On the Mechanism of Constitutive Pdr1 Activator-mediated PDR5 Transcription in Saccharomyces cerevisiae

EVIDENCE FOR ENHANCED RECRUITMENT OF COACTIVATORS AND ALTERED NUCLEOSOME STRUCTURES

Chen Gao, Luming Wang, Elena Milgrom, and W.-C. Winston Shen‡

From the Department of Biochemistry and Molecular Biology, State University of New York Upstate Medical University, Syracuse, New York 13210

Drug resistance as a result of overexpression of drug transporter genes presents a major obstacle in the treatment of cancers and infections. The molecular mechanisms underlying transcriptional up-regulation of drug transporter genes remains elusive. Employing Saccharomyces cerevisiae as a model, we analyzed here transcriptional regulation of the drug transporter gene PDR5 in a drug-resistant pdr1-3 strain. This mutant bears a gain-of-function mutation in PDR1, which encodes a transcriptional activator for PDR5. Similar to the well studied model gene GAL1, we provide evidence showing that PDR5 belongs to a group of genes whose transcription requires the Spt-Ada-Gen5 acetyltransferase (SAGA) complex. We also show that the drug-independent PDR5 transcription is associated with enhanced promoter occupancy of coactivator complexes, including SAGA, Mediator, chromatin remodeling SWI/SNF complex, and TATA-binding protein. Analyzed by chromatin immunoprecipitations, loss of contacts between histones and DNA occurs at both promoter and coding sequences of PDR5. Consistently, micrococcal nuclease susceptibility analysis revealed altered chromatin structure at the promoter and coding sequences of PDR5. Our data provide molecular description of the changes associated with constitutive PDR5 transcription, and reveal the molecular mechanism underlying drug-independent transcriptional up-regulation of PDR5.

Transcriptional regulation of the ATP binding cassette (ABC) transporter genes plays a pivotal role in the development of a drug resistance phenotype in mammalian (1, 2) and yeast cells (3–5). Constitutive transcriptional up-regulation of drug transporter genes occurs in certain mutations involving various transcriptional activators. However, the molecular mechanism underlying this enhanced transcription is not fully understood.

In Saccharomyces cerevisiae, the multiple/pleiotropic drug resistance (MDR/PDR) phenotype is primarily regulated via two transcriptional activators encoded by homologous PDR1 and PDR3 genes. These factors are responsible for activating the majority of drug transporter genes, including PDR5 (5–7). Both activators belong to the Gal4 superfamily with the Zn2Cys6 DNA binding domain (8). The amino-terminal of their DNA binding domains recognize promoters with the Pdr1/Pdr3 response element (PDRE),5 5'-TCCCGGA-3' (9), such as that with PDR5.

The Pdr5 transporter belongs to the ABC transporter superfamily. In the drug-resistant pdr1-3 and pdr3-7 strains, PDR5 transcription is the highest among target genes activated by Pdr1 and Pdr3 (10). The pdr1-3 and pdr3-7 strains are analogous to other mutants bearing gain-of-function mutations in PDR1 (11) and PDR3 (12) that up-regulate PDR5 transcription.

The pdr1-3 allele bears a F815S mutation located near the transcription activation domain at the COOH terminus of Pdr1, suggesting that the mutation may alter the function of the activation domain.

Activation of transcription in eukaryotes is mainly controlled by activator-mediated recruitments of transcription factors (13), facilitating the formation of pre-initiation complex. Recruitment of TBP is essential for pre-initiation complex formation (14, 15). TBP and TBP-associated factors (TAFs) are components of TFIIID, which is recruited to the core promoter (16). Mediator, on the other hand, is recruited to the upstream activating sequence (UAS) promoter (17) and associates with RNA polymerase II during initiation of transcription (18, 19). Moreover, the ATP-dependent chromatin remodeling SWI/SNF and the histone modification SAGA (Spt-Ada-Gen5 acetyltransferase) complexes are also recruited by activators to facilitate transcription (20–22).

Limited information is available on PDR5 transcription by Pdr1 and Pdr3 activators. It has been reported that Ada3, a component of the SAGA complex, interacts with the Pdr1 activation domain in two-hybrid analysis (23). The transcription of a GAL10-LacZ reporter gene by a fusion activator (consisted of Gal4 DNA binding and Pdr1 activation domains) was enhanced in ada3Δ and ada2Δ strains, suggesting that the association of Ada3 with the Pdr1 activation domain may inhibit Pdr1-mediated transcription (23). Whether the inhibitory effect of Ada3 also occurs on endogenous target genes of Pdr1 activator remains unclear. Furthermore, a transcriptional repressor of PDR5, termed Rdr1 (a zinc cluster protein), was suggested...
Constitutive Pdr1 Activation of Yeast PDR5 Transcription

