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Histone deacetylase inhibitors decrease *reelin* promoter methylation *in vitro*

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Abstract

We investigated the effects of agents that induce *reelin* mRNA expression *in vitro* on the methylation status of the human *reelin* promoter in neural progenitor cells (NT2). NT2 cells were treated with the histone deacetylase inhibitors, trichostatin A (TSA) and valproic acid (VPA), and the methylation inhibitor aza-2′-deoxycytidine (AZA) for various times. All three drugs reduced the methylation profile of the *reelin* promoter relative to untreated cells. The acetylation status of histones H3 and H4 increased following treatment with VPA and TSA at times as short as 15 min following treatment; a result consistent with the reported mode of action of these drugs. Chromatin immunoprecipitation experiments showed that these changes were accompanied by changes occurring at the level of the *reelin* promoter as well. Interestingly, AZA decreased *reelin* promoter methylation without concomittantly increasing histone acetylation. In fact, after prolonged treatments with AZA, the acetylation status of histones H3 and H4 decreased relative to untreated cells. We also observed a trend towards reduced methylated H3 after 18 h treatment with TSA and VPA. Our data indicate that while TSA and VPA act to increase histone acetylation and reduce promoter methylation, AZA acts only to decrease the amount of *reelin* promoter methylation.

Keywords: *reelin*, cytosine methylation, histone deacetylase, valproate, histone acetylation, schizophrenia.

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Reelin is a glycoprotein that is selectively synthesized and secreted by GABAergic neurons of multiple brain regions into the extracellular matrix of the adult brain in a constitutive manner (D'Arcangelo *et al*. 1995; D'Arcangelo *et al*. 1997; Lacor *et al*. 2000). The nucleotide and amino acid sequences of reelin are both highly conserved between mouse and human (DeSilva *et al*. 1997). Reelin is expressed in neurons during development and throughout the lifespan of rodents, non-human primates, and humans (Alcantara *et al*. 1998; Impagnatiello *et al*. 1998; Pesold *et al*. 1998; Guidotti *et al*. 2000; Rodriguez *et al*. 2000). During mammalian brain development, reelin is secreted by telencephalic marginal zone Cajal-Retzius cells and by cerebellar granule cells where it plays a critical role in the proper laminar development of the cortex and cerebellum, respectively (D'Arcangelo *et al*. 1995; Curran and D'Arcangelo 1998; Pesold *et al*. 1998). In the adult brain, reelin is synthesized by specific subtypes of GABAergic interneurons in cortex, hippocampus, and olfactory bulb and glutamatergic cerebellar granule cells (Alcantara *et al*. 1998; Pesold *et al*. 1998; Pesold *et al*. 1999; Eastwood and Harrison 2003), and evidence supports the hypothesis that reelin promotes dendritic spine plasticity via a mechanism that involves the stimulation of spine resident mRNA translation (Costa *et al*. 2003; Dong *et al*. 2003).

Interestingly, about a 50% reduction in reelin mRNA and protein levels has been detected in post-mortem brain tissue of patients diagnosed with schizophrenia in all brain areas studied to date, including several cortical regions, hippocampus, and cerebellum (Impagnatiello *et al*. 1998; Fatemi *et al*. 2000; Guidotti *et al*. 2000; Eastwood and Harrison

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Abbreviations used: AZA, aza 2-deoxycytidine; CpG, cytosine-phospho-guanine dinucleotide; CREB, cyclic AMP response binding protein; DMEM, Dulbecco's modified Eagle's medium; DNMT1, DNA methyltransferase I; GLM, univariate general linear model; HDAC, histone deacetylase; hNT, differentiated NT2 neurons; H3, histone H3; H4, histone H4; LSD, Fisher's least significant difference test; NT2, Ntera2 cells; Pax-6, paired box transcription factor 6; RA, retinoic acid; Tbr-1, transcription factor T brain 1; TSA, trichostatin A; UTX, untreated or vehicle treated; VPA, valproic acid.

