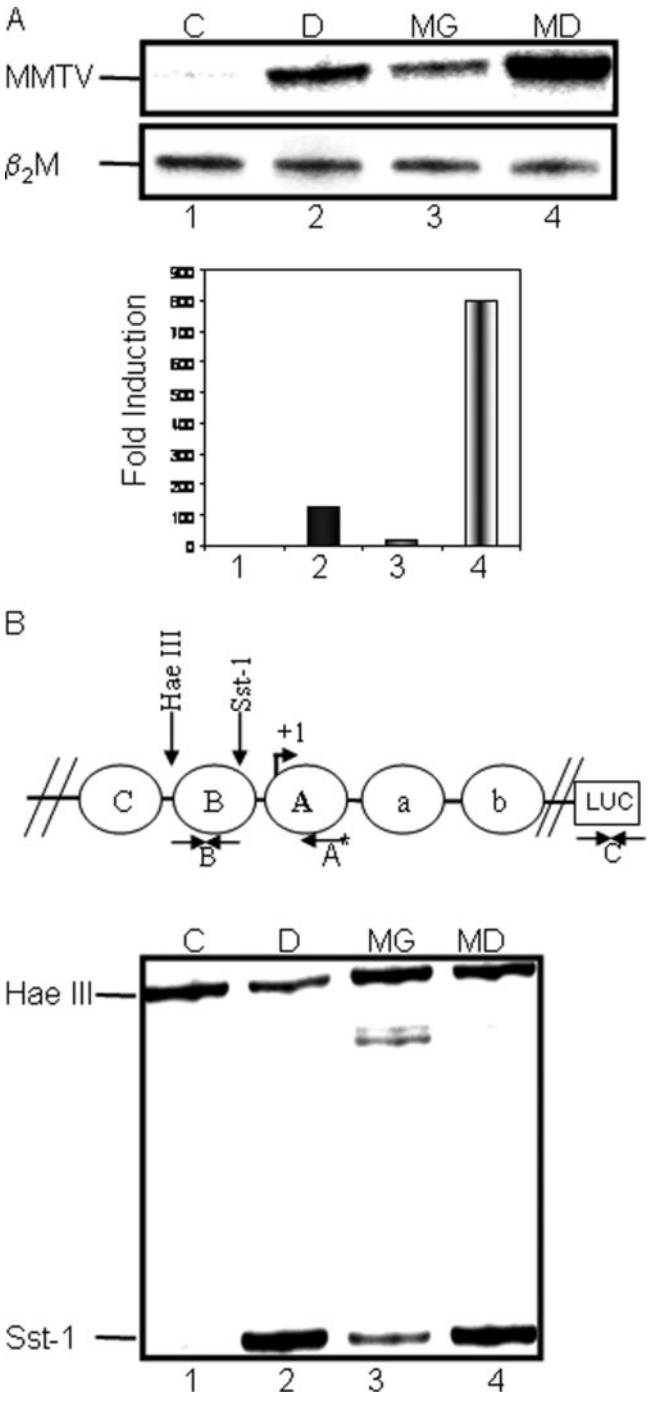

FIG. 1. Inhibiting the proteasome stimulates hormone-dependent transcription but not chromatin remodeling. (A) Proteasome inhibition induces MMTV mRNA expression. MCF-7 cells grown in phenol red-free MEM supplemented with 5% charcoal dextran-stripped FBS were treated with vehicle (C; lane 1) for 4 h, 100 nM dexamethasone (D; lane 2) for 4 h, 1 uM MG132 (MG; lane 3) for 20 h, or 1 μM MG132 (20 h) plus dexamethasone (4 h) (MD; lane 4). MMTV-LUC and β_2-microglobulin mRNA expression was monitored by semiquantitative and quantitative reverse transcription-PCR analysis. (B) Proteasome inhibition does not change hormone-dependent nuclease hypersensitivity. Schematic of the MMTV promoter fused to the luciferase gene reporter. Primer locations for analysis in nuclease hypersensitivity (A) and chromatin immunoprecipitation at the promoter (B) or coding region (C) are shown. Nuclei were isolated from MCF-7 cells treated with vehicle (lane 1) for 1 h, 100 nM dexamethasone (D; lane 2) for 1 h, 1 μM MG132 (MG; lane 3) for 23 h, or MG132 (23 h) plus dexamethasone (1 h) (MD; lane 4) and digested in vivo with SstI. DNA was purified and digested with HaeIII to provide an internal standard and as a control for the degree of nuclease accessibility in vivo. DNA was analyzed by reiterative PCR using a ^{32}P-end-labeled oligonucleotide specific for the MMTV promoter (22). Lanes 1 to 4 represent PCR products for SstI and HaeIII.

in MMTV transcription after proteasome inhibition. Proteasome inhibition of and RNA interference (RNAi) in specific 26S proteasome components results in an increase in GR-mediated MMTV transcription. This appears to be a direct effect, as elements of the 26S proteasome are detected on both the promoter and body of the gene. We report that inhibiting proteasome activity results in an increase in the global levels of trimethyl histone H3K4 and phosphorylated RNA polymerase II forms. Consistent with the increase in global levels of trimethyl histone H3K4, the trimethyl histone H3 lysine 4 marks are enriched in the body of the activated gene. Further, we show an increase in hormone-dependent association of phosphorylated RNA Pol II with MMTV chromatin fragments. Together, these findings suggest that apart from proteolysis of the receptor, the proteasome can modulate steroid hormone receptor-mediated gene transcription by modification of the chromatin structure and transcription machinery.

MATERIALS AND METHODS

Cell culture. The MCF-7 cells stably expressing the GR and the MMTV long terminal repeat (LTR) promoter fused to the luciferase gene reporter (MMTV-LUC) have been described previously (22). Cells were grown in a humidified incubator at 37°C with 5% CO_2 in minimal essential medium (MEM) supplemented with 2 mM glutamine, 100 μg/ml penicillin-streptomycin, 10 mM HEPES, 10% fetal bovine serum (FBS), and 300 μg/ml G418. For experiments, cells were seeded overnight in phenol red-free MEM supplemented with 5% charcoal-stripped calf serum and 2 mM glutamate. The next day cells were treated with proteasome inhibitor MG132 or dexamethasone at the concentrations described in the figure legends.

RNA analysis. Total RNA was extracted using TRIzol reagent (Invitrogen, Rockville, MD). For reverse transcriptase PCR (RT-PCR) analysis, cDNA was synthesized by standard protocols after DNase I treatment (Invitrogen). PCR was performed with the following pair of primers: MMTV-LUC, 5′-TCT GGA AAG TGA AGG ATA AAG TGA CGA-3′ and 5′-CCT CTT CTG TGT TTG TGT CTG CTG TTC-3′. The human β_2-microglobulin gene was amplified using primer sequences 5′-ACC CCC ACT GAA AAA GAT GA-3′ and 5′-ATC TTC AAA CCT CCA TGA TG-3′. Levels of labeled PCR transcripts were analyzed on 8% polyacrylamide denaturing gels and quantified by PhosphorImager and ImageQuant Software analysis (Molecular Dynamics, Sunnyvale, CA).

Alternatively, following reverse transcription, cDNA was used for real-time PCR employing SYBR green detection. Real-time PCR was performed in the Stratagene MX 3000P using Brilliant SYBR Green QPCR master mix (La Jolla, CA). All reactions were performed with the model MX 3000P sequence detector. Primers were designed by Primer Express Software, version 2.0 (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the differences in the amount of mRNA in each reaction. For small interfering RNA (siRNA) experiments, β-actin was used for normalization. Each RNA sample was run in triplicate and repeated in two to three independent experiments. Primers used were the following: MMTV-LUC, 5′-CCG CCG TTG TTG TTT TGG-3′ and 5′-TCC TCC GCG CAA CTT TTT C-3′; GAPDH, 5′-TCG GAG TCA ACG GAT TTG G-3′ and 5′-GGC AAC AAT ATC CAC TTT ACC AGA GT-3′; β-actin, −5′-CTC CTC CTG AGC GCA AGT ACT C-3′ and 5′ CAT ACT CCT GCT TGC TGA TCC A-3′; MLL, 5′-AGC AGG TAA ACT CTC TCC-3′ and 5′GTT CCT TCC TTG TCT TTC C-3′; and SYMD3, 5′-TCC TAA GGG AAC GCA GTC AGA-3′ and 5′CAA AGC ATA GAG TGT GTG ACC TCA A-3′.

In vivo chromatin analysis. Nuclei were isolated as previously described and subjected to limited digestion using SstI (10 U/100 μl). After in vivo digestion, DNA was purified by phenol-chloroform extraction and ethanol precipitation. Purified DNA samples were digested to completion using HaeIII (100 U/100 μl) to provide an internal standard for the in vivo cutting and to confirm that equivalent amounts of DNA were used for reiterative primer extension analysis. Purified DNA (10 μg) was amplified using reiterative primer extension, *Taq* DNA polymerase, and ^{32}P-labeled specific oligonucleotide complementary to MMTV sequences (22). Extended products were purified using phenol-chloroform extraction and ethanol precipitation. Samples were analyzed on 8% polyacrylamide gels as described previously (22).