TABLE I

| Strain                  | Genotype                                  | Source          |
|-------------------------|-------------------------------------------|-----------------|
| WCS261 (YALF-A1)        | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 pdr1–3 | Wollger et al. (34) |
| WCS262 (YALF-11)        | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 | Wollger et al. (34) |
| WCS302                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 TAF1-Myc | This study      |
| WCS303                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 pdr1–1 TAF1-Myc | This study      |
| WCS314                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 pdr1–1 GCN5-Myc | This study      |
| WCS315                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 TAFL-Myc | This study      |
| WCS318                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 AD2-Myc | This study      |
| WCS322                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 SPT20-Myc | This study      |
| WCS323                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 SPT3-Myc | This study      |
| WCS326                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 pdr1–3 SPT3-Myc | This study      |
| WCS327                  | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 SPT3-Myc | This study      |
| WCS463                  | MATα ura3 his3 leu2–3,112 trp1 Δ/HHT1-HHF1 Δ/HHT2-HHF2 pdr1–3 pNOY436 | This study      |
| WCSS464                 | MATα ura3 his3 leu2–3,112 trp1 Δ/HHT1-HHF1 Δ/HHT2-HHF2 PDR1 pNOY436 | This study      |
| WCSS506                 | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 pdr1–3-Myc | This study      |
| WCSS507                 | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1-Myc | This study      |
| WCSS508                 | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 pdr1–1 GAL11-Myc | This study      |
| WCSS509                 | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 GAL11-Myc | This study      |
| WCSS510                 | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 pdr1–1 SBR4-Myc | This study      |
| WCSS511                 | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 SBR4-Myc | This study      |
| WCSS512                 | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 pdr1–1 SRF2-Myc | This study      |
| WCSS513                 | MATα ura3–3 leu2–3,112 his3–11,15 trp1–1 PDR1 SRF2-Myc | This study      |
| FY294                   | MATα sptΔ202 his4-976 lex2Δ1 lex2-173R2 trp1Δ63 ura3–52 | Sterner et al. (35) |
| FY1462                  | MATα sptΔ202::LEU2 his4-976 lex2Δ1 lex2-173R2 trp1Δ63 | Sterner et al. (35) |
| FY1481                  | MATα his4-976 lex2Δ1 lex2-173R2 trp1Δ63 ura3–52 | Sterner et al. (35) |
| FY626                   | MATα his4-976 lex2Δ1 lex2-173R2 trp1Δ63 ura3–52 | Sterner et al. (35) |
| FY668                   | MATα his4-976 lex2Δ1 ura3–52 | Sterner et al. (35) |
| FY61                    | MATα his4-976 leu2Δ1 ura3–52 | Sterner et al. (32) |
| FY1097                  | MATα trp1Δ63 spt2Δ100::URA3 ura3–52 | Roberts and Winston (33) |
| FY1067                  | MATα trp1Δ63 ura3–52 | Roberts and Winston (33) |

Based on observations that deletion of PDR1 increased PDR5 transcription and augmented cycloheximide resistance in a PDR4-sequence-specific manner (24). However, it remains unknown whether Rdr1 directly binds to the PDR5 promoter and interferes with Pdr1/Pdr3-mediated transcription. Through screening of a panel of strains carrying deletions of zinc cluster genes, Hsh5 was identified as a transcriptional activator of multidrug resistance genes (25), although the interaction between Hsh5 and Pdr1/Pdr3 remain to be explored. Finally, in genome-wide microarray analyses, the transcription of PDR5 was not significantly affected by inactivating TAFs (26). However, inactivating transcription of PDR5 in a pair of isogenic drug-sensitive PDR1 and drug-resistant pdr1–3 strains. For the two strains, we compared the recruitment of Pdr1 activator and coactivators to the PDR5 promoter, the histone association with DNA, and the sensitivity to nucleosome digestion on PDR5. Our data depict molecular events associated with drug-independent (constitutive) PDR5 transcription, and reveal distinctions between drug-dependent and drug-independent transcriptional up-regulation of PDR5.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Growth of yeast cells in rich (YPD, yeast extract, peptone, dextrose) or synthetic media was performed according to standard procedures (31). The genotypes of yeast strains used in this study are listed in Table I (32-35). To monitor drug-induced PDR5 transcription, cycloheximide treatment at a final concentration of 0.2 μg/ml (~0.71 μl) for 45 min was used, which was far below the drug concentration required for translation inhibition (26.13 μg/ml or 100 μM). Introduction of Myc tags to the COOH termini of various transcription factors (Pdr1, Taf1, Gcn5, Spt20, Ada2, Srb4, and Snf2) was accomplished by PCR-mediated gene modifications as described previously (36). The strains WCS463/ WCS464 are a pair of pdr1-3/PDR1 strains whose endogenous histone H4 genes HHH1 and HHH2 were deleted and supplemented with a low copy plasmid-borne Myc-tagged HHH2 gene with the Myc sequence, SEKQKLISEEDL, inserted between the first and second amino acids of histone H4 encoding sequences. We generated these strains by mating pdr1–3 (WCS261) with NOY847 (which contains Myc-tagged histone H4 as the sole source of histone H4 (37)). The resulting diploid cells were sporulated and dissected to recover haploid progeny with pdr1-3 and Myc-HHH2 alleles. The haploid strains harboring the desired pdr1-3 Myc-HHH2 genotype were mated back to NOY847 and the diploid dissected to isolate the isogenic haploid pair of pdr1-3/PDR1 strains. The WCS463/WCS464 strains are segregants from diploid cells obtained after three consecutive runs of mating and dissection.

**Northern Blot Analysis**—Preparation of total RNA and Northern blot analysis were performed as described (38). Typically, 20 μg of total RNA was separated by gel electrophoresis on 1% formaldehyde-agarose gels, blotted to GeneScreen membranes (PerkinElmer Life Sciences) and UV cross-linked. Membranes were hybridized in 6× SSC, 5× Denhardt’s solution, 0.5% SDS, and 50 μg/ml denatured salmon sperm DNA at 65°C for 12 to 16 h, followed by four 15-min washes with 2× SSC and 0.1% SDS. Probes for PDR5 and SCR1 transcripts were obtained by PCR amplification from genomic DNA. SCR1 was used as an equal loading control.