2003; Veldic *et al*. 2004). However, the mechanism underlying this decrease in reelin expression remains unclear. The area surrounding the site of initiation of transcription of the *reelin* gene in both mouse and human contains a high GC content and forms a CpG island (Royaux *et al*. 1997; Tremolizzo *et al*. 2002). Cytosine methylation within CpG islands of gene promoter regions is recognized widely as an important mechanism of gene suppression (e.g. Newell-Price *et al*. 2000; Szyf and Detich 2001; Tucker 2001; Singh *et al*. 2003). Multiple mechanisms, which are not mutually exclusive, have been proposed by which the addition of methyl groups to cytosines within the promoter region of genes could down-regulate expression (Singal and Ginder 1999; Szyf and Detich 2001; Singh *et al*. 2003). One such mechanism is the direct interference with the binding of transcription factors whose recognition sites contain CpG dinucleotides. Alternatively, there is evidence that methyl CpG binding proteins recruit histone deacetylase (HDAC); histone deacetylation is believed to promote heterochromatin formation which renders transcription factor binding sites inaccessible, thereby repressing gene expression.

Thus, one potential regulatory mechanism of reelin expression is the methylation of critical cytosines in the promoter region of the gene. Consistent with this hypothesis, we have demonstrated recently that methionine treatment decreases reelin protein and mRNA in the frontal cortex of both the wild-type and heterozygous *reeler* mouse, and increases frontal cortical levels of primary methyl donor *S*-adenosyl methionine and its demethylated metabolite, *S*-adenosyl homocysteine. This treatment also increased the number of methylated cytosines in the *reelin* promoter region in DNA extracted from frontal cortex in the same mice, such that the number of methylated cytosines was negatively correlated with frontal cortical *reelin* mRNA levels (Tremolizzo *et al*. 2002). The methionine-induced attenuation of *reelin* mRNA content was reversed by valproic acid (VPA), an agent which was recently shown to be an HDAC inhibitor (Phiel *et al*. 2001). We have also demonstrated that NT2 neuronal precursor cells express reduced *reelin* mRNA and exhibit *reelin* promoter hypermethylation relative to the retinoic acid-induced hNT neurons. Increasing promoter methylation in NT2 cells reduced *reelin* promoter activity, and treatment of NT2 cells with the HDAC inhibitors VPA or trichostatin A (TSA), or with aza 2-deoxycytidine (AZA), a non-methylatable analog of cytosine, produced pronounced increases in *reelin* mRNA levels (Chen *et al*. 2002).

The possibility that a reduction in *reelin* promoter methylation is associated with the previously observed increase in *reelin* mRNA in NT2 cells by VPA, TSA, and AZA was investigated in the current studies. Consistent with this hypothesis, treatment of NT2 cells with all three agents reduced the number of methylated cytosines in the promoter region relative to untreated cells. TSA and VPA rapidly increased the acetylation of histones H3 and H4 and produced a strong tendency toward a delayed decrease in the methylation of H3, which may suggest that HDAC inhibition increases the activity of DNA demethylases, such as MBD2 (Cervoni and Szyf 2001; Szyf 2001; Detich *et al*. 2002). AZA failed to increase the acetylation of H3 and H4 or to decrease the methylation of H3 with relatively short treatment durations (18 h), and even decreased the acetylation of H3 and H4 with longer treatment durations (36 or 72 h).

Experimental procedures

Cell culture

NT2 cells (Stratagene, La Jolla, CA, USA) were maintained in DMEM/F12 supplemented with 10% fetal bovine serum and 1% penicillin, 1% streptomycin, and 1% L-glutamine. For cytosine methylation experiments, cultures were either untreated, or treated with 5 mM VPA for 48 h, 0.3 μM TSA for 24 h, or 5 μM AZA for 72 h. For histone acetylation analysis, cultures were untreated, or treated with 5 μM AZA, 0.3 μM TSA, or 5 mM VPA for 15 min, 2, 4, 8, 12 or 18 h. Additionally, some cultures were treated with 5 μM AZA for 36 or 72 h.

Bisulfite modification and cloning

Genomic DNA was isolated from the NT2 cultures using a proteinase K/sodium dodecyl sulfate (SDS) protocol (Zuccotti and Monk 1995). Harvested DNA was digested with *Eco*R1 and then denatured by incubation with 0.2 M NaOH at 37°C for 30 min. The sodium bisulfite modification was conducted using a CpGenome DNA Modification kit (Intergen, Purchase, NY, USA). Modified material was then amplified via nested PCR using primers for the B strand: outer primers −790 bp, 5′-TTTAAAATCCTCTACAAATAAAACTCTATCACT-3′, and +350 bp, 5′-TGTTTGTAATATGTAGGGAAATGAG-TATTT-3′; inner primers −527 bp, 5′-ACATCCTCCCAAAAAAA-ACAAAACACACTAA-3′, and +305 bp, 5′-TTTTTTTAGTT-TTTTGTGGTGGGTGTATAGGAA-3′. The resulting PCR products were gel-purified, and amplicons were subcloned into pGEM T-Easy (Promega, Madison, MI, USA). Multiple clones from each treatment group were sequenced by the University of Chicago Cancer Research Center DNA Sequencing Facility.

