

## Valproate Induces Replication-independent Active DNA Demethylation\*

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In this report, we demonstrate that valproic acid (VPA), a drug that has been used for decades in the treatment of epilepsy and as a mood stabilizer, triggers replication-independent active demethylation of DNA. Thus, this drug can potentially reverse DNA methylation patterns and erase stable methylation imprints on DNA in non-dividing cells. Recent discoveries support a role for VPA in the regulation of methylated genes; however, the mechanism has been unclear because it is difficult to dissociate active demethylation from the absence of DNA methylation during DNA synthesis. We therefore took advantage of an assay that measures active DNA demethylation independently from other DNA methylation and DNA replication activities in human embryonal kidney 293 cells. We show that VPA induces histone acetylation, DNA demethylation, and expression of an ectopically methylated CMV-GFP plasmid in a dose-dependent manner. In contrast, valpromide, an analogue of VPA that does not induce histone acetylation, does not induce demethylation or expression of CMV-GFP. Furthermore, we illustrate that methylated DNA-binding protein 2/DNA demethylase (MBD2/dMTase) participates in this reaction since antisense knockdown of MBD2/dMTase attenuates VPA-induced demethylation. Taken together, our data support a new mechanism of action for VPA as enhancing intracellular demethylase activity through its effects on histone acetylation and raises the possibility that DNA methylation is reversible independent of DNA replication by commonly prescribed drugs.

DNA methylation is a modification of DNA whereby methyl groups are added as part of the covalent structure of the genome, thus providing an extra layer of epigenetic information. A well documented relationship exists between DNA methylation, chromatin structure, and gene expression (1) such that methylated genes are generally transcriptionally silent. Two mechanisms have been proposed to explain this repression: the first is that methylation causes interference in the binding of transcription factors and has been shown for several proteins such as AP2 (2) and c-Myc (3). The second mechanism involves the recruitment of various repressor complexes to methylated

DNA via the binding of methylated DNA-binding proteins (MBDs).<sup>1</sup> These complexes contain proteins that have histone deacetylase and chromatin remodeling activities, leading to the formation of a more compact and transcriptionally inactive chromatin (4).

Valproic acid/Valproate/2-*n*-propylpentanoic acid (VPA) has been used for decades in the treatment of epilepsy and is also effective as a mood stabilizer and in migraine therapy. Recent data suggest that this drug, in addition to its other known classical actions, can modulate the epigenome by inhibiting histone deacetylases (HDACs) (5, 6), similar to agents such as trichostatin A (TSA) and *n*-butyrate, thus triggering an increase in gene expression. Other studies also support a role for VPA in the regulation of methylated genes. It was demonstrated that the *reelin* gene, which encodes a neuronal protein that is down-regulated in schizophrenia, is methylated in neuronal precursor cells, accompanied by minimal expression. Following treatment of these cells by 5'-aza-2'-deoxycytidine (5-aza-CdR), a known DNA methylation inhibitor, or VPA, the expression of *reelin* is induced (7). VPA can also revert the down-regulation of both *reelin* and *gad67* caused by L-methionine, an agent shown to increase DNA methylation (8). Similarly, both 5-aza-CdR and VPA induce the expression of 5-lipoxygenase (9). The data presented above suggest the possibility that VPA may also be able to trigger DNA demethylation.

The prospect of erasing established DNA methylation patterns in somatic non-dividing tissue is extremely attractive and might have important therapeutic applications. However, the generally accepted model is that the DNA methylation pattern is an irreversible reaction, maintained only by DNA methyltransferase activities. If this is the case, then demethylation could only come about when replication occurs in the absence of DNA methyltransferase. Therefore, it should be impossible to reverse DNA methylation in non-dividing postmitotic tissues such as the brain. VPA, according to this classical model, could only act by inhibiting DNA methylation during DNA synthesis. However, there are several pieces of evidence indicating that DNA demethylation can occur in the absence of cell division (10–15). In addition, a DNA demethylase activity was purified in our laboratory from the human lung cancer cell line A549 (16), and the methylated DNA-binding protein 2/DNA demethylase (MBD2/dMTase) was found to actively demethylate DNA both *in vitro* (17) and in living cells (18, 19). Other groups (20, 21) have nevertheless disputed the demethylase activity of MBD2. Since we have shown that active demethylation can be induced or inhibited by modifying histone acetylation (18, 22),

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<sup>1</sup> The abbreviations used are: MBD, methylated DNA-binding protein; MBD2/dMTase, MBD2/DNA demethylase; VPA, valproate/valproic acid; VPM, valpromide; HDAC, histone deacetylase; Ac-H3, acetyl histone H3; TSA, trichostatin A; 5-aza, 2'-deoxycytidine-5-aza-CdR; GFP, green fluorescent protein; ChIP, chromatin immunoprecipitation.

and in view of the fact that VPA is also known to be an HDAC inhibitor, we hypothesize that it might act by increasing histone acetylation and increasing the accessibility of demethylase to DNA. If VPA acts by this mechanism, it might be used to stably erase methylation of specific genes in tissues where DNA replication does not take place.

