Histone Modifications at the ABCG2 Promoter following Treatment with Histone Deacetylase Inhibitor Mirror Those in Multidrug-Resistant Cells

Kenneth K.W. To, Orsolya Polgar, Lyn M. Huff, Kuniaki Morisaki, and Susan E. Bates

Molecular Therapeutics Section, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland

Abstract

ABCG2 is a ubiquitous ATP-binding cassette transmembrane protein that is important in pharmacology and may play a role in stem cell biology and clinical drug resistance. To study the mechanism(s) regulating ABCG2 expression, we used ChIP to investigate the levels of acetylated histone H3, histone deacetylases (HDAC), histone acetyltransferases, and other transcription regulatory proteins associated with the ABCG2 promoter. Following selection for drug resistance and the subsequent overexpression of ABCG2, an increase in acetylated histone H3 but a decrease in class I HDACs associated with the ABCG2 promoter was observed. Permissive histone modifications, including an increase in histone H3 lysine 4 trimethylation (Me3-K4 H3) and histone H3 serine 10 phosphorylation (P-S10 H3), were observed accompanying development of the resistance phenotype. These changes mirrored those in some cell lines treated with a HDAC inhibitor, romidepsin. A repressive histone mark, trimethylated histone H3 lysine 9 (Me3-K9 H3), was found in untreated parental cells and cells that did not respond to HDAC inhibition with ABCG2 up-regulation. Interestingly, although all five studied cell lines showed global histone acetylation and MDR1 up-regulation upon HDAC inhibition, only those cells with removal of the repressive mark, and recruitment of RNA polymerase II and a chromatin remodeling factor Brg-1 from the ABCG2 promoter, showed increased ABCG2 expression. In the remaining cell lines, HDAC1 binding in association with the repressive Me3-K9 H3 mark apparently constrains the effect of HDAC inhibition on ABCG2 expression. These studies begin to address the differential effect of HDAC inhibitors widely observed in gene expression studies. (Mol Cancer Res 2008;6(1):151–64)

Introduction

ABCG2 is a ubiquitous ATP-binding cassette transmembrane protein that plays a significant role in absorption, distribution, and elimination of its substrate drugs (1-4). It also confers resistance in cancer cells to a variety of cancer chemotherapeutic agents such as mitoxantrone, topotecan, and methotrexate (5). ABCG2 overexpression is frequently observed in human cancer cell lines selected with various anticancer drugs (6–10).

Little is known about the molecular mechanisms regulating ABCG2 expression. The human ABCG2 gene has a TATA-less promoter, with several Sp1, AP1, and AP2 sites and a CCAAT box downstream from a putative CpG island. To date, two functional cis elements in the ABCG2 promoter, namely the hormone (11) and hypoxia (12) response elements, and a peroxisome proliferator-activated receptor response element upstream of the ABCG2 gene (13) have been reported. We recently reported that DNA methylation plays a role in the regulation of human ABCG2 in renal carcinoma cell lines (14). DNA methylation–mediated ABCG2 silencing was associated with a coordinate modification (i.e., methylation and deacetylation) of histone H3 at lysine 9 bound to the promoter, causing alterations in chromatin structure. Interestingly, in the renal carcinoma cell line UOK181, despite the fact that the promoter was found to be unmethylated, basal ABCG2 expression was only 2-fold higher than that in two ABCG2 promoter-methylated cell lines. Moreover, we observed that ABCG2 in UOK181 was significantly up-regulated by romidepsin (also known as depsipeptide or FK228), a histone deacylase (HDAC) inhibitor. Thus, a DNA methylation–independent repression of ABCG2 that is responsive to inhibition of HDACs is likely to exist. The activation of ABCG2 by romidepsin at transcript and protein levels has been reported in both renal and colon cancer cell lines (15). However, the mechanisms underlying this activation have not been elucidated.

In eukaryotic cells, DNA is tightly packed in a highly organized chromatin structure. The packaging of DNA controls the interaction of regulatory proteins with their cis elements in the promoters of genes. Chromatin structure is modulated by the covalent modifications of the NH2 termini of the core histones in nucleosomes and by the action of ATP-dependent chromatin remodeling complexes. In particular, histone...
Acetylation at the promoter of genes has been shown to be necessary, albeit not sufficient, for transcriptional activation (16-18). Acetylation of histones neutralizes the positive charges on lysine residues and disrupts nucleosome structure, allowing unfolding of the associated DNA, access by transcription factors, and changes in gene expression. Acetylation of core nucleosomal histones is regulated by the opposing activities of histone acetyltransferases (HAT) and HDACs. Histone hyperacetylation is associated with active transcription whereas histone hypoacetylation is linked to transcriptionally silent chromatin. Chromatin fractions enriched in actively transcribed genes are abundant in the acetylated form of core histones (19).

For active transcription to occur, histone-modifying complexes are thought to cooperate with chromatin-remodeling complexes to establish a local chromatin environment that is permissive for the subsequent assembly of an active preinitiation complex at the promoter (20, 21). The major chromatin remodeling factors are the SWI/SNF ATP-dependent remodeling complexes. Upon recruitment to the promoter, they can alter chromatin structure by either shifting nucleosomes along the DNA or twisting DNA to modulate the nucleosome structure (22). Brg-1 and Brm are the two ATPase subunits of the SWI/SNF complex.

The aim of this study was to investigate the relevance of histone modifications to human ABCG2 gene expression and the recruitment of transcription regulatory proteins to the ABCG2 promoter in multidrug-resistant cells. We used the chromatin immunoprecipitation (ChiP) assay to map the region of histone hyperacetylation and recruitment of other transcription factors at the human ABCG2 promoter in ABCG2-overexpressing multidrug-resistant cell lines and in some cancer cell lines selected from the National Cancer Institute drug screen, expressing various levels of ABCG2. We compared the effects of HDAC inhibition on the promoter with those resulting from selection for drug resistance. The data show that the characteristic pattern of histone acetylation at the ABCG2 promoter was altered following in vitro selection of drug resistance and after treatment with romidepsin, a HDAC inhibitor currently in clinical trials. Other histone modifications were also observed in these settings, consistent with a histone code that facilitates the up-regulation of ABCG2 in the cells.