**Chromatin Immunoprecipitation**—Chromatin immunoprecipitation (CHIP) of Myc-tagged transcription factors was performed as described (26), except the immunoprecipitation was performed using anti-Myc antibody. Specific polyclonal rabbit antibody against TBP has been described (26). The antibodies recognizing acetylated histone H3 and H4 were purchased from Upstate Cell Signaling Solutions. Antibodies recognizing acetylated histone H3 lysines 5, 8, 12, and 16 were used for detection of histone H3 acetylation levels, whereas antibodies recognizing acetylated histone H4 lysines 9 and 14 were used for detection of the histone H4 acetylation levels. Typically, DNA templates recovered from immunoprecipitation products were amplified by PCR in 20 μl reactions; this condition confers a linear reflection of relative quantity of the immunoprecipitation products. Each CHIP experiment was repeated independently at least twice to ensure the reproducibility of the results. We conducted a series of 2-fold dilutions of DNA recovered at the end of the CHIP procedure to ensure that the PCR reflected a linear correlation of CHIP products. Typically, input signals correspond to 1/200 of the total input. Taf1, Srb4, and Hht1 were used per immunoprecipitation reaction. Quantification of relative recruitment signals was performed by a Phosphor-Imager (Amersham Biosciences, model 425) and the relative ratio of IP to input signal was presented. The following sets of primer pairs were used for PCR analysis of CHIP products by various antibodies: PDR5.
Constitutive Pdr1 Activation of Yeast PDR5 Transcription

(PDR1), 5’-CTTGGCAGGAGAAAGCTC-3’ and 5’-ACCCGCCGCTTGGC-3’ and 5’-ACCCGCCAAGTACAG-3’ and 5’-ACCCGCCAAGTACAG-3’. PDR5 (PDR2 and -3), 5’-TGGATGACAGTATTACAGTT-CAGACC-3’ and 5’-GGAGGAGGCTTTGTTTATGC-3’. PDR5 (TA), 5’-CGCGCTTGGTACATGATGC-3’ and 5’-GTCGATGTAACACT-AACACAGTTG-3’. PDR5 (N-CDS), 5’-CCAGAAGCGCTGATACAG-3’ and 5’-CCCTGTCGATATGCCCTCC-3’. PDR5 (M-CDS), 5’-GAAAGCTCTGGAAGGAATCCC-3’ and 5’-CCUCCTGCGGGCAAAATCTC-3’. PDR5 (C-UTR), 5’-GACCAAACTCTCTATAGC-3’ and 5’-GGGGCTTCCCTGCCC-3’. PDR5 (G-UTR), 5’-CCTAGCTTCTGAGTT-3’ and 5’-CCGTATATGAGAAGACGGTTC-3’. PDR12 (promoter), 5’-CACACTTGTCTGACTGTT-3’ and 5’-GTAACCTGAGAACAGAG-3’.

Mapping PDR5 Chromatin Structure by Micrococcal Nucleases—The method employed for nuclease hypersensitivity analysis on the PDR5 chromatin structure was modified from previously described protocols (39, 40). Briefly, yeast cells were grown in YPD to an optical density of 0.9, and immediately fixed by the addition of a final concentration of 1% formaldehyde and incubated for 10 min. The formaldehyde treatment was terminated by the addition of a final concentration of 125 mM glycine followed by a 5-min incubation. The fixed cells were washed with double distilled water and treated with 1 mg/ml Zymolase for 8 min. Spheroplasts were resuspended in cold-buffer A (1.0 x sorbitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5.0 mM MgCl2, 1.0 mM CaCl2, 1.0 mM β-mercaptoethanol, and 0.5 mM spermidine). For nuclease digestion, 100-µl aliquots of buffer B (buffer A plus 0.2% Nonidet P-40) containing 25–400 units/ml micrococcal nuclease ( Worthington Biochemical Corp.) were prewarmed at 37 °C in microcentrifuge tubes followed by the addition of 100 µl of spheroplasts to each tube followed by a 5-min incubation at 37 °C. The reactions were terminated with 20 µl of stop buffer (250 mM EDTA, 5% SDS). Formaldehyde cross-linking was reversed by overnight incubation at 65 °C. Digested chromatin was then purified through RNAase A treatment, phenol/chloroform extraction, and isopropanol precipitation. The recovered DNA was digested to completion with HindIII (for PDR5 promoter mapping) or PstI (for PDR5 3’-UTR mapping), and separated on 1 or 1.2% agarose gels and blotted for indirect end labeling analysis. 32P-labeled probes for Southern blot hybridization were obtained from genomic PCR products of primers 5’-CATGCTTGTACGTACAGTT-CAGACC-3’ and 5’-GGGGCTTCCCTGCCC-3’. PDR5 (G-UTR), 5’-CCTAGCTTCTGAGTT-3’ and 5’-CCGTATATGAGAAGACGGTTC-3’. PDR12 (promoter), 5’-CACACTTGTCTGACTGTT-3’ and 5’-GTAACCTGAGAACAGAG-3’.

RESULTS

PDR5 Belongs to a Group of SAGA-dependent Genes—The transcription of PDR5 was found to be independent of TAFs (26). However, TAFs are present in the transcription factor complexes SAGA and TFIID, and the functions of these two complexes are closely related (27, 41). We first analyzed the role of SAGA components in the transcription of PDR5 in a wild-type strain, PDR1 strain. Drastic reduction of PDR5 transcription was observed in spt7Δ, spt20Δ, spt3Δ, spt8Δ, adaΔ, and adaΔΔ strains (Fig. 1A). In contrast, no significant change in PDR5 transcription was detectable in cells with gen5ΔΔ, indicating that GCN5 is dispensable for PDR5 transcription (Fig. 1A, right panel).