Western blot analysis

For analysis of acetylated histones H3 and H4, cells were harvested with cell scrapers (Corning, Acton, MA, USA) in lysis buffer (20 mM Tris HCl, 2 mM EGTA, 5 mM EDTA, 0.2 U/mL aprotinin, 1.5 mM L-pepstatin, 2 mM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol) and vortexed. For analysis of methylated histone H3, proteins were acid extracted. Cells were harvested in acid extraction lysis buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM, phenylmethylsulfonyl fluoride, 1.5 mM dithiothreitol) and vortexed. HCl (0.2 M) was added to the samples, which were then incubated on ice for 30 min and centrifuged at 11 000 *g* for 10 min at 4°C. The supernatant was harvested and dialyzed at 4°C against 0.1 M acetic acid twice for 1 h and against distilled H_2O for 1, 3 h, and overnight.

Protein concentrations for all samples were determined by the Lowry method (Lowry *et al*. 1951). Protein samples were diluted 1 : 1 in Lammli buffer and denatured by boiling for 5 min 10 μg aliquots of protein were loaded per well onto 4–20% Tris/Glycine gels (Invitrogen, Carlsbad, CA, USA), separtated by electrophoresis, and blotted onto nitrocellulose membranes (Invitrogen). Following the transfer, membranes were incubated overnight at 4°C in 3% non-fat dry milk in phosphate-buffered saline (PBS) with the following antibodies: anti-acetyl-histone H3 (Product #06–599; Upstate Biotechnology, Lake Placid, NY, USA), anti-acetyl-histone H4 (Product #06–598; Upstate Biotechnology), or anti-dimethyl-histone (Lys9) H3 (Product #07–212; Upstate Biotechnology) primary antibodies (all used at 1 : 1000, Upstate Biotechnology). The 06–599 antibody was raised against an H3 peptide acetylated at lysines 9 and 14 and therefore recognizes acetylated H3-K9, acetylated H3-K14 and when both are acetylated. The 06–598 antibody was raised against an H4 peptide acetylated at lysines 5, 8, 12 and 16 of histone H4. For both antibodies, there is recognition if just one site or all sites are acetylated. Finally, the 07–212 antibody recognizes predominantly the di-methyl K9 H3 and also the tri-methyl K9 histone H3 with little specificity for the mono-methylated species.