Results
Transcriptional Up-Regulation of ABCG2 in Drug-Resistant Cell Lines

ABCG2 is expressed at varying levels in unselected cell lines and is highly overexpressed in drug-selected cells at both mRNA and protein levels (Fig. 1). To evaluate the role of promoter activity in the up-regulation of ABCG2 in unselected and selected cell lines, a series of 5’ deletion reporter gene constructs were generated from the ABCG2 promoter with the 3’ end terminating at +396 bp (14). Bailey-Dell et al. (23) showed that reporter activity roughly paralleled the relative level of ABCG2 expression in their studies of choriocarcinoma cell lines. This suggested that basal expression was mediated to some extent by transactivating factors binding to the promoter.

To study the possible contribution of transactivating factors in drug resistance, we evaluated the S1M180 subtype, which overexpresses ABCG2 without gene amplification. We transfected parental and drug-resistant cells with the ABCG2 promoter-luciferase reporter constructs. Compared with parental S1 cells, reporter activity driven by the various ABCG2 promoter fragments was increased 8- to 10-fold in the resistant S1M180 cells (Supplementary Fig. S1A and S1B). This increase in reporter activity is actually modest when compared with the overwhelming up-regulation of ABCG2 in the resistant S1M180 cells. Moreover, there was a generalized up-regulation of the reporter constructs in S1M180 cells; we were not able to narrow down to a particular promoter region that contributes to the highest up-regulation. To this end, a DNA footprinting analysis was done in an attempt to identify the DNA sequences that could be involved in the up-regulation of ABCG2 in the resistant S1M180 cells. However, a convincing evidence of differential transcription factor binding between the parental and resistant cells could not be obtained (data not shown).

Transcriptional Activation of the ABCG2 Promoter by Romidepsin

The modestly increased transactivating activity noted in the reporter assay and the lack of an identifiable difference in DNA footprinting led us to seek other methods for explaining ABCG2 overexpression, particularly in the S1M180 cells lacking gene amplification. We thus explored a model system for the study of transactivation in which endogenous ABCG2 expression is acutely induced and, therefore, not subject to potential genetic changes. Previous observations revealed that ABCG2 expression is up-regulated in cells treated with romidepsin, a HDAC inhibitor (14, 15). ABCG2 mRNA (Fig. 2A) and protein levels (Fig. 2B) were induced by romidepsin supplemented with 5 μg/mL of verapamil upon 24 h treatment in S1 cells in a dose-dependent manner. Verapamil, a P-glycoprotein inhibitor, was used to prevent the efflux of romidepsin by P-glycoprotein that is expressed in the cells. A dose of 20 ng/mL of romidepsin, which gave rise to a consistent...
Histone Modifications and Up-Regulation of ABCG2

As shown in Fig. 2B, global levels of histone H3 and H4 acetylation were increased by romidepsin treatment, even at the lowest concentration used (2 ng/mL). Histone acetylation may have been saturated at higher concentrations of romidepsin because acetylation levels did not show any further increase in a dose-dependent manner. In contrast, global acetylation of histone H3 and H4 in the resistant cells was not higher than in the parental cells (Fig. 2B).

ChIP assays were done to evaluate modifications specific to the ABCG2 promoter. Higher levels of promoter-associated histone H3 acetylation were observed in both romidepsin-treated and drug-resistant cells, compared with the untreated parental cells. DNA purified after immunoprecipitation with anti-AcH3 and AcH4 antibodies was evaluated by PCR using primers targeting the proximal (P1-P4; nt −687 to +20) and distal regions (nt −1,527 to −1,268) of the ABCG2 promoter (Fig. 3A), and the promoter region of GAPDH where RNA polymerase II (Pol II) binds (ChIP assay kit manual from Active Motif). The relative amount of promoter-associated acetylated H3 and H4 was quantified by measuring the PCR band intensity in a molecular imager followed by normalization with the input. Serial dilutions of the input DNA were amplified by PCR to determine the linearity of the PCR reactions (Supplementary Fig. S2). The levels of PCR amplifications for all ChIP samples were within this linear range. No signal was obtained from immunoprecipitated samples if normal IgG was used (Fig. 3B). The proximal promoter fragment evaluated by ChIP includes three putative AP1 and seven Sp1 binding sites, and includes the putative minimal promoter for ABCG2 (23). Romidepsin treatment induced hyperacetylation of histone H3 in all four regions of the proximal ABCG2 promoter tested in S1 parental cells (Fig. 3B and C). No significant change in AcH4 was observed, but the basal levels were much higher than those for H3. The increase in Ac-K9,14 H3 in the P3 and P4 regions was found to be the most pronounced, at ~4- and 6-fold, respectively, where basal acetylation levels were the lowest. Surprisingly, the resistant S1MI80 cells also exhibited hyperacetylation of histone H3 on the proximal ABCG2 promoter (P1-P4), and at 10-fold over control in P3, an even greater extent than in romidepsin-treated cells. Of note, the subregions within the proximal promoter (i.e., P1, P2, P3, or P4) were analyzed separately despite the fact that they cannot be precisely resolved by the ChIP assay. This serves to show the reproducibility of the ChIP analyses for the proximal promoter. In contrast, the enrichment of acetylated H3 in the distal region remained essentially unchanged after romidepsin treatment and in the resistant cells, confirming the specificity of histone H3 acetylation in the ABCG2 promoter (Fig. 3B and C). Consistent differential results between the distal and the proximal (P1-P4) regions indicated that the developed ChIP assay allowed precise analysis of contiguous DNA regions positioned at distances <1,000 bp. As an internal control, histone H3 and H4 acetylation levels at the constitutive GAPDH promoter were also analyzed. The considerable acetylated H3 and H4 binding to the GAPDH promoter did not change upon romidepsin treatment or in the resistant cells. Notably, GAPDH gene expression did not respond to romidepsin treatment, as shown in Fig. 2A. The proximal promoter region P3 was chosen for all