Ahc1 is a unique subunit of the ADA complex (42), which can form a complex physically independent of SAGA (42). Rtg2 is present specifically in a SAGA-like (SLIK) complex (43). We included ahc1Δ and rtg2Δ strains in our Northern analysis of PDR5 transcription (Fig. 1A, right panel). Similar to the result with the gen5ΔΔ strain, no change in PDR5 transcription was noted in either the ahc1Δ or rtg2Δ strains. The dispensability of Ahc1 and Rtg2 for PDR5 transcription argues that the functions of SAGA complex, rather than its related complexes, are critical for PDR5 transcription. Consistent with this notion, an spt8Δ strain exhibits a dramatic loss of PDR5 transcription (Fig. 1A, left panel), and the Spt8 subunit is present in the SAGA complex but is missing in SAGA-related SLIK and SALSA complexes (43, 44). Therefore, the transcriptional regulation of PDR5 in spt7Δ, spt20Δ, spt3Δ, spt8Δ, adaΔ, and adaΔΔ strains reflect genetic requirements for the SAGA complex, but not Gen5-mediated chromatin modification.

We also observed that deletion of GCN5 does not affect cycloheximide-induced PDR5 transcription (data not shown). However, Gen5 is recruited to the PDR5 promoter in a comparable manner as SAGA components (Fig. 3B, discussed below). We concluded that Gen5 is present on the PDR5 promoter, but its presence was not essential for PDR5 transcription. This finding is reminiscent of the dispensable role of Gcn5 in GAL1 activation, even though Gen5 is recruited to the GAL1 promoter with other components of SAGA complex (29).

SAGA is an essential target of Gal4 at the GAL1,10 promoters (29, 30). Fluorescence resonance energy transfer experiments demonstrated that the Gal4 activation domain targets the Tra1 subunit of SAGA, Gal4-Tra1 interaction is required for recruitment of SAGA to the UAS, and SAGA, in turn, recruits the Mediator complex to the UAS (22). To address the role of Mediator in regulating PDR5 transcription, we analyzed the effect of inactivating Srb4 on PDR5 transcription. The Mediator subunit Srb4 is required for the integrity of the complex (45, 46). Thermal inactivation of temperature-sensitive srb4Δ-2 caused a dramatic reduction of PDR5 transcription and, in fact, the effect of the srb4Δ-2 mutation on PDR5 was noticeable even at 25 °C (Fig. 1B). These data indicated that PDR5, like GAL1, requires both SAGA and Mediator for its transcription.

It was reported that the association of Ada3, a SAGA component, with the Pdr1 activator resulted in inhibition of the Pdr1 activation domain in two-hybrid assays (23). In contrast, our studies on endogenous PDR5 transcription demonstrated that Ada2 and Ada3 subunits of SAGA were required for PDR5 transcription. Moreover, the enhanced recruitment of Ada2 to the PDR5 promoter (Fig. 3B) correlated with increased PDR5 transcription, supporting a positive role of Ada2 in Pdr1 activator-regulated PDR5 transcription. This apparent discrepancy could be because of the artificially created chromatin structure for the two-hybrid reporter genes (23) versus the endogenous PDR5 promoter.
Constitutive Pdr1 Activation of Yeast PDR5 Transcription

Fig. 2. Wild-type and mutant activators of Pdr1 are associated with the PDR5 promoter independent of drug induction. A, Northern blot analysis of PDR5 transcription with and without cycloheximide induction in PDR1 and pdr1-3 strains. Treatment of cycloheximide (CYH) at a final concentration of 0.2 µg/ml for 45 min was used. SCR1 served as a loading control. B, Northern blot analysis of PDR5 transcription in Myc-tagged PDR1 and pdr1-3 strains indicated that the Myc-tagged Pdr1 and Pdr1-3 activators were functional. No cycloheximide induction was used in this experiment. C, Myc-tagged Pdr1 and Pdr1-3 activators are constitutively recruited to the PDR5 UAS containing three PDREs that bind Pdr1 (diagrammed in Fig. 3). Top panel, recruitment of C-terminal Myc-tagged activators Pdr1 and Pdr1-3 to the UAS of the PDR5 promoter was analyzed by CHIP. Myc antibody was used and followed by PCR amplification of the PDR5 promoter DNA present in IP products. The recruitment signals of Pdr1 or Pdr1-3 to the PDR12 promoter served as negative controls. Bottom panel, quantification of recruitment signals of Pdr1 or Pdr1-3 to PDR5 UAS was presented as the relative IP/input ratio by a histogram. Error bars reflect standard deviations between three independent experiments.

Constitutive Recruitment of Pdr1 to the PDR5 Promoter—The transcription of PDR5 in a pdr1-3 drug-resistant strain is dramatically higher than that in its corresponding PDR1 wild-type strain. PDR5 transcription in the pdr1-3 strain in the absence of cycloheximide was comparable with that in the PDR1 strain in the presence of cycloheximide (Fig. 2A). Because PDR5 transcription in the pdr1-3 strain remained inducible by cycloheximide (Fig. 2A), the underlying molecular events associated with drug-independent Pdr1-3-mediated transcription of PDR5 appeared distinct from drug-dependent transcriptional induction of PDR5.

As the first step toward understanding the molecular mechanisms causing high PDR5 transcription in the pdr1-3 strain, we analyzed the recruitment of Pdr1 and Pdr1-3 proteins onto the PDR5 UAS by CHIP. We introduced Myc tags to wild-type Pdr1 and mutant Pdr1-3 at their COOH-terminal ends and integrated the tagged genes into the endogenous PDR1 and pdr1-3 loci in wild-type and drug-resistant strains, respectively (36). The Myc-tagged version of Pdr1 and Pdr1-3 proteins are functional as they can mediate transcription of PDR5 in a comparable manner to their counterparts without Myc tags (Fig. 2B), and the tagged versions of the pdr1-3 and PDR1 strains remain drug resistant and sensitive, respectively (data not shown).