The membranes were then rinsed three times in PBS and incubated with peroxidase-conjugated donkey anti-rabbit IgG (1 : 1000, Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 h in 3% non-fat dry milk. Membranes were rinsed five times in PBS and incubated for 10 min in ECL chemiluminescence solution (Amersham Biosciences). Immunoreactive bands were visualized using the Blue Flurorescence/Chemifluorescence Storm system and IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA, USA), and a peak analysis was conducted. The membranes were then rinsed in PBS, incubated with anti-β-actin Ig (1 : 6000, Sigma Chemical Co. St Louis, MO, USA) overnight at 4°C in 3% non-fat dry milk, rinsed three times in PBS, incubated with donkey anti-goat Ig (1 : 1000, Amersham Biosciences) for 1 h at room temperature in 3% non-fat dry milk, rinsed five times in PBS, incubated for 10 min in ECL chemiluminescence solution, and visualized and analyzed as described above.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using the ChIP assay kit protocol as recommenmded by the manufacturer (06–599, Upstate Biotechnology, Lake Placid, NY, USA). After treatments (24 h with vehicle, 5 mM VPA or 0.3 μM TSA), 1% formaldehyde was added to cross-link histones to DNA (10 min, 37°C). Cells were than washed, scraped and pelleted at 2000 rpm for 4 min at 4°C. The cell pellet was resuspended in SDS Lysis Buffer (Upstate Biotechnology, Lake Placid, NY, USA) with protease inhibitor cocktail and incubated on ice. Lysates were sonicated to shear DNA to lengths below 1000 bp (amplitude 60%, 8 × 10 s, Fisher Scientific Sonifier, Pittsburgh, PA, USA). From each sonicated sample, 5% was used as the input control for immunoprecipitated fragments. Diluted samples were pre-cleared with Salmon Sperm DNA/Protein A Agarose Slurry and incubated overnight with anti-acetyl-histone H3 (06–599, Upstate, ChIP grade), anti-acetyl-histone H4 (06–866, Upstate, ChIP grade) or without antibody (negative control). Complexes were bound to Protein A Agarose, washed and then eluted in 1% SDS/0.1 M $NaHCO_3$. After reversing the cross-links (5 M NaCl, 65°C, 4 h) and incubation with Proteinase K (45°C, 1 h), DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Input and test samples were resuspended in nuclease free water. One half of the sample was used for the primary PCR and then 1/50th was used in the subsequent nested reaction (1/5th of the final was used for electrophoresis). The *reelin* promoter region was PCR amplified (forward primer: −534 bp, 5′-CTTCCCTCACGCATCCTCCCAGGA-3′; reverse primer: +127 bp, 5′-CGCCGCTCGCTCATTCAGTTTTGGAG-3′). The protocol included an initial denaturation cycle (5 min, 94°C), 42 cycles of denaturation (45 s, 94°C), annealing (1 min, 67°C) and extension (2 min, 68°C), with a final extension cycle (7 min, 68°C). The PCR products were then subjected to nested PCR reaction (forward primer: −433 bp, 5′-TTTGACGT CCCTCGCAGAAGAGTC-3′; reverse primer: +74 bp, 5′-CGGAGAGAAGGCGAGAAGAAGGCG-3′. The protocol included a denaturation cycle (1 min, 95°C), 19 cycles of denaturation (30 s, 94°C), and annealing/extension (3 min, 68°C), followed by a final extension cycle (3 min, 68°C). The PCR reactions were carried out using Advantage-GC Genomic Polymerase Mix (BD Biosciences ClontechFranklin Lakes, NJ, USA).

For the β-globin locus control region/AF071080 (Li *et al*. 2004), the forward primer (+3961 bp) was 5′-AGACACTTGCTCTTTCCAGGACTT-3′ while the reverse primer (+4250 bp) was 5′-TGCCAGTATATGTGCTTCGATAGG-3′. The amplification involved an initial denaturation cycle (5 min, 94°C), 40 cycles of denaturation (15 s, 94°C), annealing (30 s, 55°C) and extension (30 s, 68°C), with a final extension cycle (7 min, 68°C). For the nested amplification (+4002 bp): 5′-ACTGCATCTGCAAGCCTTTT-3′ and reverse primer (+4218 bp): 5′-GTGCCTGATTCCGGGTACTA-3′ were used. The amplification included an initial denaturation cycle (5 min, 94°C), 19 cycles of denaturation (15 s, 94°C), annealing (30 s, 55°C) and extension (30 s, 68°C) with a final 3 min extension. The *β-globin* gene is not active in NT2 cells and is not activated by TSA or VPA. The locus control region served as a negative control for our ChIP assays.

Nested PCR amplification products were separated on 1.6% agarose gels and optical density (ROD) readings were determined using a computer-assisted densitometry program (GEL LOGIC, Kodak Edas, New Haven, CT, USA). For all experiments, the immunoprecipitated DNA template was well below the saturation level. This was verifed by electrophoresis of the primary and secondary PCR amplification products. PCR bands derived from the input DNA samples were also under the saturation level. Prior to PCR, the amount of input DNA was quantified using the pico green method with external DNA standards for generating a standard curve (Singer *et al*. 1997).

Statistical Analyses

To analyze the effects of VPA, TSA, and AZA on cytosine methylation of the *reelin* promoter in NT2 cells relative to untreated cells, a univariate general linear model (GLM) procedure was used with Treatment as a factor. Significant differences were further assessed with the Fisher's least significant difference test (LSD) test. Similarly, univariate GLMs followed by Fisher's LSD were used to investigate the effect of VPA, TSA, and AZA on cytosine methylation within binding sites for the transcription factors Sp1 and cyclic AMP response binding protein (CREB), and for the entire enhancer region of the *reelin* promoter. Data from the ChIP experiments were analyzed with one-way ANOVA and *post hoc*

Bonferroni *t*-test using SIGMASTAT Software (Systat Software, Point Richmond, CA, USA).