FIGURE 2. Transcriptional up-regulation of ABCG2 by HDAC inhibition. A and B. Dose-dependent up-regulation of ABCG2 in S1 cells after treatment with romidepsin (2, 5, 10, 20, or 30 ng/mL) for 24 h as measured by reverse transcription-PCR analysis (A) and immunoblot analysis (B). Global levels of AcH3 and AcH4 were increased upon romidepsin treatment, which seem saturated even after the lowest dose of romidepsin used (i.e., 2 ng/mL). The levels of AcH3 and AcH4 in the resistant S1MI80 cells were found to be similar to that in the parental S1 cells. GAPDH and rRNA expression was used as controls. C. Reverse transcription-PCR analysis of the up-regulation of ABCG2 in S1 cells by romidepsin with or without a 4-h pretreatment of actinomycin D (5 µg/mL).
subsequent ChIP analyses because of a more reproducible and consistent increase in promoter-associated AcH3 upon romidepsin treatment and in resistant cells. Region P4 showed greater variability in this respect.

Release of HDACs from and Recruitment of RNA Pol II to the ABCG2 Promoter Upon Its Activation

Next, we used the proximal promoter P3 region as a probe for alterations in RNA Pol II, HDACs, and HATs bound to the ABCG2 promoter, using antibodies specific for Pol II, HDACs, p300, or GCN5, in control, romidepsin-treated, and SIM180 cells. Immunoprecipitation of the ABCG2 chromatin using antibodies against HDAC1, HDAC3, HDAC6, and HDAC8 revealed the association of these HDACs with different affinity, to the proximal and distal ABCG2 promoters (and the control GAPDH promoter) in the untreated parental cells (Fig. 4A and B). The association of HDAC1 and HDAC3 with the proximal ABCG2 promoter was significantly reduced in romidepsin-treated S1 cells and in the SIM180-resistant cells, compared with that in the untreated parental S1 cells, suggesting that release of these HDACs was associated with promoter activation. HDAC8 bound weakly to the proximal ABCG2 promoter in the untreated parental S1 cells, and this binding was decreased in resistant SIM180 cells (but not in romidepsin-treated S1 cells). However, the binding of HDAC1, HDAC3, and HDAC8 to the distal ABCG2 promoter region was identical in all three groups (parental, resistant, and romidepsin-treated). In contrast, HDAC6 bound constitutively.
to both the proximal and distal ABCG2 promoter and the binding was not affected upon romidepsin treatment and in the resistant cells. Anti-HDAC2 antibody did not result in immunoprecipitation of the ABCG2 promoter from any samples. To show the reproducibility of the ChIP assays, especially for the proximal ABCG2 promoter, the association of HDAC1, HDAC2, and HDAC3 and AcH3 with different ABCG2 promoter regions and the GAPDH promoter is shown in Supplementary Fig. S3. The binding of Pol II, an indicator of ABCG2 promoter activity, to the proximal promoter in the untreated parental S1 cells was only barely detectable. In contrast, there was more Pol II binding to the P3 region in the romidepsin-treated cells (3-fold) and even more in the resistant S1MI80 cells (8-fold; Fig. 4A and B). Of note, we observed considerable Pol II binding at the ABCG2 distal promoter region in untreated S1 cells and it remained unchanged in romidepsin-treated and drug-resistant cells. These results suggested that ABCG2 gene expression correlates with the presence of Pol II at the proximal promoter of the ABCG2 gene. For the HATs, both p300 and GCN5 were found binding to the distal and the proximal P3 regions, and not changing in romidepsin-treated or in the drug-resistant cells (Fig. 4A and B). In the GAPDH control, Pol II, p300, and GCN5 bound strongly to the GAPDH promoter, whereas the HDACs (except HDAC6) were only weakly bound (Fig. 4A and B). Further, their association with the promoter was not different in the untreated parental S1, romidepsin-treated S1, or resistant S1MI80 cells.

Global levels of these HDACs and HATs were also examined in whole-cell lysates to show that the observations were not due to global nonspecific events. In contrast to the promoter-specific effects, levels of HDAC1, HDAC3, and HDAC8 were found to be similar in the parental, romidepsin-treated S1, and resistant S1MI80 cells upon immunoblot analysis (Fig. 4C).

Taken together, the results indicate that site-specific histone H3 hyperacetylation, reduced association of HDAC1 and HDAC3 (HDAC8, also, in the resistant S1MI80 cells), and recruitment of Pol II at the ABCG2 proximal promoter correlates with the transcriptional activation of ABCG2 in both romidepsin-treated and resistant cells.

**FIGURE 4.** The proximal ABCG2 promoter is associated with more AcH3 and RNA Pol II but less class I HDACs (HDAC1 and HDAC3). A. ChIP analyses targeting the proximal P3 and the distal regions of the ABCG2 promoter in parental S1, romidepsin-treated S1, and resistant S1MI80 cells, using antibodies against AcH3 (K9,14), RNA Pol II, HDAC1, HDAC2, HDAC3, HDAC6, HDAC8, GCN5, and p300. The GAPDH promoter was used as a control. B. Quantitative analyses of the occupancy of AcH3, Pol II, HDAC1, HDAC2, HDAC3, HDAC6, HDAC8, GCN5, and p300 to the ABCG2 promoter (P3 and distal region) and the GAPDH promoter in the ChIP assays in A. The results are expressed as the percentage of immunoprecipitate over total input DNA. Bars, SDs of three independent and reproducible experiments. C. Immunoblot analysis of the cellular level of AcH3, ABCG2, HDAC1, HDAC2, HDAC3, HDAC6, HDAC8, GCN5, and p300 in the parental S1, romidepsin-treated S1, and S1MI80 cells. GAPDH was used as a loading control. The three treatment groups expressed similar levels of all HDACs and HATs examined. HDAC inhibition by romidepsin results in hyperacetylation of H3 in the treated S1 cells. No appreciable global histone acetylation was observed in the resistant S1MI80 cells.
Other Histone Modifications at the ABCG2 Promoter in Drug-Resistant S1MI80 Cells

In a previous study, we showed that the activation of ABCG2 in methylated renal carcinoma cell lines was associated with the switching of histone modification from methylation to acetylation at lysine 9 on histone H3 (14). We thus asked whether the hyperacetylation of histone H3 and the corresponding up-regulation of ABCG2 in the resistant S1MI80 and romidepsin-treated S1 cells were accompanied by specific histone modifications at the ABCG2 promoter.