It is impossible to determine whether hypomethylation of a gene *in vivo* or in living cells following a drug treatment is caused either by passive demethylation, resulting from inhibition of DNA methylation during DNA synthesis (as with 5-aza-CdR), or by an active removal of the methyl group by an enzyme. We have recently described a model system (18) that allows one to distinguish between these two mechanisms. When an *in vitro* methylated CMV-GFP plasmid is transiently transfected into the human embryonal kidney cell line HEK 293, it generally remains methylated. Increasing histone acetylation induces active demethylation of the plasmid (18). Since we have shown that the plasmid does not replicate during the time frame of the experiment, nor is it methylated by DNA methyltransferases, the demethylation measured by this assay is active demethylation and not passive demethylation. We have recently used this assay to demonstrate the inhibition of active demethylation by inhibitors of histone acetylation (22).

In this report, we utilize this assay to test the hypothesis that VPA treatment induces active DNA demethylation in living cells. Our data have important potential implications on the therapeutic reversal of pathological DNA methylation in non-dividing somatic tissues.

#### MATERIALS AND METHODS

**In Vitro Methylation of Substrates**—CMV-GFP (pEGFP-C1 from Clontech; GenBank™ accession number U55763) was methylated *in vitro* with SssI CpG methyltransferase (New England Biolabs), which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence 5' . . . CG . . . 3'. Ten  $\mu$ g of plasmid DNA was incubated with 12 units of SssI in the recommended buffer containing 800  $\mu$ M S-adenosylmethionine for 3 h at 37 °C. Twelve units of SssI and 0.16  $\mu$ mol of S-adenosylmethionine were then added, and the reaction was further incubated for an additional 3 h. The methylated plasmid was recovered by phenol/chloroform extraction and ethanol precipitation, and complete methylation was confirmed by observing full protection from HpaII digestion.

**Cell Culture and Transient Transfections**—Human embryonal kidney HEK 293 cells (ATCC number CRL 1573) were plated at a density of  $7.5 \times 10^4$ /well in a 6-well dish and transiently transfected with 80 ng of CMV-GFP (methylated or mock-methylated) using the calcium phosphate precipitation method as described previously (23). Twenty-four h after transfection, cells were treated with one of the following: 1–20 mM VPA (sodium salt, Sigma), 0.3  $\mu$ M TSA (Sigma), or 5 mM valpromide (VPM) (kindly donated by Katwijk chemie bv), or left untreated. Fresh media containing VPA and VPM were replaced after an additional 24 h, and cells were harvested 72 h after transfection. To determine the effects of MBD2/dMTase knockdown on demethylation of CMV-GFP, 1.6  $\mu$ g of either a pRetroTet On MBD2 antisense expression plasmid (24) or the pRetroTet On empty vector (Clontech) was added to the CMV-GFP transfection mixture followed by treatment with 20 mM VPA. Each experiment was performed in triplicate, and experiments were performed several times using different cultures of HEK 293 cells. 5-aza-CdR was purchased from (Sigma).

**Southern Blot Analysis**—DNA was extracted from HEK 293 cells using the DNeasy tissue kit (Qiagen). DNA was first digested with 50 units of EcoRI followed by digestion with 20 units of either HpaII or MspI restriction enzymes. Samples were subjected to electrophoresis on a 1.5% agarose gel and then transferred to Hybond-N+ membrane (Amersham Biosciences). Blots were probed with a <sup>32</sup>P-labeled CMV-GFP cDNA probe (AvaII-Cfr10I fragment) synthesized using a random priming labeling kit (Roche Applied Science). Membranes were hybridized at 68 °C for 4–6 h in a buffer containing 0.5 M sodium phosphate, pH 6.8, 1 mM EDTA, 7% SDS, and 0.2 mg/ml herring sperm DNA. Following hybridization, the membranes were washed twice for 10 min in a 5% SDS, 0.04 M sodium phosphate, pH 6.8, 1 mM EDTA solution and then four times for 10 min in the same solution containing 1% SDS. The demethylation assay measures the fraction of CMV-GFP molecules

that were demethylated using HpaII restriction enzyme, which cleaves unmethylated CCGG but does not cleave methylated CCGG sequences. The methylated GFP DNA remains intact following HpaII digestion and is identical to the fragment obtained following EcoRI digestion (see Fig. 2C, Me), whereas the unmethylated GFP DNA is cleaved by HpaII, resulting in a 0.5-kb fragment (see Fig. 2C, U). We scanned each HpaII-digested lane and measured the intensity of the total signal hybridizing with the GFP probe in the same HpaII lane (including the unmethylated U and methylated Me fragments); this value is equal to 100% of CMV-GFP molecules in the lane. We then determined the intensity of the unmethylated signal per HpaII lane and divided this value (U) by the total signal for CMV-GFP (U + M) in the same HpaII lane. The percent demethylation is thus calculated as (U/(U + Me))  $\times$  100. The results of three independent experiments were quantified and averaged.