ChIP. MCF-7 cells were treated with dexamethasone or proteasome inhibitor (MG132) as specified in the figure legends. The ChIP assay was performed by using a ChIP kit (Upstate) as described previously (22). Briefly, cells were cross-linked with 1% formaldehyde and sonicated to generate DNA fragments with an average of 500 bp. Immunoprecipitation was performed overnight (8 to 12 h) at 4°C with various antibodies denoted in the figure legends. DNA-protein complexes were heated at 65°C for 4 h to reverse the formaldehyde cross-linking, and protein was digested with proteinase K for 1 h at 45°C. DNA was purified using a QIAGEN QIAquick PCR purification kit (Valencia, CA). Immunoprecipitated DNA sequences were detected and quantified by real-time PCR using specific primers for the MMTV promoter NUC B region and the luciferase gene coding region (LUC).

For ChIP analysis, real-time PCR was used to determine the amount of immunoprecipitated DNA using the following primers: MMTV NUC B region (promoter), 5′-GGT TAC AAA CTG TTC TTA AAA CGA GGA T-3′ and 5′-CAG AGC TCA GAT CAG AAC CTT TGA-3′; MMTV-LUC (coding region), 5′-CCG CCG TTG TTG TTT TGG-3′ and 5′-TCC TCC GCG CAA CTT TTT C-3′.

RNA interference. MCF-7 cells were transfected with siRNA pools (100 pmol; Dharmacon) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 h after transfection, and total RNA and whole-cell lysates were collected.

Antibodies and Western blotting. After being washed twice with phosphate-buffered saline, cells were pelleted by centrifugation. For whole-cell extracts, cells were lysed as previously described (22). Ten to 100 μg of protein was resolved by 6 to 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinyl difluoride membrane (Amersham). Proteins were immunoblotted using the following antibodies: anti-GR BUGR2 (B. Gametchu, Medical College of Wisconsin, Milwaukee, WI); BRG-1 (H-88), p300 (C-20), Cdk9 (H-169), Cdk7 (C-19), cyclin T (H-245), cyclin H (C-18), RNA Pol II (N-20), and normal serum immunoglobulin G (IgG) (Santa Cruz Biotechnology); RNA Pol II carboxy-terminal domain (CTD) antibodies 8WG16 (nonphosphorylated CTD), H14 (serine 5 phosphorylation specific), and H5 (serine 2 phosphorylation specific) (Covance); 20S proteasome subunit α2 (HC3) (Biomol, International); 19S proteasome subunit S1 and Set 9 (Upstate); p45/SUG1 (BD Transduction Laboratories); histone H3K4 trimethyl lysine, MLL, SYMD3, and WDR5 (ABCAM); β-actin (Sigma); and MLL1, ASH2, and RbBP5 (Bethyl).

RESULTS

Proteasome inhibition increases transcription independent of chromatin remodeling. To test whether proteasome activity is required for transcription in mammalian cells, we used the specific proteasome inhibitor MG132 to inhibit proteasome activity in MCF-7 breast cancer cells stably integrated with the well-characterized GR-responsive MMTV promoter (22). Hormone-dependent activation of the GR leads to an increase in MMTV mRNA expression levels. RT-PCR analysis confirmed that dexamethasone treatment, but not vehicle, induces MMTV-LUC transcript levels by 125-fold (Fig. 1A, lanes 1 and 2). Treatment with the proteasome inhibitor MG132 alone induced mRNA 20-fold (Fig. 1A, lanes 1 and 3). Addition of hormone and proteasome inhibitor increases mRNA expression 800-fold (Fig. 1A, lanes 1 and 4). The GR dynamically interacts with response elements on the MMTV promoter to alter local chromatin structure and regulate hormone-dependent transcription. To examine whether proteasome inhibition alters chromatin remodeling, we determined the extent of restriction enzyme cleavage in the NUC B region of the MMTV promoter (Fig. 1B). Consistent with previous findings, hormone activation of the GR induces local alteration in chromatin structure by 6.5-fold (Fig. 1B, lanes 1 and 2). Interestingly, although MG132 alone induces alteration of chromatin struc-

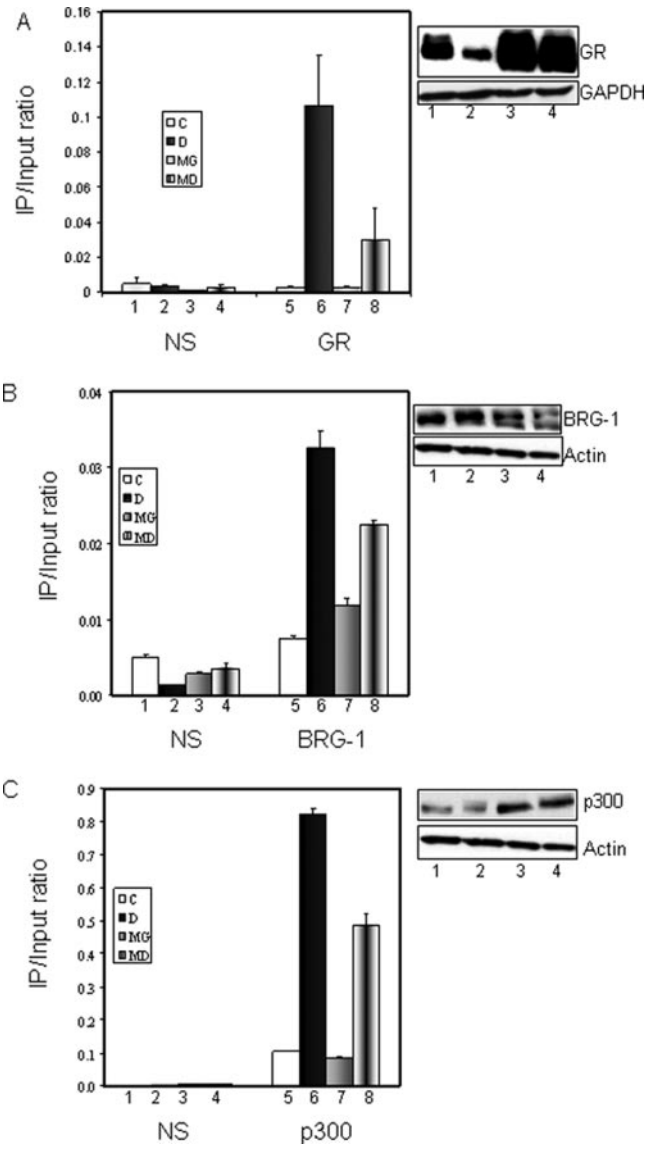

FIG. 2. Inhibiting the proteasome does not increase GR or cofactor association with the promoter. Proteasome inhibition decreases GR and cofactor association (BRG-1 and p300) with MMTV chromatin fragments. Results of chromatin immunoprecipitation are shown. Cells were treated as described in the legend to Fig. 1B. DNA was immunoprecipitated with antibody against GR, BRG-1, or p300 or with normal serum IgG (NS) as a control. Immunoprecipitated MMTV DNA was analyzed by real-time PCR using specific primers for the MMTV promoter. The panel A inset shows whole-cell extracts immunoblotted with antibodies against GR and GAPDH as loading controls. The panel B inset shows nuclear extracts immunoblotted with antibodies against BRG-1 and β-actin, and the inset in panel C shows nuclear extracts immunoblotted with antibodies against p300 and β-actin as controls. IP, immunoprecipitation. Other abbreviations are the same as those in the legend to Fig. 1.