As analyzed by the CHIP technique using Myc antibody, the recruitment signals of Pdr1 and Pdr1-3 to the PDR12 promoter, which is not subject to regulation by PDR1 (47, 48), were at least 10-fold lower than those to the PDR5 UAS sequences. These data indicate that Pdr1 and Pdr1-3 activators bind the PDR5 promoter in a specific manner. Interestingly, Pdr1 and Pdr1-3 binding to the PDR5 UAS remained virtually unchanged in the presence and absence of drug induction (Fig. 2C). These data argue against the possibility that Pdr1 binding per se is a rate-limiting step in drug-dependent PDR5 transcription. Nevertheless, slightly higher binding signals of Pdr1-3 relative to Pdr1 onto the PDR5 promoter were observed, which suggested that the drug-independent hyperactive PDR5 transcription in the pdr1-3 strain is associated with increased occupancy of transcription factors on the PDR5 promoter.

Drug-independent Enhanced Coactivator Recruitment to the PDR5 Promoter in the pdr1-3 Strain—We analyzed the recruitment of TFII D, SAGA, Mediator, and SWI/SNF complexes to the PDR5 promoter. We Myc-tagged representative subunits of TFII D, SAGA, Mediator, and SWI/SNF complexes, and performed CHIP analyses on the recruitment of these transcription factors to the PDR5 TATA box and UAS promoter (Fig. 3). TBP and Taf1 (a TFII D-specific TAF), representing TFII D, and Spt20, Spt3, Adn2, and Gcn5, representing SAGA components, were analyzed. In parallel, Srb4 and Gal11, subunits located at different domains of the Mediator complex, and Snf2, the ATPase subunit of the SWI/SNF complex, were analyzed. All Myc-tagged transcription factors were functional; moreover, drug resistance and relative levels of PDR5 transcription in PDR1 versus pdr1-3 strains remained unchanged in the tagged strains (data not shown). The recruitment data of these transcription factors onto the PDR5 promoter are summarized in Fig. 3E, which clearly demonstrated a 2–3-fold increase in recruitment of all analyzed transcription factors, except Taf1 in pdr1-3 relative to PDR1 strains. The low Taf1 recruitment to the PDR5 promoter relative to the recruitment of TBP is typical of a TAF-independent promoter (47). Strikingly, in no case did we observe significant variation in the transcription factor recruitment with and without cycloheximide (Fig. 3E). We concluded that in the absence of drug induction, Pdr1-3-mediated PDR5 transcription involved enhanced coactivator recruitment. In contrast, drug-dependent PDR5 transcription was not regulated at the step of transcription factor recruitments.