To analyze the effect of VPA, TSA, and AZA on acetyl H3, acetyl H4, and methyl H3 content relative to untreated cells using different treatment durations, the ratio of the peak area of the histone band divided by the peak area of the β-actin band was calculated for each replication of each treatment at each duration. Univariate GLMs with Treatment, Duration, and Treatment × Duration as factors were conducted for each individual measure. Additionally, we investigated the effect of 36 and 72 h treatment with AZA on acetyl H3 and of 72 h treatment with AZA on acetyl H4 relative to untreated cells. These data were analyzed with independent *t*-tests.

Results

Methylation of *reelin* promoter cytosines

Three cytosines were found to be methylated ('hot spots') in nearly every clone regardless of treatment; these bases were not included in the statistical analyses. These 'hot spots' were located at positions −138, −133 and +22 relative to the RNA start site. A GLM followed by Fisher's LSD revealed that the number of methylated cytosines in the *reelin* promoter region of NT2 cells was dramatically reduced (> threefold) by treatment with VPA (1.9 ± 0.49), TSA (2.3 ± 0.33), or AZA (1.30 ± 0.34) relative to untreated cells (7.20 ± 0.92) ($F_{3,45} = 89.1$, $p < 0.001$) (Fig. 1). None of the treatment conditions were statistically different from each other. The reduction in cytosine methylation induced by VPA, TSA, and AZA did not appear to be specific for individual cytosines within the promoter region (Figs 2*a–d*), nor was it observed in the putative binding sites for the transcription factors Sp1 ($F_{3,45} = 1.69$, $p = 0.182$), Tbr1 (no methylation in any condition), or CREB ($F_{3,45} < 1.0$). More precise mapping of these binding sites and a functional analysis of their activity with deletion mutants is currently underway. Analysis of the enhancer region of the *reelin* promoter (from −303 to −135 bp), also showed a significant reduction in the level of methylation elicited by VPA, TSA, and AZA relative to untreated cells ($F_{3,45} = 29.2$, $p < 0.001$) (Fig. 1*b*). Interestingly, we observed methylated cytosines which were present in CpNpG trinucleotides as well as methylation in more conventional CpG dinucleotides. This finding has been reported by ourselves (Chen *et al*. 2002) and others (Ramsahoye *et al*. 2000; Haines *et al*. 2001) and may reflect the activity of multiple DNA methyltransferases with overlapping specificities. Collectively, each of the various treatments reduced the degree of methylation of the *reelin* promoter as compared to the non-treated group (UTX) and this reduced methylation correlates with increased expression of the corresponding *reelin* mRNA (Chen *et al*. 2002). This is particularly interesting in that VPA and TSA act as histone deacetylase inhibitors while AZA is an inhibitor of DNA methylation, suggesting a common final end-point.

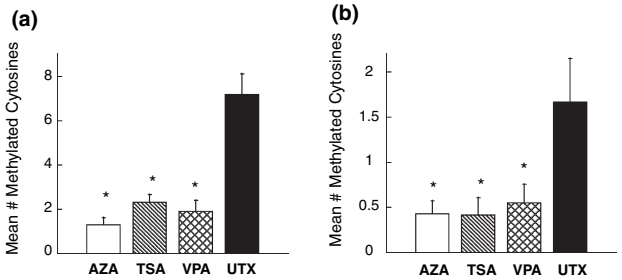

Fig. 1 Cytosine methylation frequency within the *reelin* promoter of NT2 cells following treatment with aza-2′-deoxycytidine (AZA, $n = 14$ clones), trichostatin A (TSA, $n = 12$ clones), valproic acid (VPA, $n = 11$ clones), or no treatment (UTX, $n = 12$ clones). All three treatments significantly decreased the amounts of methylation in (a) the entire amplified region (−527 to +305 bp) and in (b) the enhancer region of the promoter (−304 to −136 bp). *$p < 0.01$.