**Bisulfite Mapping**—Bisulfite mapping was performed exactly as described (22).

**Chromatin Immunoprecipitation (ChIP) Assays**—CMV-GFP transfections and VPA treatments were performed essentially as described previously except that HEK 293 cells were seeded in 10-cm plates (2/sample) at a density of  $5 \times 10^5$ /plate, and 0.5  $\mu$ g of methylated GFP was used. ChIP assays were performed by following the Upstate Biotechnology ChIP assay kit protocol (catalog number 17-295). Chromatin was immunoprecipitated using anti-acetylated histone H3 antibody (Upstate Biotechnology, catalog number 06-599) as recommended by the manufacturer. PCR reactions were repeated exhaustively using serial dilutions (1–1:10,000) of template to determine the linear range of the PCR, and a final dilution of 1/100 was used. The following primers were used to amplify a 500-bp region of the GFP gene: 5'-CAAGGGC-GAGGAGCTGTT-3' (sense) and 5'-CGGCCATGATATAGACGTTG-3' (antisense) at an annealing temperature of 58 °C.

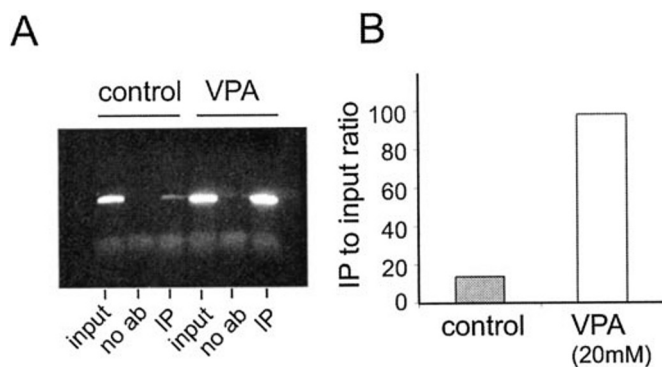
**Western Blot Analysis**—GFP Western blots were performed exactly as in Ref. 18. For MBD2 Western blots, nuclear extracts were prepared from HEK 293 cells as described (25). Twenty-five to 50  $\mu$ g of nuclear proteins was loaded onto a 10% SDS-PAGE and transferred to nitrocellulose membrane. Anti-MBD2 monoclonal antibody (IMG147, Imgenex, San Diego, CA) was used at a dilution of 1:200 in a solution of Tris-buffered saline (0.05 M Tris, 0.2 M NaCl, pH 7.6) + 0.5% Tween 20 + 5% milk overnight at 4 °C. The blot was then reacted with peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) at a 1:20,000 dilution. The enhanced chemiluminescence detection kit (Amersham Biosciences) was used for all Western blots. Membranes were also stained with 0.1% Amido Black (in 25% isopropanol, 10% acetic acid) or probed with an antibody to  $\beta$ -actin to confirm equal loading of protein in each lane.  $\beta$ -actin Western blots were performed using a 1:5000 dilution of anti- $\beta$ -actin monoclonal antibody (Sigma catalog number A-5316) followed by mouse secondary antibody as just described for MBD2. Both the Western blots and Amido-stained membranes were quantified using NIH Image 1.62 software, and the GFP/MBD2 signal was normalized to the total protein in each lane.

#### RESULTS

**The Association of CMV-GFP with Acetylated Histones Is Increased in the Presence of VPA**—We first validated that VPA induces histone acetylation of transiently transfected ectopically methylated CMV-GFP in our system, using ChIP assays with anti-acetyl-histone H3 (anti-Ac-H3). The results presented in Fig. 1A show that, whereas a minimal fraction of the transfected methylated GFP gene is associated with Ac-H3 in control cells (lane 3), the fraction of transfected methylated CMV-GFP associated with Ac-H3 increased 7-fold (lane 6) following a 48-h treatment with 20 mM VPA (Fig. 1B). Thus, VPA is effective in inducing histone acetylation around the transiently transfected methylated CMV-GFP gene.