We first examined the global levels of histone modifications in parental cells upon romidepsin treatment; in the resistant cells, histone modifications were examined and compared with the untreated parental S1 by immunoblot analysis with a panel of antibodies against various modified forms of histone H3. As shown in Fig. 5A, romidepsin treatment remarkably increased the global level of Ac-K9,14 H3, increased only slightly that of Me3-K4 H3, did not affect Me3-K9 H3, but decreased the level of P-S10 H3 to nearly undetectable levels. The significant repression of the global histone H3 Ser-10 phosphorylation by HDAC inhibition by romidepsin resulted in a robust hyperacetylation of H3 in the histone extracts. B. ChIP analyses targeting the proximal P3 region of the ABCG2 promoter or the GAPDH promoter in parental S1, romidepsin-treated S1, and resistant S1MI80 cells, using antibodies against Ac-K9,14 H3, Me3-K4 H3, Me3-K9 H3, and P-S10 H3. C. Quantitative analyses of the association of the various modified forms of histone H3 at the proximal ABCG2 promoter as in B. The results are expressed as the percentage of immunoprecipitate over total input DNA. Bars, SDs of three independent experiments.

**FIGURE 5.** Global and ABCG2 promoter-specific histone modifications in resistant S1MI80 and romidepsin-treated parental S1 cells. A. Immunoblot analysis of the cellular expression of the various modified forms of histone H3. Histone was extracted from the cells for immunoblot analysis. Equal loading of histones was ascertained by immunoblotting using anti-total H3 antibody. HDAC inhibition by romidepsin resulted in a robust hyperacetylation of H3 in the histone extracts. B. ChIP analyses targeting the proximal P3 region of the ABCG2 promoter or the GAPDH promoter in parental S1, romidepsin-treated S1, and resistant S1MI80 cells, using antibodies against Ac-K9,14 H3, Me3-K4 H3, Me3-K9 H3, and P-S10 H3. C. Quantitative analyses of the association of the various modified forms of histone H3 at the proximal ABCG2 promoter as in B. The results are expressed as the percentage of immunoprecipitate over total input DNA. Bars, SDs of three independent experiments.
methylated with ABCG2 expression. Elevated levels of Ac-K9,14 H3, Me3-K4 H3, and P-S10 H3 have been linked to a permissive chromatin state that favors active transcription (28-33). Compared with the parental S1 cells, a consistent and robust increase in these permissive histone modifications were observed at the proximal ABCG2 promoter in the romidepsin-treated S1 cells and in the S1MI80-resistant subline (Fig. 5B and C). The increased histone H3 S10 phosphorylation at the ABCG2 promoter is remarkable in the face of reduction in the global H3 S10 phosphorylation level as noted above. On the other hand, a significantly lower level of Me3-K9 H3, a modification that marks silent heterochromatin (34-36), was found at the ABCG2 promoter, particularly in the resistant cells.

As a control, analysis of histone modifications at the GAPDH promoter was also conducted. Not surprisingly, given its ubiquitous expression, high levels of the permissive histone modifications (Ac-K9,14 H3 and Me3-K4 H3) but minimal levels of a repressive histone modification (Me3-K9 H3) were detected in the GAPDH promoter (Fig. 5B). Consistent with its selection as a control gene, the histone modifications at the GAPDH promoter were similar in romidepsin-treated and resistant cells, compared with the parental cells.

Inhibition of HDACs by Romidepsin Did Not Up-Regulate ABCG2 in All Cell Types

Taken together, the results above show that romidepsin is able to up-regulate ABCG2 in S1 cells through gene-specific alterations of chromatin, particularly an increase in Ac-K9,14 H3, Me3-K4 H3, and P-S10 H3, but a decrease in Me3-K9 H3. Similar changes were observed in drug-resistant cells (S1MI80 and combined with the enhanced transactivating activity noted in Supplementary Fig. S1 may begin to explain the up-regulation of ABCG2 in S1MI80. We next examined histone modifications in a number of other cell lines and their drug-resistant counterparts. We first asked whether ABCG2 induction was a universal response to treatment with an HDAC inhibitor. S1, H460, MCF-7, SF295, and SW620 cells were treated with a range of different doses of romidepsin (0-30 ng/mL) for 24 h. Again, verapamil was added to prevent P-glycoprotein expression from limiting the effect of romidepsin. As a control, MDR1 expression was also assessed because it has been found to be activated in a number of different cell types by HDAC inhibitors, including sodium butyrate, trichostatin A, and romidepsin (15, 37-39). As depicted in Fig. 6, romidepsin induced a dose-dependent up-regulation of MDR1 in all cell lines tested. A dose-dependent increase in ABCG2 was also noted in S1 and H460 cells. In contrast, ABCG2 expression was down-regulated in MCF-7 and SF295 and not altered in SW620 after romidepsin treatment, despite the fact that a consistent activation of MDR1 was observed in these cell types. In a previous study, we showed that ABCG2 promoter methylation could constrain gene induction in response to romidepsin treatment (14); it should be noted that the ABCG2 promoter was unmethylated in all cell lines used in this study (data not shown).

Thus, at doses readily capable of up-regulating MDR-1, romidepsin did not induce ABCG2 in all cell lines. Why ABCG2 was repressed in MCF-7 and SF295 upon romidepsin treatment is not clear. However, repression of selected genes has been observed following HDAC inhibitor treatment (40). The cell type–specific changes in ABCG2 did allow us to look at the specific changes at the ABCG2 promoter in this study.