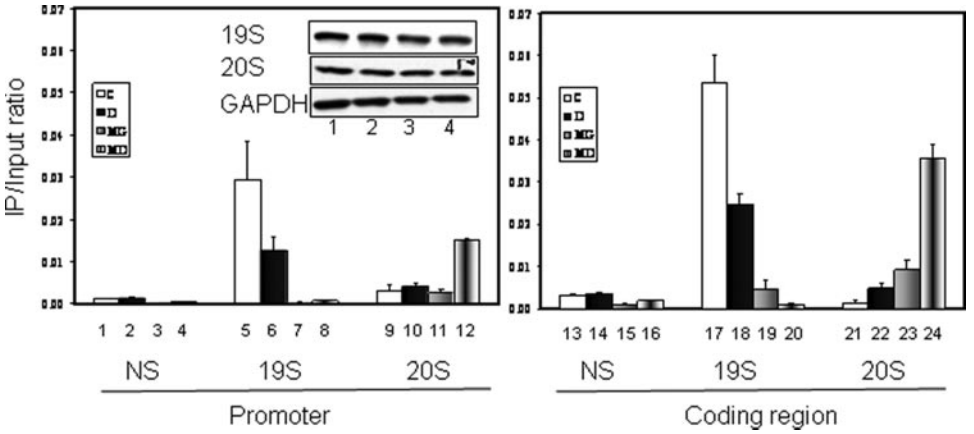

FIG. 3. Redistribution of proteasome subunits on the MMTV locus upon proteasome inhibition. Chromatin was immunoprecipitated with antibodies against the 19S (S1; Upstate) or the 20S [α2 (HC3); Biomol] proteasome subunits. Normal serum IgG (NS) was used as a control. Immunoprecipitated (IP) DNA/protein complexes were analyzed by real-time PCR using specific primers for the promoter and coding region. The inset in the promoter graph shows whole-cell extracts immunoblotted with antibodies against the 19S complex, the 20S complex, and GAPDH as a control. The abbreviations are the same as those described in the legend to Fig. 1.

ture by 2.5-fold, there is no additional alteration of chromatin structure in cells treated with MG132 and dexamethasone compared to dexamethasone alone (Fig. 1B, lanes 2, 3, and 4). As with other steroid receptors, the hormone-bound GR is rapidly degraded via proteasomal proteolysis. Inhibiting proteasomal proteolysis blocks degradation of the GR and increases receptor but not GAPDH levels (Fig. 2A; top, GR; bottom, GAPDH; lane 2 versus lanes 3 and 4). We then asked whether under these conditions the GR was associated with the response elements on the MMTV promoter by ChIP analysis. Analysis of GR-immunoprecipitated chromatin by PCR indicated a 40-fold increase in GR occupancy at the MMTV promoter after hormone treatment compared to the control (Fig. 2A, bar 6). Proteasome inhibition alone did not increase GR occupancy on the promoter (Fig. 2A, bar 7). Surprisingly, treatment with hormone after proteasome inhibition increased GR occupancy by only 10-fold (10) compared to the control, despite the increase in GR protein under these conditions (Fig. 2A, bar 8 and lane 4). In contrast to GR, normal serum IgG (NS) does not immunoprecipitate any MMTV DNA (Fig. 2A, bars 1 to 4). Glucocorticoid receptor-mediated activation and BRG-1-dependent chromatin remodeling of the MMTV promoter are intimately linked (14, 51). Additionally, other receptor coregulators, including p300, facilitate GR activation of the MMTV promoter (1, 28). We next examined the occupancy of these factors at the MMTV promoter after proteasome inhibition. Proteasome inhibition does not significantly change BRG-1, whereas p300 protein levels increase (Fig. 2B and C; top, BRG-1 or p300; bottom, actin; lanes 1 and 2 versus lanes 3 and 4). Similar to GR, ChIP analysis showed that treatment with dexamethasone increases promoter occupancy by these chromatin-modifying machines. In contrast, proteasome inhibition decreased their occupancy, suggesting that the changes in chromatin structure observed under these conditions may be independent of the BRG-1 complex (Fig. 2B and C). No PCR product with immunoprecipitated DNA was observed on the GAPDH promoter, suggesting that the recruitment of these factors is specific for the MMTV promoter (data not shown). These observations suggest that increased chromatin remodeling or coactivator recruitment, resulting from elevated GR levels, was unlikely as a mechanism mediating the increased mRNA expression upon proteasome inhibition.

Redistribution of proteasome subunits on the MMTV locus upon proteasome inhibition. There is a growing body of evidence to suggest a role for the proteasome in hormone receptor function apart from proteolysis of the receptors (23). Proteasome subunits such as Sug1/Rpt6/TRIP1 are transcriptional coactivators, assisting nuclear receptors in recruitment of RNA Pol II and initiation of transcription (27). To test whether specific proteasome subunits are linked to transcription at the MMTV locus (Fig. 1B), we analyzed binding of the 26S proteasome components to MMTV chromatin. Treatment with either the hormone or the proteasome inhibitor did not result in significant changes in 19S or 20S protein expression. As a control, GAPDH levels do not change (Fig. 3, inset). Antibodies against the 19S complex are S1 (recognizes ATPases independent of 20S [APIS complex]), which immunoprecipitated DNA from the promoter and coding region in cells treated with vehicle (Fig. 3, bars 5 and 17), and dexamethasone (bars 6 and 18), although binding of 19S decreases with hormone (compare bars 5 and 6 or 17 and 18). Inhibiting the proteasome evicts the 19S subunit from both the promoter and the coding region in either the presence or absence of hormone. In contrast to the 19S subunit, the 20S subunit showed little association with the promoter or coding region in the absence of either MG132 or hormone (Fig. 3, 20S promoter; bars 9, 10, and 21). However, upon proteasome inhibition there was a significant enrichment of the 20S subunit at the coding region, particularly in the presence of hormone (Fig. 3, coding region; bars 23 and 24). Similarly, there was an accumulation of the 20S on the promoter (Fig. 3, promoter; bar 12) after proteasome inhibition. These observations suggest a dynamic redistribution of proteasome subunits occurs concomitantly with the hormone-induced increase in MMTV transcription when the proteasome activity is inhibited.

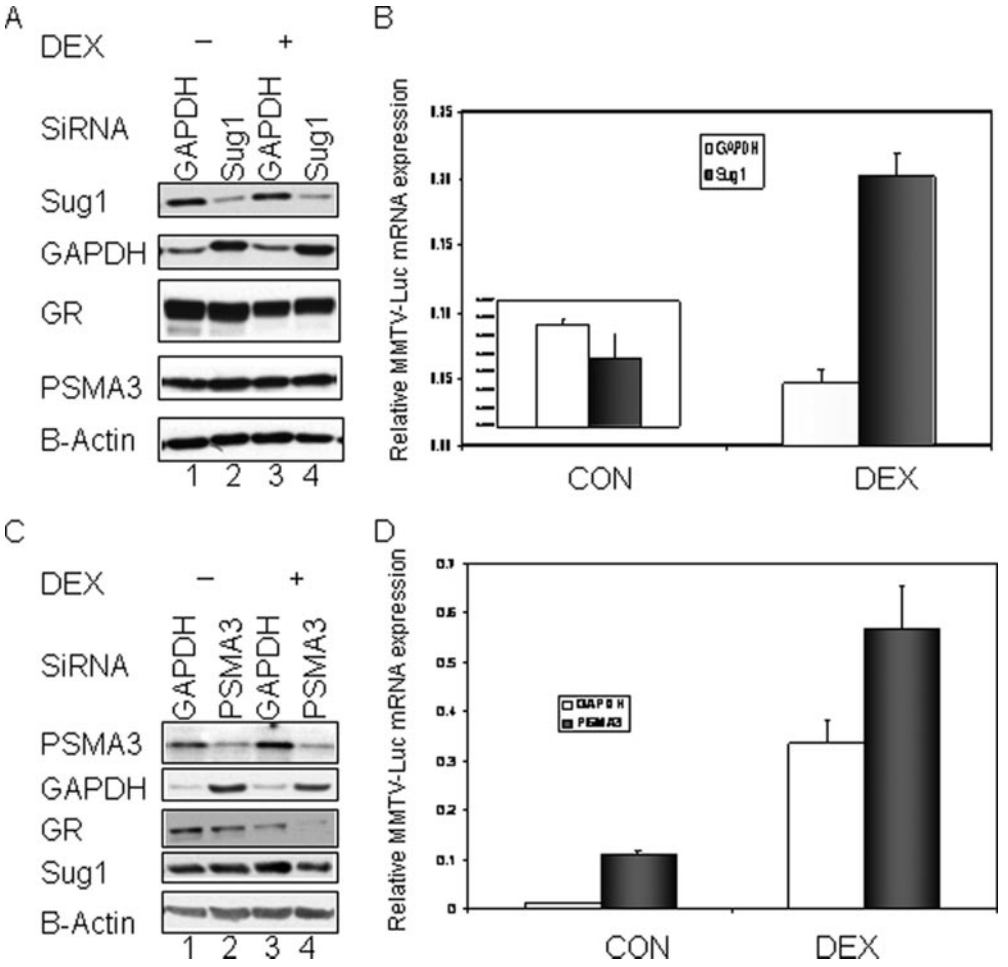

FIG. 4. RNAi of specific proteasome subunits enhances MMTV transcription. Depletion of proteasome subunits by RNAi increases the levels of MMTV mRNA expression. MCF-7 cells were transfected with either control siRNA (GAPDH) or siRNA targeted to the Sug1 or PSMA3 proteasome subunit. Whole-cell lysates were collected for Western blotting of various proteins (A and C). Total RNA was isolated, and MMTV mRNA expression was analyzed by quantitative reverse transcription-PCR (B and D). DEX, dexamethasone; CON, control.