**VPA Stimulates Active Demethylation of Ectopically Methylated CMV-GFP in a Dose-dependent Manner**—We next tested the hypothesis that VPA triggers active DNA demethylation of transiently transfected methylated CMV-GFP, similarly to TSA, an HDAC inhibitor (18). To measure active demethylation, we must use a system in which the DNA of interest (*i.e.* CMV-GFP) does not undergo replication. The CMV-GFP plasmid does not bear an origin of replication and should not replicate in HEK 293 cells; this was validated in a previous study (18). We have shown that methylated CMV-GFP does not





**FIG. 1. Histone acetylation within CMV-GFP is induced by VPA.** As shown in A, HEK 293 cells were transfected with *in vitro* methylated GFP plasmid and treated with a final concentration of 20 mM VPA or left untreated (*control*). Cells were formaldehyde cross-linked after 72 h and subjected to a ChIP assay using an antibody against Ac-H3. A 500-bp region of the CMV-GFP sequence was amplified from the DNA samples by PCR as described under "Materials and Methods." A representative PCR is shown: *input*, 10% of total DNA prior to immunoprecipitation; *no ab*, reactions carried out in the absence of antibody; *IP*, immunoprecipitations with anti-Ac-H3 antibody. As shown in B, the experiment in A was quantified (NIH Image 1.63), and results are presented as the ratio of GFP DNA amplified from the Ac-H3 immunoprecipitate relative to the corresponding input.

replicate in HEK 293 cells using the restriction enzymes *DpnI* and *XbaI* (18). *DpnI* cleaves the sequence GATC only when the adenine is methylated on both strands (26). Because mammalian cells do not bear a methylase that methylates the adenine in GATC, replication of the plasmid in HEK cells will render it resistant to *DpnI* digestion. *XbaI*, on the other hand, does not cleave the sequence TCTAGATC (which is present in CMV-GFP) when the adenine is methylated (27). Replication of the plasmid in mammalian cells will render it sensitive to *XbaI* digestion. Our data demonstrated that CMV-GFP is completely digested with *DpnI* and resistant to *XbaI* digestion (18), indicating that CMV-GFP does not replicate in HEK 293 cells, and therefore, any demethylation observed is occurring actively.

In this study, we first determined the effects of increasing doses of VPA on the demethylation of methylated CMV-GFP (Fig. 2). DNA was isolated from HEK 293 cells transfected with methylated CMV-GFP DNA (whose full methylation status was confirmed prior to transfection (Fig. 2A)) for 72 h and treated with 0–20 mM VPA. Cells were also treated with 0.3  $\mu$ M TSA as a positive control since we have shown that this leads to nearly 100% demethylation (18). DNA was first linearized with the *EcoRI* restriction enzyme followed by digestion with *MspI* (which cleaves the sequence CCGG) or *HpaII* (which cleaves the sequence CCGG only when it is not methylated). The demethylated, *HpaII*-digested 529-bp fragment (*U*), and the methylated undigested DNA (*Me*) were quantified within each sample (to control for possible loading differences), and the percent methylation for total CMV-GFP DNA in each lane was determined as  $(Me/(U + Me)) \times 100$ . As can be seen in Fig. 2, C and E, there is a minimal amount of CMV-GFP demethylation in untreated HEK 293 cells, most likely due to the background histone acetylation of the transfected plasmid, which allows access to demethylase even in the absence of histone deacetylase inhibitors as has been shown previously (18). The addition of VPA results in a dose-dependent active demethylation of methylated CMV-GFP, which is illustrated by the increase in the ratio of the 529 bp *HpaII* fragment (*U*) to the undigested DNA (*Me*). The addition of 20 mM VPA results in almost complete demethylation, similar to 0.3  $\mu$ M TSA. Fig. 2B confirms that unmethylated CMV-GFP, transfected under identical conditions, does not get *de novo* methylated in our system, either in the presence or in the absence of 20 mM VPA,

suggesting that VPA does act on DNA methyltransferase activity.