Permissive and Repressive Histone Modifications at the ABCG2 Promoter Parallel ABCG2 Expression

To explore the failure of romidepsin to up-regulate ABCG2 in MCF-7, SF295, and SW620 cells following its treatment, we examined histone modifications following romidepsin. Global histone acetylation was increased in every cell line tested (Supplementary Fig. S4). ChIP assays were thus done on the ABCG2 promoter to determine whether the pattern of histone modification was altered after romidepsin. There was an increase in Ac-K9,14 H3 at the proximal ABCG2 promoter in all five cell lines (Fig. 7A and B). Although this was a permissive modification, it was not sufficient to support ABCG2 up-regulation. Elevation of the permissive modification Me3-K4 H3 and reduction in the repressive mark Me3-K9 H3 at the proximal promoter were observed only in S1 and H460 cells, which directly correlated with the induction of ABCG2.
Also observed in Fig. 5, increased P-S10 H3 was noted in S1 cells treated with romidepsin. In contrast, changes in these histone modifications (i.e., Me3-K4 H3, Me3-K9 H3, and P-S10 H3) were not observed in cells that did not show ABCG2 induction following romidepsin treatment (i.e., MCF-7, SF295, and SW620 cells; Fig. 7A and B). There was a perfect match between these histone marks and gene induction following romidepsin treatment. These results show that inhibition of HDACs alone is insufficient to alter the transcription-repressive pattern of histone modification (i.e., Me3-K9 H3; Fig. 7A and B) present in the untreated cells. Interestingly, compared with the parental cells, the pattern of histone modifications favoring gene activation (i.e., increase in Me3-K4 H3 and decrease in Me3-K9 H3) found in S1MI80-resistant cells was also shown in the ABCG2-overexpressed drug-selected resistant H460 MX20, MCF-7 FLV1000, and SF295 MX2000 sublines examined in our study.

ABCG2 Up-Regulation Is Not Due to Histone Loss from the Promoter

A recent study reported that nucleosome loss occurs at many active promoters and is proportional to the transcription initiation rate (41). In another study, the nucleosome loss from the PHO5 promoter associated with its transcriptional activation is preceded by a transient increase in histone H3 acetylation (42). To determine whether ABCG2 up-regulation

FIGURE 7. Histone modifications at the ABCG2 promoter in a panel of drug-selected resistant and romidepsin-treated parental cells. A. Levels of Ac-K9,14 H3, Me3-K4 H3, Me3-K9 H3, and P-S10 H3 at the proximal ABCG2 promoter (P3). ChIPs were done with the parental, romidepsin-treated parental, and the resistant cells. B. Quantitative analyses of the association of the various modified forms of histone H3 to the proximal ABCG2 promoter by ChIP analysis in A. The results are expressed as the percentage of immunoprecipitate over total input DNA used. Bars, SDs of three independent experiments.
in the resistant cells and in romidepsin-treated S1 and H460 cells was associated with histone loss and to verify the accuracy of the estimate of histone modifications shown in Fig. 7, occupancy of total and unmodified histone H3 at the ABCG2 promoter was determined by ChIP assays using antibody against the COOH terminus of histone H3. Neither romidepsin-treated cells nor resistant cells showed a loss of total histone H3 at the ABCG2 promoter (Supplementary Fig. S5).

Factors Associated with the Responsive and Unresponsive ABCG2 Gene after Romidepsin Treatment

To explore further the epigenetic factors determining ABCG2 response to romidepsin treatment, a panel of candidate proteins was examined for their differential association with either the responsive or unresponsive ABCG2 promoter by ChIP (Fig. 8) and their global expression by immunoblot analysis (Supplementary Fig. 6). As depicted earlier in S1 cells (Fig. 4), the induction of ABCG2 was correlated closely with the increased association of Pol II at the proximal promoter, which was only observed in those cell lines whose ABCG2 could be up-regulated by romidepsin (i.e., S1 and H460). Similarly, in these lines, an increased association of the chromatin remodeling factor known to be associated with the SWI/SNF complex, Brg-1, to the ABCG2 promoter was consistently observed. Finally, a significant reduction of HDAC1 and Sp1 association with the ABCG2 proximal promoter was observed in S1 and H460 cells following romidepsin treatment. These are likely to be critical differences, given that in cells that do not respond to romidepsin treatment (i.e., MCF-7, SF295, and SW620), no significant difference in the association of HDAC1, Sp1, Brg-1, or Pol II with the ABCG2 promoter was found following romidepsin treatment. Thus, the persistence of HDAC1 and Sp1 binding, and the lack of Brg-1 association with the promoter, seem to be involved in the romidepsin unresponsiveness of ABCG2 in MCF-7, SF295, and SW620.

Discussion

Overexpression of the ABCG2 gene is frequently found in cancer cell lines selected with a number of anticancer drugs, including mitoxantrone, topotecan, SN-38, or flavopiridol (6–10). However, little is known about the mechanisms underlying its up-regulation. Earlier studies identified gene amplification and chromosome translocation as mechanisms for the increased expression of the ABCG2 gene in drug-resistant cell lines (43). The use of alternative 5' promoters at the ABCG2 gene in drug-selected cells may offer another novel mechanism of ABCG2 up-regulation (44), a finding similar to observations in MDR-1 where rearrangement of the 5' region of MDR-1 resulted in capture of that gene by another promoter (45). In this study, we examined epigenetic mechanisms for the overexpression of ABCG2 in multidrug-resistant cell lines and in unselected cell lines treated with a HDAC inhibitor. This includes the hyperacetylation of histone H3 (K9,14) at the proximal ABCG2 promoter, the concomitant increase in trimethylation of histone H3 (K4), and the decrease in trimethylation of histone H3 (K9).

Using a series of ABCG2 promoter-luciferase reporter gene vectors with progressive NH2 terminus deletion, our study was initiated with an aim to identify promoter sequences necessary for transactivation of ABCG2. A modest increase in transactivation was observed in the resistant S1MI80 cells, compared with the parental S1 cells. However, the reporter assay failed to identify 5'-flanking sequence cis-elements involved in the preferential expression of ABCG2 in S1MI80 cells. To this end, DNA footprinting experiments done using S1 and S1MI80 cell lysates did not offer convincing differential transcription factor binding (data not shown). Thus, we turned to an inducible system to identify regulatory elements relevant for ABCG2 activation.