RNAi depletion of specific proteasome subunits enhances transcription. An important question was whether the above described findings using proteasome inhibitor were direct effects of the proteasome subunits on transcription or pleiotropic effects of the proteasome inhibitor. We addressed this question by depleting specific proteasome subunits by RNAi and examining the effect on hormone-dependent gene expression. Sug1 is one of the six AAA-ATPases at the base of the 19S regulatory particle. PSMA3 is the proteasome subunit alpha type 3 of the 20S core particle. Western blot analysis confirmed Sug1 siRNA was effective in depleting Sug1 in the absence and presence of hormone (Fig. 4A, lanes 1 to 4). Depletion of Sug1 does not affect GAPDH or PSMA3 expression (Fig. 4A). Conversely, transfection of control siRNA against GAPDH does not change Sug1 or PSMA3 protein expression (Fig. 4A and C, Sug1 and PSMA3; compare lanes 1 and 3 to 2 and 4). Additionally, depletion of Sug1 does not block hormone-dependent decreases in GR protein levels (Fig. 4A, GR; compare lanes 1 and 2 to 3 and 4). Proteasome inhibition increases expression of MMTV-LUC transcript, concomitant with a decrease in binding of the 19S complex at both the promoter and the body of the gene (Fig. 1A and 3). Depletion of Sug1 enhances hormone-dependent MMTV expression, consistent with the effect of proteasome inhibition (Fig. 4B, DEX). Notably, basal MMTV expression does not significantly change after Sug1 depletion (Fig. 4B, CON, inset). As shown in Fig. 3, there is a redistribution of the proteasome subunits on the MMTV locus after proteasome inhibition, suggesting different roles of these subunits in MMTV transcription. To this end, unlike Sug1, depleting the PSMA3 subunit increases basal transcription, with a smaller effect on overall hormone-dependent transcription (compare CON and DEX in Fig. 4C and D). Similar to Sug1, depletion of the PSMA3 subunit does not block hormone-dependent decrease in GR protein (Fig. 4C, GR; compare lanes 1 and 2 to 3 and 4). Thus, inhibition of the proteasome or depletion of protein subunits by RNAi leads to a hormone-dependent increase in gene expression.

Proteasome inhibition modulates expression of chromatin-modifying and RNA polymerase II regulators. The ChIP experiment showing that GR association with the MMTV promoter after proteasome inhibition does not increase suggests that receptor proteolysis/turnover may not be the key feature

in the increase in the MMTV transcript after proteasome inhibition. Further support of this idea is provided by the observation that specific subunits of the proteasome negatively regulate MMTV transcription. These observations suggest that the proteasome can modulate hormone-mediated gene expression by regulating another factor(s) (apart from the receptor) that controls the transcription process. Additional analysis of the MMTV transcript, including nuclear run-on and mRNA stability, also suggests a possible role for the proteasome in postinitiation transcriptional processes (see Fig. S1B in the supplemental material). To get a general view of factors that can account for the increase in transcription, we turned to transcript profiling to provide a genome-wide view of transcriptional regulators that could be involved in modulating gene expression after proteasome inhibition (see Table S1 in the supplemental material). Total RNA samples from cells treated with vehicle, dexamethasone, or proteasome inhibitor with or without hormone were hybridized onto Agilent 60mer oligomicroarrays. Transcript profiles were compared between hormone treatment and proteasome inhibitor alone or with hormone. We found that a large percentage of genes changed by the combined hormone treatment and proteasome inhibition were transcriptional regulators (data not shown). We concentrated on examining genes in this category that would support a role of the proteasome in hormone response (see Table S1 in the supplemental material). In particular, the microarray experiments reveal a hormone- and proteasome inhibitor-dependent increase in transcripts encoding the MLL1 and RbBP5, a member of the MLL complex, a histone H3 lysine 4-specific methyltransferase (see Table S1 in the supplemental material) (9). This finding was particularly interesting to pursue, since a recent study has linked specific proteasome subunits to histone H3K4 methylation (10).

Proteasome inhibition modulates expression of the MLL histone methyltransferase. The specific increase in MLL mRNA, but not other histone methyltransferase mRNAs, seen in the microarray suggested that proteasome inhibition might cause an increase of some, but not all, histone methyltransferases (Fig. 5A). Additional analysis of other histone methyltransferases associated with histone H3 methylation showed a modest increase in Set 9 but not the histone methyltransferase SMYD3 (Fig. 5A). Analysis of the MLL complex proteins showed an increase in RbBP5 levels after proteasome inhibition, whereas other members of the complex, WDR5 and ASH2, did not significantly change (Fig. 5A, lanes 1 and 3). RT-PCR analysis confirmed that inhibiting the proteasome resulted in an increase in MLL mRNA expression, as predicted from the transcript profiling (Fig. 5B). As observed for the protein expression, proteasome inhibition decreases SMYD3 mRNA expression (Fig. 5C). Furthermore, in contrast to SMYD3, depleting either Sug1 or PSMA3 causes a hormone-dependent increase in MLL gene expression (Fig. 5D and E, Sug1 and PSMA3). These data suggest a direct and specific regulation of MLL by the 26S proteasome in the presence of hormone.

Proteasome inhibition increases trimethyl histone H3K4 at the MMTV locus. We pursued the consequences of elevating MLL by examining the levels of histone H3 lysine 4-specific methylation, as it is widely correlated with active chromatin in multiple systems (reviewed in reference 46). In the next set of experiments, we asked whether there were global changes in histone H3K4me3-specific methylation after proteasome inhibition. Inhibiting the proteasome led to a modest increase in cellular levels of trimethyl histone H3K4 (H3K4me3 in Fig.

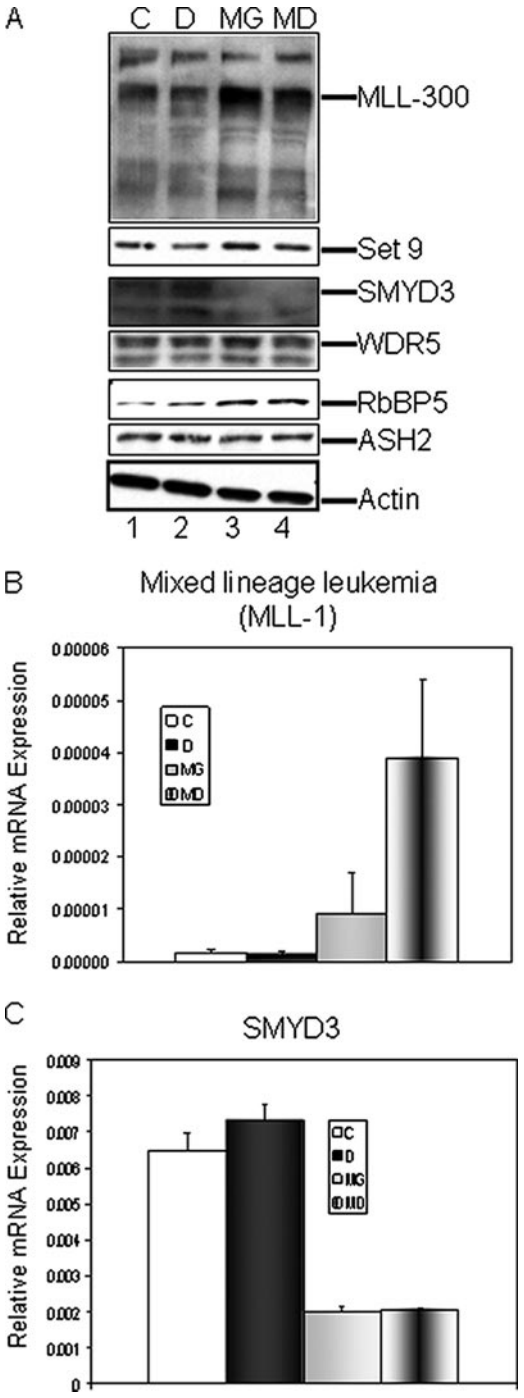

FIG. 5. Proteasome inhibition increases MLL expression. MCF-7 cells were treated as described in the legend to Fig. 1B. (A) Western blot of protein extracts to monitor MLL expression and MLL1 structural components. (B and C) Proteasome inhibition increases MLL, but not SMYD3, mRNA expression. (D and E) RNAi of specific proteasome subunits specifically enhances MLL (left panel), but not SMYD3 (right panel), mRNA expression. DEX, dexamethasone; CON, control. Other abbreviations are the same as those described in the legend to Fig. 1.