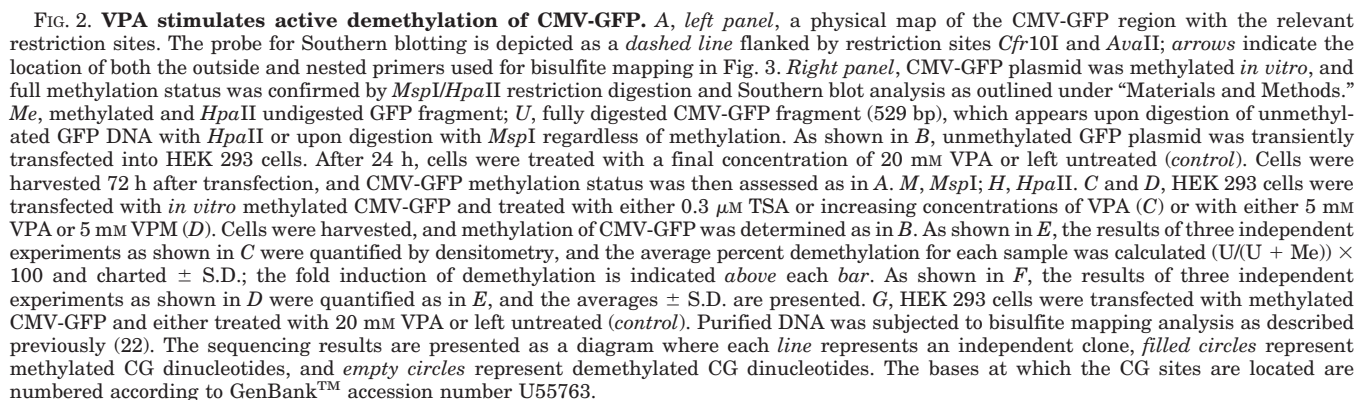
To determine whether this demethylation is dependent on HDAC inhibitory properties of VPA, we used our assay to compare the effects of VPA with those of its analogue VPM, which is not an HDAC inhibitor (5). Fig. 2, D and F, illustrates that, although 5 mM VPA results in almost 50% demethylation, the same concentration of VPM has virtually no effect. Together, the above data support the hypothesis that VPA, through its ability to induce histone hyperacetylation, is an inducer of active DNA demethylation.

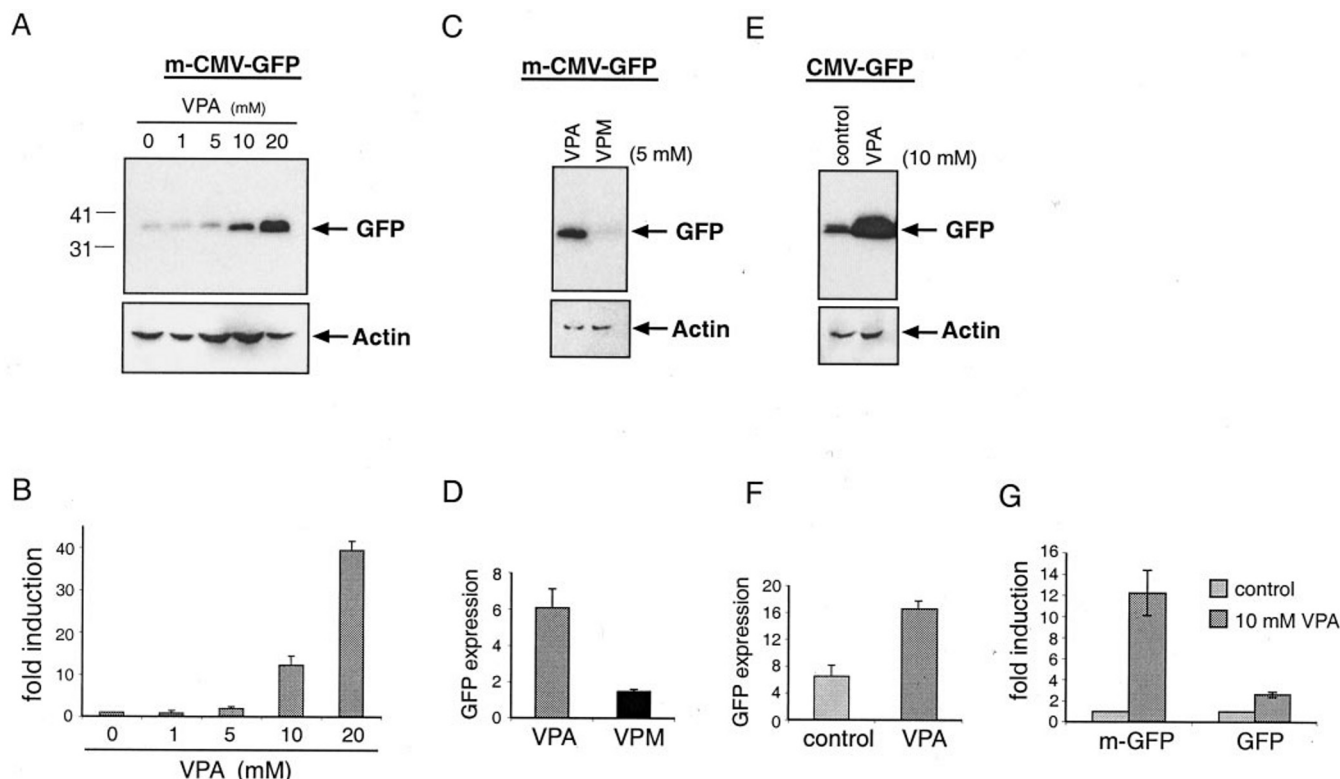
We also confirmed our results by an independent bisulfite analysis, which determines the methylation pattern at single base resolution (28). The results of these analyses, presented in Fig. 2G, reveal that methylated CMV-GFP retains its methylation pattern in untreated HEK 293 cells (*filled circles* represent methylated CpGs). In contrast, a 20 mM VPA treatment results in almost complete demethylation of all the methylated CpG sites tested in CMV-GFP (*open circles*), with the exception of three different CpG sites in several clones. It is not clear why these CpGs are not demethylated. One explanation is that the binding of a specific protein to a methylated CpG site protects it from demethylation. However, if this were the case, one would expect the consistent methylation of the same CpG in the majority of clones. Since the methylated sites we observe occur more or less randomly in different clones, a more likely explanation is simply slight inaccuracy on the part of the demethylase enzyme.