Loss of Contact between Histones and DNA of PDR5 in pdr1-3—Hyperacetylation of histones is generally associated with activated transcription (50). We attempted to compare the acetylation levels of the PDR5 gene in pdr1-3 versus PDR1 strains, employing antibodies recognizing acetylated histone H3 (lysines 4 and 9) and H4 (lysines 5, 8, 12, and 16). CHIP experiments were performed using antibodies against acetylated histone H3 and H4 to evaluate the acetylation level of the entire PDR5 gene, ranging from the 5’ promoter (UAS with three Pdr1/Pdr3 response elements, PDR5s; and TATA box containing core promoter), coding sequences (CDS), to the 3’-UTR (see the diagram in Fig. 3). Unexpectedly, the apparent histone H3 and H4 acetylation levels at the PDR5 promoter region in pdr1-3 were one-third to one-half of those observed in the PDR1 strain, regardless of drug induction (Fig. 4A). Interestingly, the same pattern extended from the PDR5 promoter to the following 4.5-kb coding sequences. In contrast, there was no difference in apparent acetylation levels at the 3’-UTR of PDR5 (Fig. 4A), which was the promoter region for the adjacent
Fig. 3. Drug-independent enhanced recruitments of coactivators to the PDR5 promoter. CHIP was used to detect the recruitment of representative subunits of TFIID, SAGA, Mediator, and SWI/SNF complexes to the PDR5 promoter in PDR1 versus pdr1-3 in the presence and absence of cycloheximide (CYH). The diagram depicts the PDR5 gene indicating the positions of the TATA box, UAS with three PDREs that bind Pdr1, coding sequences, and the 3'-UTR. Three PDREs on the PDR5 promoter marked as open circles and the TATA box marked as a square are located at positions -923, -205, -143, and -60 relative to the transcription start site, respectively. The PDR5 coding sequences (+174 to +4710) are drawn in a smaller scale relative to that in the promoter region. Arrows marked with control, TATA, and UAS represent the primer sets used in this figure and additional arrows depict more primer sets used in Fig. 4. Treatment of cycloheximide at a final concentration of 0.2 μg/ml for 45 min was used for PDR5 induction. A, recruitment of TFIID components TBP and Taf1 to the PDR5 TATA box region. B, recruitment of SAGA components Spt20, Spt3, Ada2, and Gcn5 to the PDR5 UAS region. C, recruitment of the Mediator components Srb4 and Gal11 to PDR5 UAS. D, recruitment of the SWI/SNF component Snf2 to PDR5 UAS. E, summary of the recruitments of representative subunits of TFIID, SAGA, Mediator, and SWI/SNF complexes to the PDR5 promoter in PDR1 versus pdr1-3 in the presence and absence of cycloheximide. The recruitment of each transcription factor is presented as the relative IP/input ratio. Variations on each relative IP/input ratio between three independent experiments are within 15% (not shown).
Loss of histone-DNA contact of *PDR5* occurs at promoter and coding sequences. A, apparent lower acetylation levels on the *PDR5* promoter in the *pdr1-3* strain revealed by CHIP analyses using antibodies recognizing acetylated histone H3 and H4 proteins. The histone H3 and H4 acetylation levels on the *PDR5* gene were analyzed in *PDR1* versus *pdr1-3* strains starting from Pdr1 activator binding sites (*PDREs*), core promoter (*TATA*), and CDS, to the 3'−UTR. Primer sets used for PCR after recovery of IP products from CHIP experiments are the same as diagramed in Fig. 3. The same IP products were analyzed for acetylation levels on the *PDR12* promoter as a control. Treatment of cycloheximide (CYH) at a final concentration of 0.2 μg/ml for 45 min was used for *PDR5* induction. Quantification of the histone H3 and H4 acetylation levels is presented as the relative IP/input ratio by histograms. B, relative levels of histone H4 associated with DNA at the *PDR5* gene determined by Myc antibody recognizing Myc-tagged histone H4 as the only source of histone H4. *PDRE*, *TATA*, CDS, and UTR are defined in A. CYH treatment and quantification of data are the same as described in A. The same IP products were analyzed for histone H4 levels on the *PDR12* promoter as a control. Standard variations on each relative IP/input ratio between three independent experiments are within 15% (not shown).
FIG. 5. Micrococcal nuclease digestion revealed the altered chromatin structure of PDR5. A, analysis of MNase susceptibility of the PDR5 promoter region in the PDR1 versus pdr1-3 strains. Distinctive differences between PDR1 and pdr1-3 strains in the hypersensitive sites located at the PDR5 TATA box and transcription start site are marked with asterisks. The increasing concentrations of MNase marked by triangles represent 0, 25, 50, and 100 units per ml of the enzymes in MNase digestion. ND stands for naked DNA. MNase used for digestion of naked DNA was 10 units. The PDR5 transcription start site is marked as +1. Solid lined ovals depict positioned nucleosome structures, whereas dotted lined ovals depict more dynamic and less well positioned nucleosome structures in the UAS region. B, analysis of MNase susceptibility of the PDR5 promoter region in the PDR1 with and without cycloheximide induction. Differences in the intensity of bands corresponding to the MNase-hypersensitive sites located at PDR5 UAS are marked with asterisks. The units of MNase used were the same as described in A. Treatment of cycloheximide (CYH) at a final concentration of 0.2 µg/ml for 45 min was used for PDR5 induction. C, analysis of micrococcal nuclease susceptibility of the PDR5 3' UTR in PDR1 versus pdr1-3 strains. The MNase hypersensitivity sites detected in the PDR1 strain define four nucleosome structures (depicted by four ovals and marked as nuc-w, nuc-x, nuc-y, and nuc-z, respectively). Distinctive differences between PDR1 and pdr1-3 strains in the MNase-hypersensitive sites located toward the end of the PDR5 translation stop codon are marked with asterisks. The increasing concentrations of MNase marked by triangles are the same as indicated in A. PDR5 3' UTR indicates the location of CHIP analyses presented in Fig. 4.
ORF (open reading frame) YOR154W (see the diagram in Fig. 5B). By comparison, acetylation levels at the PDR12 promoter region were virtually identical between pdr1-3 and PDR1 strains (Fig. 4A).

We then tested if hyperacetylated histones at the PDR5 promoter are detectable in pdr1-3 relative to PDR1 strains after 3 min instead of 45 min of cycloheximide treatment. The results were indistinguishable from those presented in Fig. 4A (data not shown). Therefore, cycloheximide induction does not appear to affect acetylation of histones on PDR5 in either PDR1 or pdr1-3 strains.

Histones at the PHO5 promoter have been shown to lose contact with the activated PHO5 promoter (51–53). Moreover, loss of histones are associated with activation of PHO5 (54). We thus considered two possible interpretations of the apparent lower acetylation levels with the higher PDR5 transcription in pdr1-3 strains. First, the data could in fact reflect lower histone acetylation levels per se on histones present at the PDR5 in the pdr1-3 strain. Alternatively, lower apparent acetylation levels could reflect a loss of histones as in the case of PHO5 and PHO8.

To address the level of histones associated with PDR5 DNA in pdr1-3 versus PDR1 strains, we generated a pair of pdr1-3 and PDR1 strains whose endogenous histone H4 genes were deleted and complemented with a centromere-based plasmid-bearing Myc-tagged histone H4 gene, HHF2, as the only source of histone H4 in the cells (37). The drug resistance and PDR5 transcription in the Myc-tagged histone H4 pdr1-3 and PDR1 strains remained unaltered, equivalent to their parental pdr1-3 and PDR1 strains (data not shown). We then performed CHIP assays with Myc antibodies to analyze the histone levels cross-linked to the DNA sequences of the PDR5 gene in the pairwise isogenic pdr1-3 and PDR1 strains bearing Myc-tagged histone H4. As shown in Fig. 4B, the amount of histone H4 cross-linked to the DNA sequences of the PDR5 promoter region through the end of coding sequences (C-CDS) in the pdr1-3 strain was approximately a quarter to one-third of those observed in the PDR1 strain. Strikingly, when the PDR5 3’-UTR was analyzed, virtually no difference in histone H4 levels was noticeable between pdr1-3 and PDR1 strains. No loss of histone at the PDR12 promoter (not regulated by Pdr1) was observed in the pdr1-3 strain. Cycloheximide induction of PDR5 did not accompany any significant change at the histone levels on PDR5 in either PDR1 or pdr1-3 strains (Fig. 4B). These data suggest that alteration of nucleosome structure as a result of histone loss plays a critical role in drug-independent PDR5 transcription. We concluded that loss of histones on PDR5 could account, to a large extent, for the lower apparent acetylation levels in pdr1-3 strain.