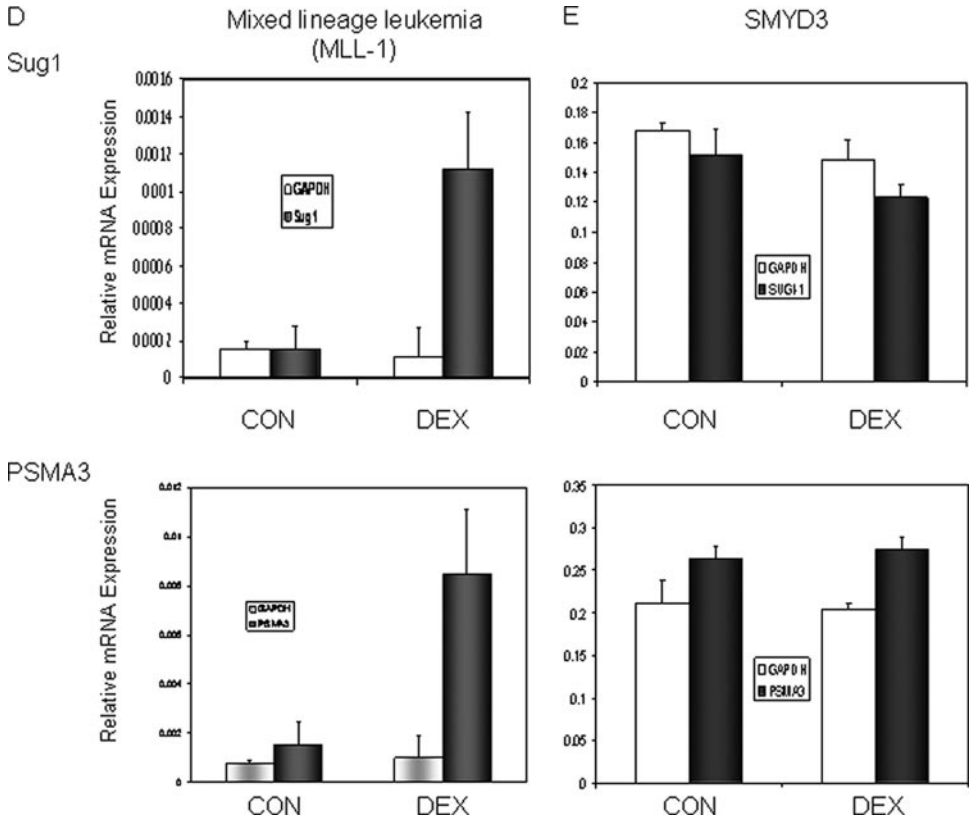

FIG. 5—*Continued*.

6A, lanes 1 and 3). As a control, we examined the levels of H3 with an antibody specific to the C terminus of H3, which revealed that the levels of histones were similar across treatment conditions, a result also supported by the Coomassie stain (Fig. 6A). Chromatin immunoprecipitation analysis with antibodies against histone H3K4me3 demonstrated that hormone exposure did not alter the levels of H3K4me3 on the promoter or the body of the locus (Fig. 6B, compare bar 5 with bar 6 and 13 with 14). In contrast, inhibiting the proteasome enriches histone H3K4me3 modification by threefold at the promoter and coding regions of the gene compared to the control (Fig. 6B, compare bar 5 with bar 7 and 13 with 15), and the enrichment is significantly enhanced sixfold by hormone (Fig. 6B, compare bar 7 with bar 8 and 15 with 16). As a control, we immunoprecipitated DNA with the antibody specific to the C terminus of H3 to show there was no loss of histone H3 after proteasome inhibition (Fig. 6C). We then asked whether the enrichment of the H3K4 trimethyl mark was a consequence of MLL binding to the MMTV locus after proteasome inhibition. ChIP experiments using an antibody against MLL1 showed that proteasome inhibition enriches MLL at the promoter by 2.5-fold in the absence of hormone and 1.5-fold with hormone compared to control (Fig. 6D, compare bar 5 with bars 7 and 8). MLL was enriched 1.5-fold at the coding region after proteasome inhibition alone (Fig. 6D, compare bar 13 with bar 15).

Proteasome inhibition modulates expression of RNA polymerase II. The trimethyl H3K4 mark is generally linked to transcriptionally active chromatin, and this in part could account for the increase in MMTV expression after proteasome inhibition independent of hormone (Fig. 1A). However, the increase in hormone-dependent MMTV expression after proteasome inhibition is not proportional to the trimethyl H3K4 mark, suggesting modulation of additional transcriptional regulators by the proteasome. Prominent among this group is a set of transcripts induced after proteasome inhibition and hormone treatment encoding factors that allow productive elongation of the transcript by RNA Pol II. The group included TFIIH (Cdk7), positive transcription elongation factor (P-TEFb-cyclin K), and a factor that stimulates the elongation rate of Pol II, eleven-nineteen lysine-rich in leukemia (ELL2). The significant changes in transcript profiles of genes encoding factors that facilitate the elongation rate of RNA Pol II prompted us to examine the status of RNA Pol II after proteasome inhibition. The proteasomes are highly concentrated within regions of genes with stalled polymerase complexes, which have recently been shown to be hyperphosphorylated (15, 48). We therefore asked whether inhibiting the proteasome alters global distribution of phosphorylated pools of Pol II. Western blot analysis revealed that Pol II was predominantly in a hypophosphorylated form in cells treated with vehicle or dexamethasone (Fig. 7A, lanes 1 and 2), whereas inhibiting the proteasome increases the global levels of the hyperphosphorylated form of Pol II (Fig. 7A, lanes 1, 3, and 4). Treatment with proteasome inhibitor results in only a small increase in the TFIIH complex (CycH/Cdk7) that phosphorylates serine 5 (S5P) compared to the control (Fig. 7B, lanes 3 and 4). In the P-TEFb complex, which phosphorylates serine 2,

treatment with MG132 increases Cdk9 but not the cyclin T component (Fig. 7B, lanes 3 and 4). Interestingly, transcript profiling had revealed an increase in cyclin K, another component of the P-TEFb complex (data not shown). It seems likely that the small increases in the expression of the kinase complexes observed will not fully account for the large changes in phosphorylated RNA Pol II pools. While we have not directly examined the kinase activity under these conditions, we are intrigued by the possibility that proteasome inhibition modulates the activity or the assembly of these complexes. Because of the dramatic changes in Pol II pools after proteasome inhibition, we examined by ChIP the binding of RNA Pol II to the MMTV locus. We first used an antibody against nonphosphorylated CTD (8WG16). Treatment with hormone increases Pol II association with the promoter and coding region two- and fourfold, respectively, compared to the control (Fig. 7C, bars 5 and 6 as well as 13 and 14). Compared to the control, proteasome inhibition alone or with hormone did not significantly change the density of the nonphosphorylated Pol II at the MMTV locus (Fig. 7C, bars 5 and 7 as well as 8, 15, and 16). Thus, the density of the nonphosphorylated Pol II at the MMTV locus after proteasome inhibition does not predict the increase in MMTV transcript under these conditions.

Phosphorylation of the carboxy-terminal domain of the large subunit of Pol II correlates with the transition from initiation to elongation complexes and is implicated in the coupling of various transcriptional processes, including a number of RNA-processing events that lead to production of mature mRNA molecules (39). If this is true, the phosphorylated Pol II could play a role in the hormone-dependent increase in MMTV expression after proteasome inhibition. To define a role for the phosphorylated Pol II in MMTV expression after proteasome inhibition, we used CTD kinase inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which blocks both Cdk7 and Cdk9 at specific concentrations. Because DRB has a general effect on Pol II-dependent transcription, treatment with DRB blocks hormone-dependent transcription by 55% (Fig. 8A, lanes 2 and 6). Notably, hormone-dependent transcription induced after proteasome inhibition is blocked 90% by DRB, suggesting a role of the phosphorylated Pol II in this process. To confirm a role of the phosphorylated Pol II in MMTV expression, we evaluated the density of the phosphorylated polymerases on the MMTV locus by ChIP. As shown on Fig. 8B, inhibiting the proteasome enhances hormone-dependent recruitment of Ser5-phosphorylated Pol II both at the promoter and at the body of the gene (Fig. 8B, S5P; bars 8 and 20). Proteasome inhibition enhances the density of Ser2 and phosphorylated Pol II primarily at the body of the gene under these conditions (Fig. 8B, S2P; bars 12 and 24). Thus, in contrast to the nonphosphorylated RNA Pol II, the hyperphosphorylated forms of Pol II on the MMTV locus are consistent with the observed hormone-dependent increase in transcription when the proteasome is inhibited.