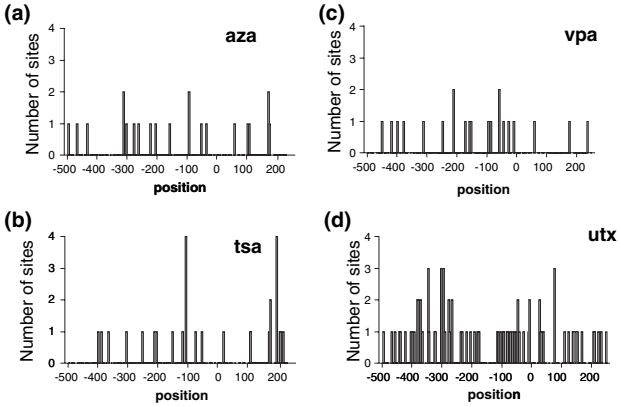

Fig. 2 Distribution map of methylated cytosines along the *reelin* promoter in NT2 cells treated with either (a) AZA ($n = 14$) (b) TSA ($n = 12$), or (c) VPA ($n = 11$), or (*d*) UTX ($n = 12$).

Western blot analysis of acetyl H3, acetyl H4, and methyl H3

Analysis of the ratio of the area of the acetyl H3 band/area of the β-actin band yielded a significant main effect of Treatment ($F_{3,48} = 10.5$, $p < 0.001$). *Post hoc* analysis revealed that TSA (2.3 ± 0.2) and VPA (2.0 ± 0.2), but not AZA (0.73 ± 0.21), produced a robust (two to threefold) increase of acetyl H3 relative to untreated cells (0.92 ± 0.21) (Figs 3*a* and *b*). Neither the main effect of Duration ($F_{5,48} = 1.04$, $p > 0.05$), nor the Treatment–Duration interaction ($F_{15,48} < 1.0$) approached statistical significance, indicating that there was no difference in the magnitude of the effect of TSA and VPA across the different time points. Thus, the maximum effect of TSA and VPA in increasing acetyl H3 was obtained with a treatment duration as brief as 15 min.

Similarly, analysis of the ratio of the area of the acetyl H4 band/area of the β-actin band yielded a significant main effect of Treatment ($F_{3,48} = 9.6$, $p < 0.001$), but there was no effect of Duration ($F_{5,48} < 1.0$) nor a Treatment–Duration