The HDAC inhibitor romidepsin has been shown to activate ABCG2 expression in a number of cell lines (14, 15) and actinomycin D pretreatment confirmed an effect at the level of RNA transcription. We examined the chromatin modifications occurring at the ABCG2 promoter by ChIP assay (24). First, we showed a significant and specific increase in histone acetylation at the proximal ABCG2 promoter both in resistant S1MI80 cells and in parental S1 cells upon treatment with romidepsin. This increase in histone acetylation occurred concurrently with RNA Pol II binding to the proximal ABCG2 promoter, marking it as transcriptionally active. The region studied corresponds to the
region of the \textit{ABCG2} promoter previously reported to have the highest activity in promoter fragment studies (23). A decrease in the amount of class I HDACs (HDAC1 and HDAC3; HDAC8 also in resistant S1M80 cells) and Sp1 specifically associated with the proximal \textit{ABCG2} promoter was observed in both resistant S1M80 cells and parental S1 cells following romidepsin treatment. Because Sp1 is known to recruit HDAC1, the two probably come off from the promoter together (46). HDAC1 is thought to repress gene transcription through both deacetylation of core histones and deacetylation of various transcription factors (47). The increased acetylation of a gene promoter encoding a drug resistance transporter has also been observed for \textit{MDR-1} following selection in doxorubicin or etoposide (48). Histone hyperacetylation of \textit{MDR-1} was also noted following treatment with the HDAC inhibitor trichostatin A and some chemotherapeutic agents, including daunorubicin and etoposide (49). HDAC6, another HDAC investigated in this study, was found constitutively binding to both proximal and distal \textit{ABCG2} promoter and the \textit{GAPDH} promoter and its binding was not affected by romidepsin treatment or by selection for drug resistance. HDAC6 is a subtype of the HDAC family that deacetylates α-tubulin. The reason for its constitutive promoter binding is unknown. Nonetheless, the inclusion of the data for HDAC6 illustrates the specificity and significance of reduced HDAC1 and HDAC3 binding to the proximal \textit{ABCG2} promoter in the up-regulation of \textit{ABCG2}. On the other hand, all the HATs (p300 and GCN5) studied bound constitutively to the \textit{ABCG2} promoter and the binding was not affected by romidepsin treatment or by selection for drug resistance. The constitutive binding can be accounted for by the fact that these HATs do form a large complex with other components of the basal transcriptional machinery and they also form indirect association with chromatin via various histone-binding proteins. Taken together, the results indicate that the dissociation of HDAC complexes rather than the recruitment of more HATs was important to facilitate histone hyperacetylation at the proximal \textit{ABCG2} promoter to up-regulate \textit{ABCG2}. Furthermore, dissociation of the HDAC complexes (including HDAC1, HDAC2, and Sp1) seems to be a prerequisite for gene induction, as cell lines without \textit{ABCG2} gene induction after romidepsin treatment showed none of these findings.

Although acetylation and deacetylation of histone proteins have been shown to induce a relaxed and competent or a condensed and inactive chromatin, respectively (50, 51), methylation of histones at key lysine residues seems to act cooperatively to generate a specific histone code to determine the chromatin configuration and thereby the transcriptional state (52-55). Specifically, methylation of histone H3 at lysine 4 is usually enriched at active hyperacetylated euchromatin domains, and is proposed to have a pivotal role in gene activation by providing a binding platform for different transcription factors (32). On the other hand, trimethylation of H3 at lysine 9 (Me3-K9 H3) has been associated with silent hypoacetylated heterochromatin regions and is considered a repressive mark. In our study, ChIP assays revealed fairly high levels of Me3-K9 H3 at the \textit{ABCG2} promoters of all the untreated parental cell lines tested. The up-regulation of \textit{ABCG2} in resistant cells and in some parental cells treated with romidepsin tracked closely with reductions of this repressive histone modification in the promoter. This was accompanied by a stronger association of permissive histone marks, Me3-K4 H3 and P-S10 H3 (the latter for S1 cells only), with the \textit{ABCG2} promoter. Active transcription was favored by this histone code, as evidenced by the increased binding of RNA Pol II to the same promoter region. Control experiments showed that the increase in chromatin accessibility over that region could not be explained by nucleosome loss and supported the importance of histone modifications at the \textit{ABCG2} proximal promoter. Taken together, the results show that activation of \textit{ABCG2}, whether due to drug selection and chronic overexpression or to treatment with an HDAC inhibitor, is associated with acetylation of histone H3, enhancement of permissive histone marks, reduction of repressive histone marks, increased RNA Pol II binding, and reduced HDAC1 and Sp1 binding. These findings are consistent with results observed at promoters of other activated genes (16, 17, 28, 34).

We have previously observed the induction of \textit{ABCG2} mRNA by romidepsin in colon and renal cancer cell lines (15). Among the five cancer cell lines selected for this study, romidepsin induced \textit{ABCG2} mRNA expression in two cell lines, S1 and H460, but not in the others, MCF-7, SF295, and SW620. Inhibition of HDAC resulted in both global and \textit{ABCG2} promoter–specific histone H3 hyperacetylation in all five cancer cell lines. Intriguingly, \textit{MDR1} mRNA encoding the ABC transporter P-glycoprotein was consistently induced as well. Thus, \textit{MDR1} induction seems to be much more general than \textit{ABCG2} induction. In MCF-7, SF295, and SW620 cells, inhibition of HDACs is not sufficient to generate the same pattern of transcription-permissive histone modifications, or to activate \textit{ABCG2}. Changes toward the permissive histone marks at the \textit{ABCG2} promoter (i.e., an increase in Me3-K4 H3 and P-S10 H3; a decrease in Me3-K9 H3) did not occur after romidepsin treatment. Notably, Me3-K4 H3 is more efficiently acetylated by known HATs than the unmodified one (56), thereby reinforcing the histone hyperacetylation. Thus, the lack of Me3-K4 H3 at the \textit{ABCG2} promoter in MCF-7, SF295, and SW620 after romidepsin treatment may have prohibited the up-regulation of \textit{ABCG2}. At least in yeast, it has been shown that increased histone acetylation at promoters is not sufficient for enhanced transcription by transcriptional activators (57).