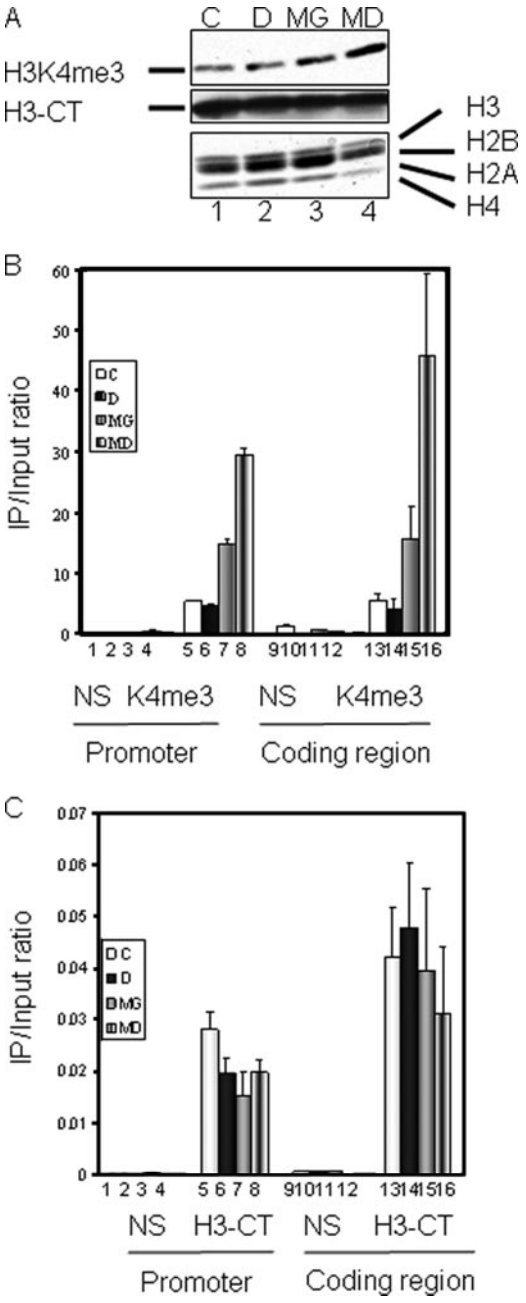

FIG. 6. Proteasome inhibition increases occupancy of trimethyl histone H3K4 and MLL at the MMTV locus. MCF-7 cells were treated as described in the legend to Fig. 1B. (A) Acid-extracted histones were immunoblotted with an antibody against trimethyl histone H3K4. An antibody directed against the C terminus of histone H3 (H3-CT) was used as a loading control. Coomassie blue stain represents another loading control for total histones (bottom panel). (B) MMTV chromatin was immunoprecipitated with antibodies against trimethyl histone H3K4. Samples were analyzed in triplicate, and data were expressed as the ratio of immunoprecipitated complexes to input DNA (IP/input ratio) ± standard errors of the means. The result is representative of a minimum of two biological replicates. (C) As a control, MMTV chromatin was immunoprecipitated with an antibody directed against the C terminus of histone H3. (D) MLL association with the MMTV locus. MMTV chromatin was immunoprecipitated with an antibody against MLL. Abbreviations are the same as those described in the legend to Fig. 1.

DISCUSSION

The 26S proteasome complex tightly regulates receptor turnover and transcription of many steroid hormone receptors, including the GR (36). Previous work showed that proteasome inhibition increases glucocorticoid receptor-mediated tran-

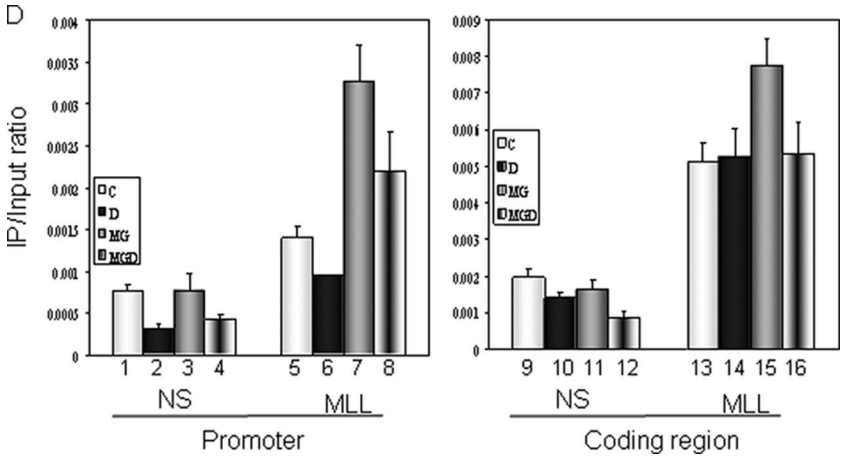

FIG. 6—*Continued*.

scriptional activity from the MMTV promoter (8). Here we have extended the study to understand the mechanism by which proteasome inhibition influences GR-mediated gene transcription. Although GR levels are increased in the cell, ChIP analysis monitoring GR occupancy revealed a decrease in GR loading on the MMTV promoter after proteasome inhibition (Fig. 1). This is consistent with the previous observation that proteasome inhibition decreases the mobility of the receptor within the nucleus, and this could lead to less GR on the promoter (8, 43, 49). Our observation is further supported by fluorescence recovery after photobleaching experiments showing no correlation between GR protein levels and transcriptional changes at the MMTV array in cells treated with proteasome inhibitor (49). Additionally, we show that neither the BRG-1 complex nor p300 are increased at the promoter after proteasome inhibition, suggesting that hormone-dependent promoter chromatin hypersensitivity after proteasome inhibition does not account for the increase in MMTV expression. These initial observations led us to propose that apart from receptor turnover, the proteasome can regulate components of the transcriptional machinery and chromatin structure modifications that modulate the hormone response.

The role of the 26S proteasome in recycling of receptor/transcriptional complexes has been suggested as the main mechanism involved in controlling gene expression (36, 42). For the GR, proteasome inhibition results in increased gene expression, suggesting that proteolytically linked recycling of the receptor is not a key mechanism for the observed increase in gene expression. Nonproteolytic activities of the proteasome such as coactivator recruitment have been proposed to be important for transcriptional regulation. As such, the differential occupancy by the 19S and 20S proteasome subunits on the promoter and the transcribed region of the gene has significant regulatory potential. One way the proteasome subunits can act at the DNA template is by facilitating activator-coactivator interactions required for the assembly of the transcription complex and activation of productive transcription. An interesting possibility is that the 19S ATPase complex can facilitate chromatin-modifying machines, allowing alteration in chromatin structure and transcription to occur as demonstrated for the SAGA complex (26). However, at the MMTV locus, chromatin remodeling is highly dependent on the BRG-1 but not the p300 hypoxanthine-aminopterin-thymidine complex. Indeed, there is a reduction in levels of 19S ATPase at the MMTV promoter in the presence of hormone. Subsequently we find that depletion of Sug1 results in an increase in hormone-dependent transcription. In contrast to Sug1, the 20S complex is present at the 3′ end of the gene, consistent with hormone-dependent and -independent transcription. This observation supports recent studies in yeast showing that the presence of the 20S at the 3′ end of the gene facilitates readthrough of the transcription termination site (15). Perhaps on the MMTV locus the 20S proteasome can decrease termination and facilitate hormone-dependent and -independent transcription, as seen in cells treated with proteasome inhibitor. RNAi experiments corroborate a role for the 20S proteasome as depletion of the PMSA3 subunit affects basal transcription. Our results are consistent with recent reports showing that the 20S is associated primarily with the 3′ ends of certain highly transcribed genes in yeast (2, 45). Additionally, the dynamic interplay between the 19S and 20S proteasome subunits at transcriptionally active loci was recently shown to dictate differential assembly of transcriptional complexes and activator-dependent transcription in embryonic stem cells and the human immunodeficiency virus type 1 (HIV-1) LTR (25, 50). In embryonic stem cells, the loss of the 19S subunit did not impede recruitment of the 20S subunit at transcriptionally active loci, suggesting the subunits can be targeted to different regulatory regions. This might then allow the recruitment of different transcriptional complexes and activities to modulate transcriptional output (50). A specific prediction would be that the 20S complex could form a preinitiation complex that could lead to permissive transcription of certain loci in embryonic stem cells (50). The finding that depletion of the 19S and 20S subunits has differential effects on basal MMTV transcription is echoed in a recent study on the HIV LTR locus. As shown for GR-mediated transactivation of the MMTV, ablation of the 19S ATPases affected TAT-mediated transcription of the HIV LTR without affecting basal transcription (25). As shown for MMTV, knockdown of the 20S enhanced basal transcription of the HIV LTR independent of TAT, analogous to the effect seen for GR. The authors attribute these effects to a switch between the proteolytic and