Altered Nucleosome Structure of PDR5 in pdr1-3 Strain—To further test the effect of loss of histones on the nucleosome structure of PDR5, we analyzed the chromatin structure at both the PDR5 promoter and 3’-UTR regions by subjecting permeabilized PDR1 and pdr1-3 cells to micrococcal nuclease (MNase) digestion (Fig. 5). Micrococcal nuclease preferentially cuts inter-nucleosomal linker regions, and GC-rich sequences are more resistant to MNase digestion (55). We observed a distinctive difference in MNase sensitivity only at the PDR5 TATA box and the transcription start site in pdr1-3 versus PDR1 strains (Fig. 5A). In PDR1, a strong and distinct band depicting a MNase-hypersensitive site close to the PDR5 TATA box was present upon low concentrations of MNase digestion, which indicated a less well defined inter-nucleosomal linker region. In pdr1-3, the relative intensity of this band was much weaker even upon higher concentrations of MNase digestion, which suggested that loss of histones on PDR5 indicates a less well defined inter-nucleosomal linker region. In pdr1-3, a strong and distinct band depicting a MNase-hypersensitive site close to the PDR5 TATA box was present upon low concentrations of MNase digestion, which indicated a less well defined inter-nucleosomal linker region. The changed MNase hypersensitivity at this site presumably reflected distorted nucleosome structures as a result of enhanced TBP binding in the pdr1-3 strain presented in Fig. 3A. Crystal structure of TBP/TATA box complex has revealed significant topological changes at the DNA structures surrounding the TATA box upon TBP binding (56). Consistent with the presence of altered nucleosomal structures at the PDR5 core promoter in pdr1-3, an additional MNase-hypersensitive site was observed that was located close to the PDR5 transcription start site in the pdr1-3 strain (Fig. 5A).

In contrast to the core promoter region, no significant change in MNase sensitivity at the PDR5 UAS region encompassing three Pdr1/Pdr3 activator binding sites was detectable in PDR1 versus pdr1-3 strains (Fig. 5A). Multiple weak bands representing MNase-hypersensitive sites were detectable at the PDR5 UAS region, which is in contrast to the digestion pattern of well positioned nucleosome structures separated by approximately 150 bp (57) at the end of the PDR5 coding sequences in PDR1 (Fig. 5C). These data suggested that in both PDR1 and pdr1-3 strains, the nucleosome structures at the PDR5 UAS region are more dynamic and less well positioned than that in the coding sequences.

Even though we observed significant loss of histone H4 at PDR5 UAS in pdr1-3 (Fig. 4), no noticeable change in the MNase digestion pattern at the PDR5 UAS promoter region was observed between PDR1 and pdr1-3 strains (Fig. 5A). However, upon cycloheximide induction moderate changes in the intensity of bands corresponding to MNase hypersensitivity sites at the PDR5 UAS region in PDR1 were detectable (Fig. 5B). These data suggested that subtle changes occurred to UAS bound Pdr1 activator upon cycloheximide induction, which in turn affected interactions between Pdr1 and its binding sites.

The difficulties in detecting significant changes of MNase digestion pattern at the PDR5 UAS region between PDR1 and pdr1-3 as well as upon cycloheximide induction may reflect the intrinsic limitations of the MNase sensitivity assays. For instance, the PDR5 UAS region does not appear to contain well positioned nucleosomes even in the PDR1 strain. It is also possible that the relatively GC-rich nature of PDR5 UAS sequences might have affected the efficiency of MNase digestion at this region. Interestingly, the UAS of the divergent GAL1,10 promoters is likewise GC-rich (58) and there is no well positioned nucleosome detectable by MNase in the vicinity (59).

Based on the MNase hypersensitivity sites observed in PDR1, four clear nucleosome structures located close to the end of PDR5 coding sequences could be defined (marked as nucle-w, nuc-x, nuc-y, and nuc-z in Fig. 5C). In the pdr1-3 strain, we observed major changes in locations and intensity of MNase-hypersensitivity sites clustered at nuc-w and nuc-x located at the end of PDR5 coding sequences, and a less pronounced change at nucleosome z (Fig. 5C). In contrast, there was no detectable alteration in MNase hypersensitivity sites between PDR1 and pdr1-3 strains at nucleosome y located immediately downstream of the end of PDR5 coding sequences (3’-UTR). These data were consistent with the CHIP analyses (Fig. 4), which indicated that there was no detectable difference in histone association with DNA and histone acetylation levels at the 3′-UTR between PDR1 and pdr1-3. Taking together the results of CHIP and MNase hypersensitivity assays, we conclude that the nucleosome structure of PDR5 is altered as a result of histone loss in the pdr1-3 strain.