Moreover, a recent study reported that a combination of high H3 acetylation (Ac-K9,14 H3) and H3 K4 methylation (Me3-K4 H3) levels define distinct euchromatin regions allowing for stable expression of an integrated transgene (58). Integration into a region that is poor in or lacking Ac-K9,14 H3 and Me3-K4 K3 conferred progressive silencing of the transgene. Figure 9 presents a schematic detailing the proposed epigenetic code involving the \textit{ABCG2} promoter.

To further explore the epigenetic factors determining \textit{ABCG2} expression, a panel of candidate proteins was examined for their differential association with either responsive or unresponsive \textit{ABCG2} promoter upon romidepsin treatment. The transcriptional repressor HDAC1, after romidepsin treatment, remained in association with the unresponsive \textit{ABCG2} promoter in MCF-7, SF295, and SW620 cells, but not with the activated \textit{ABCG2} promoter in S1 and H460 cells (Fig. 8). In addition, an increased association of the chromatin remodeling

\textbf{Figure 9 presents a schematic detailing the proposed epigenetic code involving the \textit{ABCG2} promoter.}
Histone Modifications and Up-Regulation of ABCG2

FIGURE 9. A model for the up-regulation of ABCG2 in multidrug-resistant cells by permissive histone modifications and recruitment of chromatin remodeling factor. In untreated parental cells, prominent association of the repressive histone mark, Me3-K9 H3, with the ABCG2 promoter locks the ABCG2 expression at low levels. In resistant cells, the repressive marks are replaced by permissive histone marks, Ac-K9,14 H3, Me2-K4 H3, and P-S10 H3, followed by the recruitment of a chromatin remodeling factor (Brg-1) and RNA Pol II, and the release of class I HDACs from the ABCG2 promoter. The switch to an open chromatin configuration also occurs in a limited number of cell lines upon treatment with romidepsin, up-regulating the expression of ABCG2. However, in other cell lines where ABCG2 is not affected by romidepsin treatment, the repressive histone mark Me3-K9 H3 is retained, although more AcH3 (K9,14) are found binding to the promoter. HDACs and likely other corepressors remain bound to the promoter, suppressing the ABCG2 expression. Co-Rep, corepressors; Co-Act, coactivators; TF, transcription factor.

In summary, we have shown that the ABCG2 promoter has a reproducible pattern of histone modification that is altered following ABCG2 overexpression due to the development of drug resistance or due to HDAC inhibition. Increased levels of permissive histone marks are accompanied by reduced levels of repressive marks. Our data strongly suggest that these histone modifications play an important role in the regulation of ABCG2 expression in multidrug-resistant cells. Further, unlike MDR1, the induction of ABCG2 following romidepsin exposure is cell line specific, a finding likely due to differences in the components of the repression complexes present at the gene regulatory region in a given cell line. It is intriguing that certain cell types respond to HDAC inhibition with increased histone acetylation but without ABCG2 gene induction. Whereas differential display (59) or microarray analyses (60, 61) identify only 2% to 10% of expressed genes as sensitive to HDAC inhibitors, in this case, we have a gene responding to HDAC inhibition in one cell line but not in another. The factors that govern these differential responses have not been determined, but the ABCG2 promoter studies presented here provide a platform for evaluating such factors. It will be important to determine whether the failure to respond to HDAC inhibitors lies in their lack of access to gene-specific HDACs or, once binding is accomplished, a failure to manipulate the regulatory complex. The data presented here clearly show that romidepsin is able to bind and inhibit HDACs elsewhere in the cells. Understanding the differential effects may reveal the dominant epigenetic mechanisms associated with ABCG2 expression and potentially provide insight into strategies needed to accomplish the induction of therapeutically important genes by HDAC inhibition. Multiple cancer treatment strategies have been proposed, exploiting the unique effects of HDAC inhibition on gene induction. Examples include the improved therapeutic target that could result from induction of CD25 for sensitization of cells to interleukin 2–targeted therapy such as denileukin diftitox (62), cancer antigens for immunotherapy (63), and the sodium-iodine symporter for transport of radioactive iodine (64). This line of investigation may also lead to understanding why some malignancies are very sensitive to HDAC inhibitors whereas others are not, and for understanding drug resistance mediated by epigenetic changes.

Materials and Methods

Tissue Culture

Human colon cancer cell line S1 and its resistant subline S1MI 80 have been described previously (8). SW620, MCF-7, H460, and SF295 cells were chosen from the National Cancer Institute Tumor Drug Screen for this study. MCF-7 FLV1000, H460 MX20, and SF295 MX2000 were resistant sublines developed from their parental counterparts by stepwise selection in increasing concentrations of selecting agent and maintained in 1,000 mmol/L flavopiridol, 20 mmol/L mitoxantrone, and 2,000 mmol/L mitoxantrone, respectively. The cell lines were maintained in IMEM (S1, S1MI80, MCF-7, and MCF-7 FLV1000) or RPMI medium (SW620, SF295, SF295 MX2000, H460, and H460 MX20) supplemented with 10% fetal bovine serum, 100 units/mL streptomycin sulfate, and 100 units/mL penicillin G sulfate, and incubated at 37°C in 5% CO2.

Drug Treatment

To test the effect of HDAC inhibition on ABCG2 expression, cells were treated with 2, 5, 10, 20, or 30 ng/mL of romidepsin (also known as depsipeptide, FR901228, or NSC630176; Developmental Therapeutics Program, National Cancer Institute) supplemented with 5 μg/mL of verapamil (Sigma) for 24 h. Verapamil was added to prevent the efflux of romidepsin mediated by P-glycoprotein up-regulation. Stock solutions of romidepsin and verapamil were dissolved in DMSO and water, respectively. For the inhibition of RNA synthesis study, S1 cells were treated with 5 μg/mL actinomycin D (Sigma) 1 h before the addition of romidepsin.