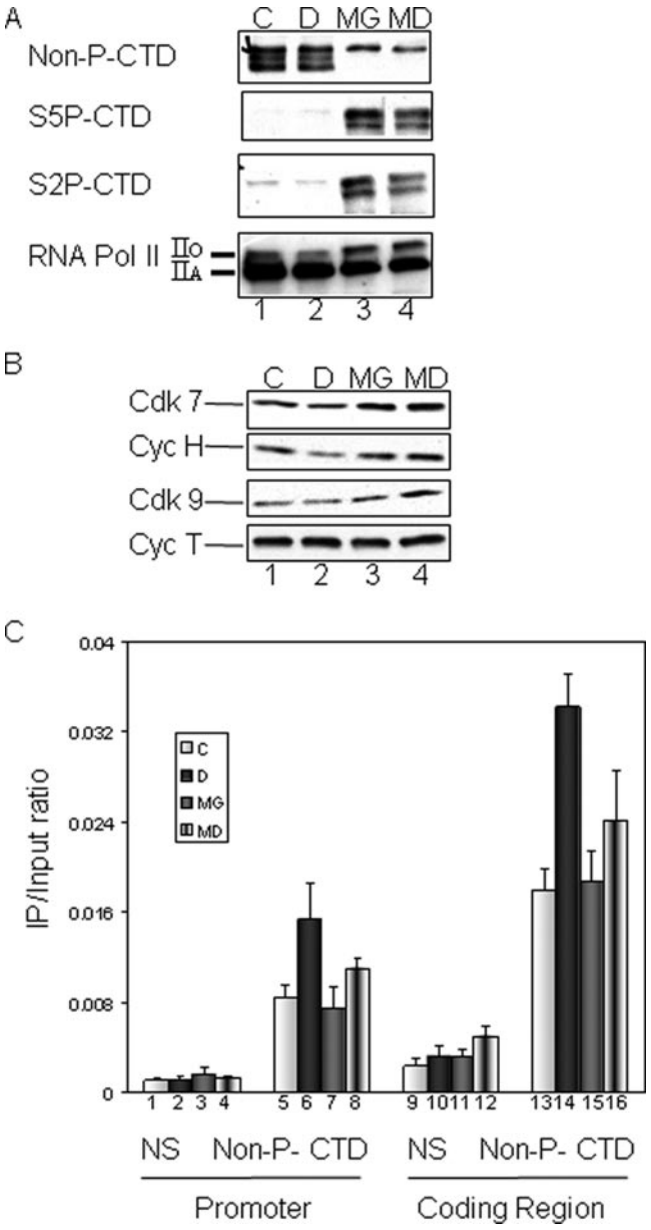

FIG. 7. Proteasome inhibition increases hyperphosphorylated forms of Pol II. (A) RNA polymerase II was evaluated by Western blotting using specific antibodies. (B) Western blot of whole-cell lysates to monitor expression of TFIIH (Cdk7/cyc H) and P-TEFb (Cdk9/cyc T) complexes. (C) Proteasome inhibition does not increase the hypophosphorylated form of Pol II associated with the MMTV locus. Normal serum IgG (NS) was used as a control. Abbreviations are the same as those described in the legend to Fig. 1.

nonproteolytic effects of the proteasome subunits. Specifically, the 19S is involved in activator turnover, whereas the 20S may be involved in initiation and elongation processes and control of the mature transcript production. Thus, the redistribution of the proteasome subunits at the MMTV locus after proteasome inhibition may facilitate formation of different transcriptional or coregulator complexes. Such complexes would then modulate hormone-dependent and -independent transcriptional output by as-yet-uncharacterized mechanisms. In contrast to the MMTV locus, a recent study has shown that the 20S proteasome beta subunit LMP2 physically interacts with the p160 coactivators and enhances estrogen receptor-mediated transcription of the pS2 gene (54). However, similar to our study and the studies cited above, the authors showed that the 20S subunit is specifically involved in the transcriptional elongation, supporting a role of the 20S at the 3′ end of the gene. Taken together, the evidence currently available supports a role of specific proteasome subunits in receptor-mediated transcriptional regulation.

Proteasome inhibition results in global changes in trimethyl histone H3K4, a mark recently shown to be associated with an active chromatin structure that is permissive to transcription (29, 41). Additionally, the trimethyl histone H3K4 is associated with genes that maintain a poised chromatin state, such as the β-globin locus (44). Histone modifications can alter chromatin structure by acting as recognition marks for factors that recognize specific modifications and alter nucleosome structure (6). For example, the chromodomain helicase binding protein 1 (CHD1), a member of the SNF2-like family of ATPases that mobilize nucleosomes, specifically recognizes the methyl histone H3K4 mark (13, 47). Notably, in human cells the nucleosome remodeling factor (NURF), another member of the ATP-dependent chromatin remodeling complex, seems to specifically recognize the trimethyl histone H3K4 mark (53). While we have not looked directly at NURF in the context of these experiments, our previous studies suggest it is not able to remodel the promoter (11). An important caveat is that in the previous studies the promoter would not have been expected to have any significant trimethyl histone H3K4, and thus its contribution is unknown.

The increase in both mRNA and protein expression for MLL, a histone H3K4me3-specific histone methyltransferase, in cells treated with proteasome inhibitor and RNAi of proteasome subunits is intriguing. This suggests that the proteasome functions to regulate MLL expression, although the mechanism is not clear. The increase in MLL expression and the presence of the trimethyl H3K4me3 on hormone-activated genes suggests a role of MLL in hormone response. MLL regulates mainly homeobox genes, but recent reports show that MLL regulates p27 (Kip 1) and p18 (ink4C) genes involved in suppression of cell growth and proliferation (34). This function agrees well with our current finding that suggests that proteasome function can modulate hormone and biological response by changing factors that regulate transcription. In contrast to MLL, SMYD3, known to increase cell proliferation, is inhibited by proteasome inhibition (18).

The observation that chromatin hypersensitivity and gene expression are increased independent of the hormone implies that the proteasome may function to regulate basal transcription (Fig. 1B, lane 3). These data suggest that the proteasome, in the absence of hormonal stimulation, functions to maintain a closed chromatin environment at the MMTV promoter. The mechanisms by which this occurs are presently unknown, but we note that the MG132-dependent increase in transcription is accompanied by a modest recruitment of BRG1 at the promoter independent of hormone (Fig. 2B). Interestingly, there are concomitant increases in the chromatin structure sensitivity, gene expression, and H3K4me3 levels at the MMTV locus, suggesting that specific histone modifications at certain loci can

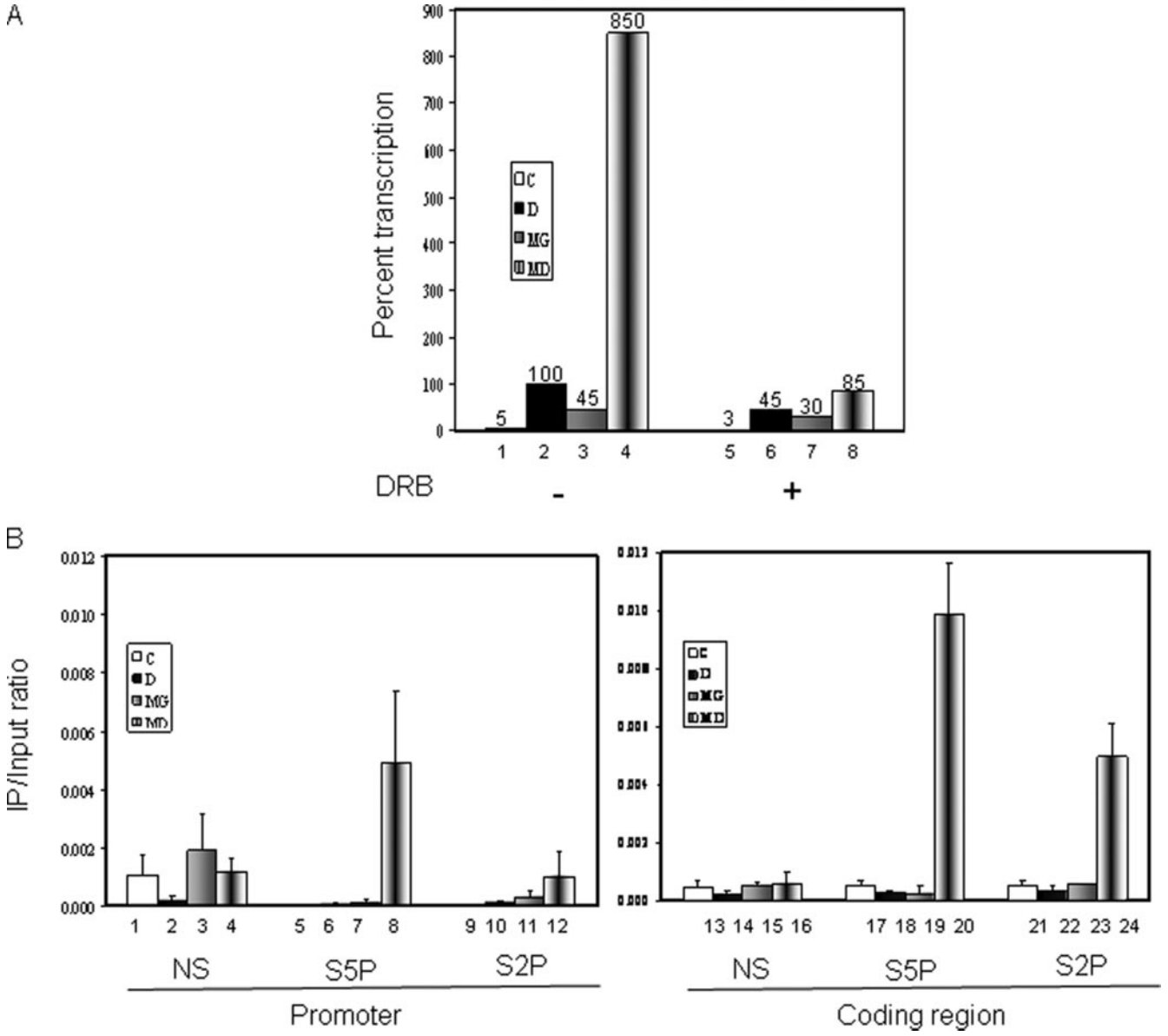

FIG. 8. Role of the hyperphosphorylated RNA of Pol II in hormone-dependent transcription. (A) The kinase inhibitor DRB blocks the increase in MMTV mRNA expression. (B) Hyperphosphorylated forms of Pol II associate with the MMTV locus. ChIP analysis showed the occupancy of different phosphorylated forms of Pol II on the MMTV locus. Normal serum IgG (NS) was used as a control. Abbreviations are the same as those described in the legend to Fig. 1.

initiate hormone-independent aberrant gene expression (Fig. 6B). Furthermore, this may specifically involve the 20S proteasome, since RNAi of this subunit increases gene expression independent of hormone (Fig. 4C and D). Our findings echo a recent report showing that histone modifications are important in modulating hormone-independent gene expression implicated in androgen insensitivity in tumor cells (19).