DISCUSSION

In this study, we describe the molecular events associated with the high transcriptional activity of the Pdr1-3 activator. The results classify PDR5 as a SAGA-dependent gene. This group of genes encompass ~10% of the yeast genome, including
GAL1 (27, 41). They contain a TATA box, and are associated with response to stress (28). In contrast to the majority of yeast genes, TATA-containing and stress-related genes are preferentially regulated by SAGA rather than by TFIID (27). PDR5 belongs to the group of stress-related, TATA-containing SAGA-dependent genes based on the following characteristics. First, the PDR5 promoter is responsive to drug induction and contains a consensus TATA sequence located 61 bp upstream of the transcription start site (9, 60). Second, significant recruitment of TBP, but not TFIID-specific Tat1, is associated with the PDR5 promoter in both PDR1 and pdr1-3 strains (Fig. 3A). PDR5 transcription is not significantly affected by inactivation of TAFs (36). Third, PDR5 transcription requires components of SAGA complex essential for SAGA integrity (35) (Fig. 1A) and these components are recruited to the PDR5 promoter (Fig. 3B).

The parallel between PDR5 and GAL1 goes beyond the requirement of TBP and SAGA and being TAF-independent. First, Gal4 and Pdr1 are both large proteins that possess a Zn2Cys6 DNA-binding domain at their NH2 termini. Second, Gal4 and Pdr1 are both large proteins that posses a requirement of TBP and SAGA and being TAF-independent. PHO5 distinct functions in addition to histone acetylation at the histone H2 (64, 65), and SAGA can perform mechanistically consistent with this notion, SAGA is required for ubiquitination of PDR5 and these components are recruited to the promoter in both PHO5 (Fig. 2A) and GAL1 (63) promoters.

PDR5 transcription can occur normally in a gcn5Δ strain (Fig. 1A). However, significantly increased recruitments of Gcn5 and other SAGA components were detected on the PDR5 promoter in the pdr1-3 strain (Fig. 3B). These data suggest that Gcn5 could be recruited to the PDR5 promoter along with other SAGA components even though its function is not absolutely required. It is possible that the SAGA complex might play multiple functions in regulating PDR5 transcription. Consistent with this notion, SAGA is required for ubiquitination of histone H2 (64, 65), and SAGA can perform mechanistically distinct functions in addition to histone acetylation at the PHO5 promoter (66). Furthermore, microarray analyses of cells bearing spt3Δ or gcn5Δ alleles indicate that Gcn5-mediated acetylation cannot fully account for the requirement for the SAGA complex at genomic transcription (27).

PDR5 can be induced by cycloheximide treatment; however, we did not observe effects of cycloheximide on transcription factor recruitment (Fig. 3), histone acetylation levels (Fig. 4A), and histone DNA contact (Fig. 4B) in PDR1 and pdr1-3 strains. Consistently, we did not observe changes nucleosome structure at the PDR5 core promoter in the PDR1 strain upon cycloheximide induction. Nevertheless, there were detectable, albeit moderate, changes at the PDR5 UAS in the PDR1 strain upon cycloheximide induction (Fig. 5B). These observations suggest that transcriptional up-regulation of PDR5 via drug-independent versus drug-dependent mechanisms are distinct. Significant changes of the nucleosome structures at the PDR5 core promoter are associated with drug independent pdr1-3-mediated PDR5 transcription (Fig. 5A), whereas cycloheximide-dependent PDR5 transcription appears to only moderately affect nucleosome structures at PDR5 UAS (Fig. 5B).

We show by CHIP the recruitment of both Pdr1 and Pdr1-3 activators to the PDR5 UAS region occurs in the absence of drug. These results are in agreement with the in vivo footprint experiments showing the protection of Pdr1 binding sites at the PDR5 promoter being constitutive (61). Our CHIP data suggest the increased recruitments of the Pdr1 activator and various transcriptional coactivators onto the PDR5 promoter in pdr1-3 are, at least partially, responsible for the drastic increase in PDR5 transcription in the pdr1-3 strain without drugs. Significantly, we did not observe any further increases in transcription factor recruitment onto the PDR5 promoter upon drug treatment in either PDR1 or pdr1-3 strains, despite the fact that PDR5 transcription can be induced by drug treatment in both strains. Therefore, the enhanced transcription factor recruitment associated with Pdr1-3-mediated PDR5 transcription is constitutive. This finding underscores the fundamental difference in PDR5 transcription between drug-dependent and drug-independent hyperactive PDR5 transcription.

Through CHIP and micrococcal nuclease sensitivity assays, we conclude that the steady state chromatin structures of the PDR5 gene are altered in the pdr1-3 strain relative to the PDR1 strain (Figs. 4 and 5). Regarding CHIP analyses, we also utilized antibodies against unacetylated histone H3 or H4 to evaluate relative histone-DNA association on the PDR5 gene in PDR1 versus pdr1-3 strains. The data (not shown) are in agreement with those obtained using the Myc antibody against Myc-tagged histone H4 presented in Fig. 4B.

The altered nucleosome structure of PDR5 in pdr1-3 is not limited to the PDR5 promoter region but also occurs at the coding sequences (Figs. 4 and 5C). As an example, Tup1-Ssn6 co-repressor complex can modulate rearrangements and orders of nucleosomal arrays on the FLO1 promoter over a 5-kb upstream intergenic region of the promoter (67). Thus, it is possible that chromatin remodeling transcription factor complexes, such as components of SWI/SNF, are recruited at the PDR5 promoter and engaged in long-range remodeling, thereby contributing to the observed changes of chromatin structures at sites other than the promoter region. Another possibility is that histone chaperone Asf1 may be required in modulating the nucleosome structure at coding sequences, in addition to its reported essential functions in chromatin disassembly upon activation of PHO5 and PHO8 promoters (54). It is also conceivable that transcription elongation factors associated with elongating RNA polymerase II are required for relaxing chromatin structure at the PDR5 coding sequences. In conclusion, we provide here clear evidence for enhanced recruitment of coactivators and altered nucleosome structures as a mechanism for Pdr1-mediated constitutive PDR5 transcription in S. cerevisiae.

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