**The Expression of Methylated CMV-GFP Is Induced by VPA and Is Dose-dependent**—Several studies have demonstrated that VPA treatment can lead to activation of unmethylated genes by inducing histone acetylation (5), activating the Wnt signaling pathway (5, 29), or activating AP-1-dependent transcription (30). Certain methylated genes are also induced by VPA treatment, but it was not determined whether demethylation is involved. We therefore determined whether demethylation of CMV-GFP by VPA changes its state of expression using a Western blot analysis with anti-GFP antibody.

As shown in Fig. 3, A and B, transiently transfected methylated CMV-GFP is almost fully repressed in untreated HEK 293 cells (*lane 1*) as expected. The small amount of GFP protein observed corresponds to the minimal demethylation occurring in untreated cells (Fig. 2C). Following treatment with 1–20 mM VPA, a dose-dependent induction of CMV-GFP expression is observed. Thus, the doses of VPA that lead to the increased demethylation of CMV-GFP also induce gene expression. VPM, which does not induce histone acetylation, has no effect on GFP protein levels (Fig. 3, C and D), indicating that the ability of VPA to promote both the demethylation (Fig. 2) and expression of CMV-GFP is dependent upon its HDAC inhibitory activity.

The induction of expression by VPA might be a consequence of demethylation, or alternatively, demethylation and expression are independent effects of the HDAC inhibitory activity of VPA. To address this issue, we compared the induction of methylated CMV-GFP and unmethylated CMV-GFP following identical transfections and 10 mM VPA treatment. If demethylation were not required for the effects of VPA on expression, then one would predict a similar fold activation of methylated and unmethylated CMV-GFP. The induction of unmethylated CMV-GFP by VPA is shown in Fig. 3, E and F, and the comparison with the methylated plasmid is presented in Fig. 3G. This experiment demonstrates that VPA induces the expression of methylated CMV-GFP (12-fold) to a greater extent than the unmethylated counterpart (3-fold). Taken together, our data are consistent with the hypothesis that VPA induces the expression of methylated genes through its effects on both





**FIG. 3. The expression of methylated CMV-GFP is induced by VPA.** As shown in A, C, and E, HEK 293 cells were transiently transfected with either methylated CMV-GFP plasmid (*m-GFP*) (A and C) or unmethylated CMV-GFP (*GFP*) (E) and treated with the indicated concentrations of VPA or VPM. Total cell extracts were prepared using standard protocols and resolved on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene fluoride membrane; GFP protein was detected using rabbit polyclonal IgG (Santa Cruz Biotechnology, sc-8334), and  $\beta$ -actin was detected with a mouse monoclonal antibody (Sigma catalog number A-5316). *Upper panel*, GFP; *lower panel*,  $\beta$ -actin control. As shown in B, experiments such as those shown in A were performed in triplicate, and GFP protein levels were quantified by densitometry and normalized to the amount of total protein per lane as determined by Amido Black staining. Results are presented as fold induction relative to the level of CMV-GFP expression in the absence of VPA. D and F, quantification of triplicate experiments as shown in C and E, respectively. The averages  $\pm$  S.D. are presented in arbitrary units  $\times 10^3$ . G, comparison of the CMV-GFP expression obtained upon treating either m-CMV-GFP- or CMV-GFP-transfected cells with 10 mM VPA. The graph displays data combined from the experiments shown in B and F.

histone acetylation and DNA demethylation, the latter of which is unique to methylated genes.