Another important feature correlated with the enhanced gene expression after proteasome inhibition is the increase in global pools of phosphorylated RNA Pol II. RNA Pol II phosphorylation is essential for a number of transcriptional processes that lead to successful mature transcript (37, 40). That Pol II is hyperphosphorylated upon proteasome inhibition is consistent with recent findings suggesting that polymerase phosphorylation, particularly at Ser5, inhibits polymerase ubiquitylation and increases transcriptional efficiency (48). Notably, despite the increase in global levels of hyperphosphorylated Pol II after proteasome inhibition, the transcriptional effect is exclusively hormone dependent, suggesting cooperativity of these forms of Pol II with additional factors that modulate transcriptional responses. Such cooperativity between the hyperphosphorylated forms of RNA Pol II and other transcriptional regulators would support differential regulation of receptor target genes after proteasome inhibition. Our findings are consistent with recent reports showing that the requirement of phosphorylated Pol II by p53 target genes is gene

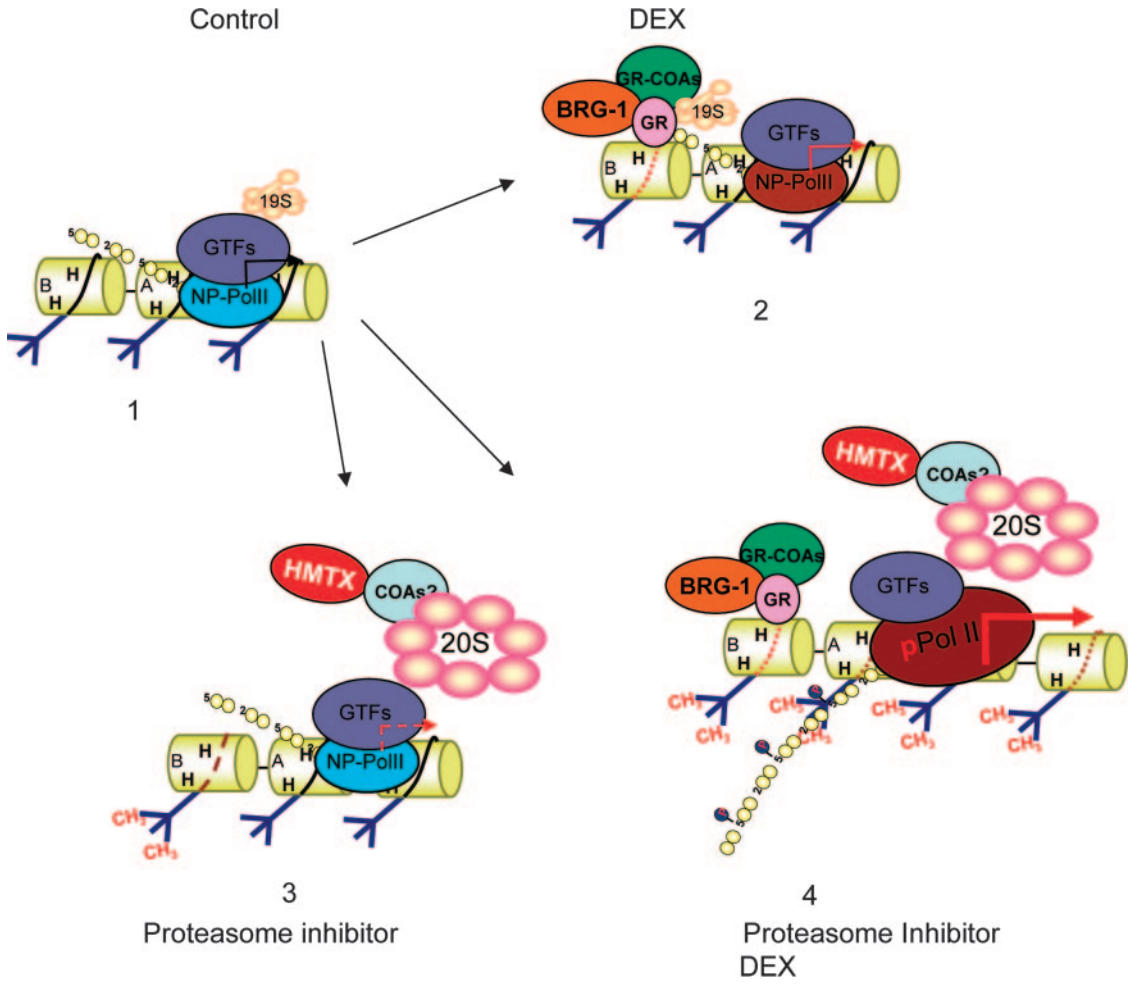

FIG. 9. Model for enhanced receptor-mediated transcriptional output by regulating chromatin modifications and RNA Pol II machinery upon proteasome inhibition. 1. In the absence of hormone or proteasome inhibition, the 19S subunit and nonphosphorylated Pol II associate with MMTV DNA, with no transcription. 2. Addition of hormone evicts the 19S and activates the GR to recruit chromatin remodeling complexes BRG-1 and coactivators (COAs; p300) to remodel chromatin, enhancing hormone-dependent occupancy of nonphosphorylated RNA Pol II and MMTV transcription. 3. In the absence of hormone (GR), proteasome inhibition results in basal transcription and partial chromatin remodeling by enhancing expression of specific histone methyltransferases that target histone lysine 4 methylation to modify chromatin, perhaps in concert with other histone-modifying enzymes and alternative ATP-dependent chromatin-remodeling complexes. The 20S proteasome is linked to basal transcription independent of hormone. 4. In the presence of hormone, proteasome inhibition increases transcriptional output by elevating the levels of the elongation-competent hyperphosphorylated RNA Pol II associated with the promoter and expression of specific histone methyltransferases that target histone lysine 4 methylation to modify chromatin, perhaps in concert with other histone-modifying enzymes that correlate with transcriptionally active genes. HMTX, unknown histone methyltransferase; GTFs, general transcription factors; B, nucleosome B; H, histone H3; A, nucleosome A; DEX, dexamethasone.

specific and dependent on the type of stimuli (16). An interesting observation is the lower levels of nonphosphorylated CTD at the promoter and the coding region of the MMTV gene after proteasome inhibition. From Western blotting analysis, it is clear that global pools of RNA Pol II forms change after proteasome inhibition. Interestingly, some studies have attributed decreases in hormone response after proteasome inhibition to the lack of polymerase loading on the promoter of the target gene. For example, a recent study showed that proteasome inhibition suppresses progesterone receptor-mediated gene expression and attributed this to a decrease in RNA polymerase II recruitment, but the study did not analyze hyperphosphorylated forms of the polymerase (7). On the same lines of evidence, Pol II was not detected in 60% of transcription-competent gene promoters using the same antibody as that in our study (21). The authors attributed the lack of correlation between transcription and Pol II occupancy to the efficiency of immunoprecipitation of chromatin fragments with this antibody. Our observations may explain the diverse effects of proteasome inhibition on steroid hormone receptor-mediated gene transcription. Indeed, global analysis of gene expression by microarray indicates differential effects of proteasome inhibition on GR target genes (H. K. Kinyamu, J. Collins, S. Grissom, P. Hebbar, and T. K. Archer, unpublished data). Finally, our data support the hypothesis that the 20S proteasome complexes are present at sites of active transcription in conjunction with Pol II phosphorylated forms (2, 45).

In summary, our findings posit dynamic interplay between

steroid hormone receptor-mediated gene transcription and proteasome activity that links proteasome activity with histone modifications and Pol II transcriptional machinery in mammalian cells (Fig. 9). This postinitiation transcriptional role for the proteasome in regulating receptor-mediated gene expression represents a powerful mechanism for receptors to regulate a diverse array of genes involved in numerous physiological processes.

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