Semiquantitative Reverse Transcription-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen). RNA (1 μg) was reverse transcribed using PowerScript
Reverse Transcriptase (Clontech). Amplification of cDNA was done using primers specific for ABCG2: 5'-CAATGGGATCAGTTAACCCTG-3' (forward) and 5'-GAGGCTGAGAATTGAGAAA-3' (reverse), MDR-1: 5'-CAGACAGCGACTG-AGATCTAGAACGGACT-3' (forward) and 5'-CCGCGCAGAGCTGAAAGACACT-3' (reverse); and GAPDH: 5'-ACCCAGTCATCGACTGAC-3' (forward) and 5'-TCCACACCCGTTGTGCTGA-3' (reverse). Amplification of GAPDH cDNA served as an internal control. PCR amplification was done at an annealing temperature of 55°C for 28 cycles (ABCG2, 25 cycles (GAPDH), or 30 cycles (MDR-1)) to yield 584, 330, and 440 bp products, respectively. The PCR products were resolved on 2% agarose gel and stained with ethidium bromide. Gel images were captured and the band intensity was quantitated as using the App_Collage PPC4.0 analysis software program.

Luciferase Reporter Assays
A series of human ABCG2 promoter constructs with progressive deletions at the 5'-ends has been described previously (14). The ABCG2 promoter/firefly luciferase fusion genes (200 ng DNA) were transfected in pairs of parental and resistant cells using Fugene 6 (Roche). The pGL3-Basic (promoterless) plasmid, encoding firefly luciferase (Promega), was used to determine the basal levels. In each experiment, the phRG-Basic plasmid (50 ng), encoding Renilla luciferase (Promega), was cotransfected for normalization purposes. Luminescence was measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). Reporter activity was normalized by calculating the ratio of firefly/ Renilla values. Each construct was tested in three independent transfections. Results were expressed as mean ± SD.

Acid Extraction of Histones
Core histone proteins were extracted from the cells according to guidelines recommended by Abcam, Inc. Histones were acid extracted for 16 h at 4°C by adding ice-cold 0.2 mol/L HCl (4 × 10^7 cells/mL), washed, and finally resuspended in 100 μL of distilled water.

Western Blot Analysis
Whole-cell lysates prepared from S1 and its resistant subline S1M80 were separated by SDS-PAGE and subjected to immunoblot analysis with the respective antibodies (AcH3, HDAC1, and HDAC2, Upstate; ABCG2, Kamiya Biomedical Company; and HDAC3, HDAC6, and HDAC8, Santa Cruz Biotechnology). The antigen-antibody complex was detected using Taq DNA polymerase (Bioline) and 1 μL of either immunoprecipitated DNA, a normal IgG control, or a 1:10 dilution of input chromatin. Experimental reactions were done to determine optimal PCR conditions so that the yield of PCR products was dependent on the amount of input DNA (data not shown). The conditions for the PCR reactions were as follows: 94°C for 3 min, 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. Band intensity was quantitated as mentioned above. Fold enrichment in each immunoprecipitation was determined by quantifying the intensities of the PCR product in immunoprecipitated DNA versus input DNA (total chromatin). Only 10% of the total input was used in the PCR reactions and they were run as the “input” lanes in gel electrophoresis. Percentage enrichment was calculated accordingly. ChIP assays were repeated thrice using different chromatin preparations.

ChIP Assays
ChIP was done using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer’s instructions, with some modifications. Briefly, proteins were cross-linked with DNA in the cultured cells using 1% formaldehyde for 10 min at 37°C and quenched with 0.125 mol/L glycine for 5 min at room temperature. The cells were then rinsed in ice-cold PBS containing 5 mmol/L sodium butyrate (Sigma), scraped, and resuspended in a lysis buffer (Active Motif) with the addition of complete protease inhibitor cocktail (Roche). The DNA-protein complexes were sheared using an enzymatic shearing kit (Active Motif) under conditions that gave a range in DNA fragments from 200 to 600 bp, as determined by agarose gel electrophoresis. ChIP was then carried out overnight at 4°C with one of the following antibodies: AcH3 (Ac-K9,14 H3), AcH4 (Ac-K5,8,12,16 H4), HDAC1, HDAC8 (Upstate), RNA Pol II, HDAC2, HDAC3, HDAC6, p300/CBP, GCN5, bromelain-related gene 1 (Brg-1; Santa Cruz), Sp1, or cJUN (Active Motif). To examine the extent of histone modifications associated with the ABCG2 promoter, ChIP assays were repeated using antibodies against the various modifications, Me2-K4 H3, Me3-K9 H3, and P-S10 H3 (Upstate). Immunoprecipitated DNA was resuspended in 100 μL of TE buffer. The amount of immunoprecipitated DNA was assessed by semi-quantitative PCR, using primers spanning the distal region (distal, nt −1,572 to −2,628) and proximal regions (P1, nt −687 to −579; P2, nt −428 to −271; P3, nt −293 to −139; and P4, nt −146 to +20) of the ABCG2 promoter (Fig. 3A; ref. 14), and compared with the amount of input DNA before immunoprecipitation. Subregions within the proximal promoter (i.e., P1, P2, P3, or P4) were examined individually, although they cannot be precisely resolved by the ChIP assay, to show further the reproducibility of the analysis at the proximal promoter.

Amplification of the immunoprecipitated DNA was achieved using Taq DNA polymerase (Bioline) and 1 μL of either immunoprecipitated DNA, a normal IgG control, or a 1:10 dilution of input chromatin. Experimental reactions were done to determine optimal PCR conditions so that the yield of PCR products was dependent on the amount of input DNA (data not shown). The conditions for the PCR reactions were as follows: 94°C for 3 min, 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. Band intensity was quantitated as mentioned above. Fold enrichment in each immunoprecipitation was determined by quantifying the intensities of the PCR product in immunoprecipitated DNA versus input DNA (total chromatin). Only 10% of the total input was used in the PCR reactions and they were run as the “input” lanes in gel electrophoresis. Percentage enrichment was calculated accordingly. ChIP assays were repeated thrice using different chromatin preparations.

References


Histone Modifications at the ABCG2 Promoter following Treatment with Histone Deacetylase Inhibitor Mirror Those in Multidrug-Resistant Cells