**MBD2/dMTase Is a Demethylase Enzyme Involved in the Active Demethylation Induced by VPA**—We have previously hypothesized that the open chromatin structure brought about by histone acetylation favors access of demethylase enzymes to the DNA (18, 22). We determined whether MBD2/dMTase, the only demethylase characterized to date, is involved in the demethylation induced by VPA by measuring the effect of antisense knockdown of MBD2/dMTase. HEK 293 cells were transfected with methylated CMV-GFP, together with an MBD2 antisense expression construct (AS-MBD2) (24) or an empty vector as a control. Following treatment with 20 mM VPA, cells were harvested and subjected to Southern blot analysis as in Fig. 2. The results shown in Fig. 4A and quantified (average of three experiments) in Fig. 4B indicate that antisense knockdown of MBD2/dMTase inhibits demethylation induced by VPA by  $\sim 70\%$ . A Western blot analysis using an anti-MBD2 antibody confirmed that MBD2/dMTase levels were knocked down by 50% by the antisense treatment (Fig. 4, C and D). These data indicate that MBD2/dMTase mediates, at least in part, the active demethylation triggered by VPA. The residual (30%) demethylation remaining after antisense knockdown of MBD2/dMTase could be due to either incomplete knockdown (as demonstrated in the Western blot analysis) or the presence of other as of yet uncharacterized demethylases.

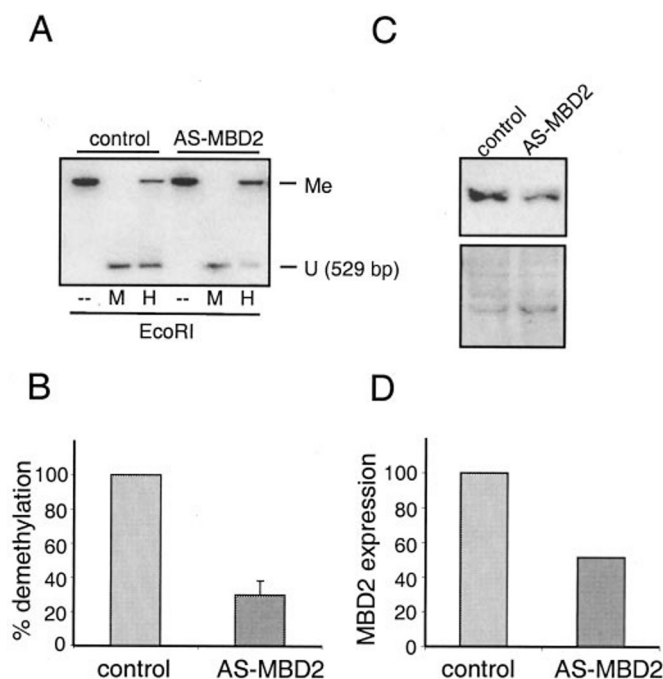
#### DISCUSSION

VPA is a well tolerated drug that has been used for many years in the treatment of epilepsy and bipolar disorder. Al-

though several modes of action have been proposed to explain the therapeutic effects of VPA, this report describes a novel mechanism that could potentially be involved. Our studies imply that VPA activates methylated genes by stimulating active, replication-independent demethylation. It has been shown before that VPA can induce hypomethylation in the rat liver (31), similar to the DNA methylation inhibitor 5-aza-CdR, but it was not clear whether it does so by inhibiting DNA methylation or by other mechanisms. This report tested the hypothesis that DNA methylation is a reversible process, and therefore, that drugs that affect DNA methylation patterns might do so either by inhibiting DNA methylation or by promoting demethylation. We took advantage of an assay developed previously in our laboratory (18), which measures active demethylation in living cells, to test whether VPA can stimulate active demethylation.

Using a ChIP assay, we first demonstrate that VPA induces acetylation of H3 histones associated with methylated CMV-GFP (Fig. 1), which is in accordance with previous studies demonstrating the HDAC inhibitory activity of VPA (5, 6). Second, we show that VPA stimulates active demethylation of ectopically methylated and transiently transfected CMV-GFP DNA (Fig. 2, C and E). Third, we illustrate that demethylation is dependent on histone acetylation since the analogue VPM, which is not an HDAC inhibitor, has no effect (Fig. 2, D and F). Bisulfite mapping analysis confirms these data and indicates that VPA stimulates the erasure of the entire methylation pattern of CMV-GFP (Fig. 2G). We propose that VPA stimulates demethylation by inducing acetylation of H3 histones





**FIG. 4. MBD2/dMTase is involved in the active demethylation induced by VPA.** In A, HEK 293 cells were transfected with *in vitro* methylated CMV-GFP, together with either an AS-MBD2 expression plasmid or an empty vector (*control*) and treated with 20 mM VPA. Cells were harvested 72 h after transfection, and the methylation status of GFP DNA was determined as described under "Materials and Methods" and in Fig. 2. *Me*, methylated and *HpaII* undigested CMV-GFP; *U*, unmethylated and *HpaII* fully digested CMV-GFP (529 bp). *M*, *MspI*; *H*, *HpaII*. In B, the results of three independent experiments as shown in A were quantified by densitometry, and the average percent of demethylation,  $[U/(U + Me)] \times 100$ , was calculated. Results are presented relative to the control, which was normalized to 100%. C, AS-MBD2 expression plasmid or empty vector (*control*) were transfected into HEK 293 cells; cells were harvested 72 h after transfection, and an MBD2 Western blot was performed as outlined under "Materials and Methods." Upper panel, MBD2 Western; lower panel, Amido Black-stained membrane. In D, the experiment in C was quantified, and results are shown relative to the control, which was normalized to 100%.

associated with CMV-GFP and increasing the accessibility of demethylase to the DNA. Previous studies have demonstrated that 3 mM VPA are required to induce significant histone acetylation (5, 6), and similarly, we observe 50% demethylation at 5 mM. Although this value is slightly higher than the serum concentrations (1 mM) achieved in patients treated with VPA (32), we do begin to see a small increase in demethylation at 1 mM. The difference with our study is that is that the cells are only exposed to VPA for 48 h; it is therefore possible that a longer treatment with lower VPA concentrations would produce similar effects. In support of this, it has been shown that both the methylated genes for *reelin* and *gad67* are up-regulated in rats treated with tolerated doses of VPA (300 mg/kg) for 15 days (8). The demethylation we observe further increases up to 20 mM VPA. It is unclear whether at higher concentrations, VPA acts exclusively as a histone deacetylase inhibitor or whether it may trigger another as of yet unknown process leading to enhanced demethylation.

Fourth, we show that VPA induces the expression of methylated CMV-GFP (Fig. 3, A and B), illustrating that VPA concurrently affects both active demethylation of DNA and gene expression. Furthermore, we demonstrate that demethylation directly contributes to the effects on gene expression (Fig. 3G). These data are consistent with a previous report illustrating that the methylated 5-lipoxygenase gene can be activated by VPA in non-proliferating cells, whereas 5-aza-CdR is only ef-

fective in cells that divide (9). However, this study did not determine whether VPA induced demethylation. Finally, we show that MBD2/dMTase is required for VPA-induced demethylation, supporting the hypothesis that demethylase activity is required for the demethylation triggered by VPA.

MBD2/dMTase has been shown to act as an active demethylase enzyme *in vitro* (16), it is able to stimulate methylated genes in transfection experiments (18, 19, 33), and its expression is associated with demethylation of endogenous genes (34). In contrast, some groups reported previously that they failed to observe demethylase activity *in vitro* for MBD2 (20), although the reason behind this discrepancy is unclear. *Mbd2*<sup>-/-</sup> knockouts also did not exhibit global differences in DNA methylation (21); however, this study did not examine the demethylation of specific genes. Therefore it is possible that MBD2/dMTase is involved in specific demethylation events, which would be consistent with our data.

Thus, VPA is the first therapeutic agent shown to be able to erase DNA methylation patterns in a replication-independent manner by stimulating the accessibility of a demethylase enzyme. An important point to note is that although our assay measures demethylation that is independent of replication, the HEK 293 cells we use are actively dividing. Thus, we do not know for certain whether postmitotic tissues possess the same machinery involved in chromatin organization and demethylation. However, we do know that MBD2/dMTase is expressed in postmitotic tissues such as the brain (35, 36) and liver (37). Furthermore, previous studies have demonstrated that VPA can induce both histone acetylation and the expression of methylated genes (*reelin*, *gad67*, 5-lipoxygenase) in non-dividing tissues or cells at therapeutically relevant concentrations (8, 9). Although further studies are required to determine whether these genes become demethylated, the abovementioned findings together with our data support the possibility that VPA might be used to modify DNA methylation patterns in postmitotic tissues such as the brain. This observation has important therapeutic and biological implications since a number of genes such as *FMR1* (38), *NF 2* (39), and *reelin* (8) were shown to be silenced in the brain by mechanisms that might involve DNA methylation. The only available demethylating drug is 5-aza-CdR, which needs to be incorporated into DNA and inhibit DNA methyltransferase during cell division. Such a mechanism makes this drug, as well as other DNA methyltransferase inhibitors, ineffective in the brain. Several other HDAC inhibitors such as phenylbutyrate, depsipeptide, and suberoylanilide hydroxamic acid are known; however, they are currently only in use in clinical trials (40). Interestingly, suberoylanilide hydroxamic acid is able to cross the blood-brain barrier and improves motor deficits in a mouse model of Huntington's disease (41). However, VPA has the advantage in that it is already an established drug whose long term effects are known. If it is possible to take advantage of well tolerated HDAC inhibitors, such as VPA, to stimulate the erasure of DNA methylation by resident demethylases, then it might be feasible to therapeutically activate genes silenced by DNA methylation in the brain and other non-dividing tissues.

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