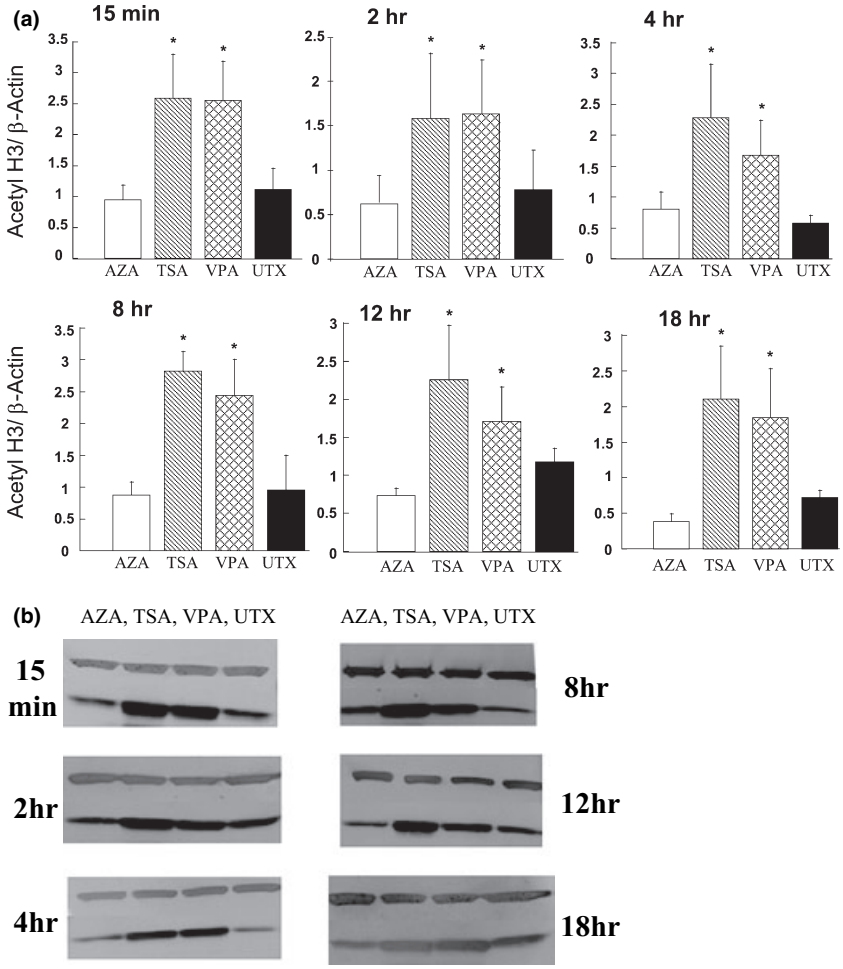

Fig. 3 Treatment of NT2 cells with TSA and VPA, but not AZA, rapidly increased acetylation of histone H3 relative to untreated cells at all timepoints. (a) Ratio of the area of the acetyl H3 band over the area of the β-actin band in protein samples harvested from NT2 cells following 15 min (top left), 2 h (top middle), 4 h (top right), 8 h (bottom left), 12 h (bottom middle), or 18 h (bottom right) treatment with either AZA, TSA, or VPA, and in untreated cells (each $n = 3$). Images were quantified with IMAGEQUANT software. *$p < 0.01$. (b) Representative western immunoblots of acetyl H3 (lower band) and β-actin (upper band) using protein samples harvested from NT2 cells following 15 min (upper left), 2 h (middle left), 4 h (bottom left), 8 h (upper right), 12 h (middle right), or 18 h (bottom right) treatment with either AZA, TSA, or VPA, and in untreated cells.

interaction ($F_{5,48} < 1.0$) (Figs 4*a* and *b*). Once again, *post hoc* analysis revealed that both TSA (1.9 ± 0.3) and VPA (1.9 ± 0.3) robustly potentiated levels of acetylated H4 (≈ threefold) relative to untreated cells (0.63 ± 0.3), whereas AZA was ineffective (0.68 ± 0.3) (Fig. 4*a* and *b*). Similar to acetylated H3, the lack of a Treatment–Duration interaction indicates that the maximum increase in acetylated H4 levels by TSA and VPA occurred with a treatment duration of 15 min. As can be seen in Fig. 4*b*, the anti-acetyl H4 antibody exhibited some cross-reactivity, most probably with acetylated histone H2B. Interestingly, this was only observed in the TSA and VPA treatment conditions, which increased levels of acetylated histones H3 and H4.

Thus, AZA failed to increase acetylated H3 and H4 with treatment durations up to 18 h. Whereas the potentiation of acetylated H3 and H4 levels in cells treated with TSA or VPA probably reflects direct HDAC inhibition, any increase in acetylated H3 and H4 by AZA might be an indirect effect produced by the inhibition of HDAC activity following inhibition of DNMT1 activity. We reported previously a significant increase in *reelin* mRNA expression in NT2 cells by treatment with AZA for 24 h, however, the mRNA increase was more robust following a treatment for 72 h. Additionally, the treatment with AZA which produced the decrease in *reelin* promoter methylation observed in Fig. 1 also lasted 72 h. We therefore investigated the effect of 36 and 72 h treatments with AZA on H3 acetylation state (Fig. 5) and 72 h treatment on H4 acetylation (Fig. 6). Contrary to our expectations, we observed significant decreases in acetylated H3 following 36 h (AZA, 0.62 ± 0.25; UTX, 1.7 ± 0.41; $t(4) = 3.80$, $p < 0.05$) and 72 h (AZA, 0.33 ± 0.21; UTX, 1.3 ± 0.01; $t(4) = 7.50$, $p < 0.01$) treatment with AZA (Figs 5*a* and *b*) and in acetylated H4 following 72 h (AZA, 0.24 ± 0.11; UTX, 0.65 ± 0.11; $t(4) = 4.44$, $p < 0.05$) treatment with AZA (Figs 6*a* and *b*).

Both TSA (0.45 ± 0.11) and VPA (0.6 ± 0.47), but not AZA (1.05 ± 0.65), exhibited a tendency to decrease levels of methylated histone H3 relative to untreated cells (0.99 ± 0.51) following 18 h treatment (Figs 7*a* and *b*). This trend was not apparent following a 2 h treatment duration (AZA, 0.57 ± 0.39; TSA, 0.81 ± 0.34; VPA, 0.79 ± 0.23; UTX, 0.96 ± 0.2). However, neither the main effects of Treatment ($F_{3,24} < 1.0$) or Duration ($F_{1,24} < 1.0$), nor the

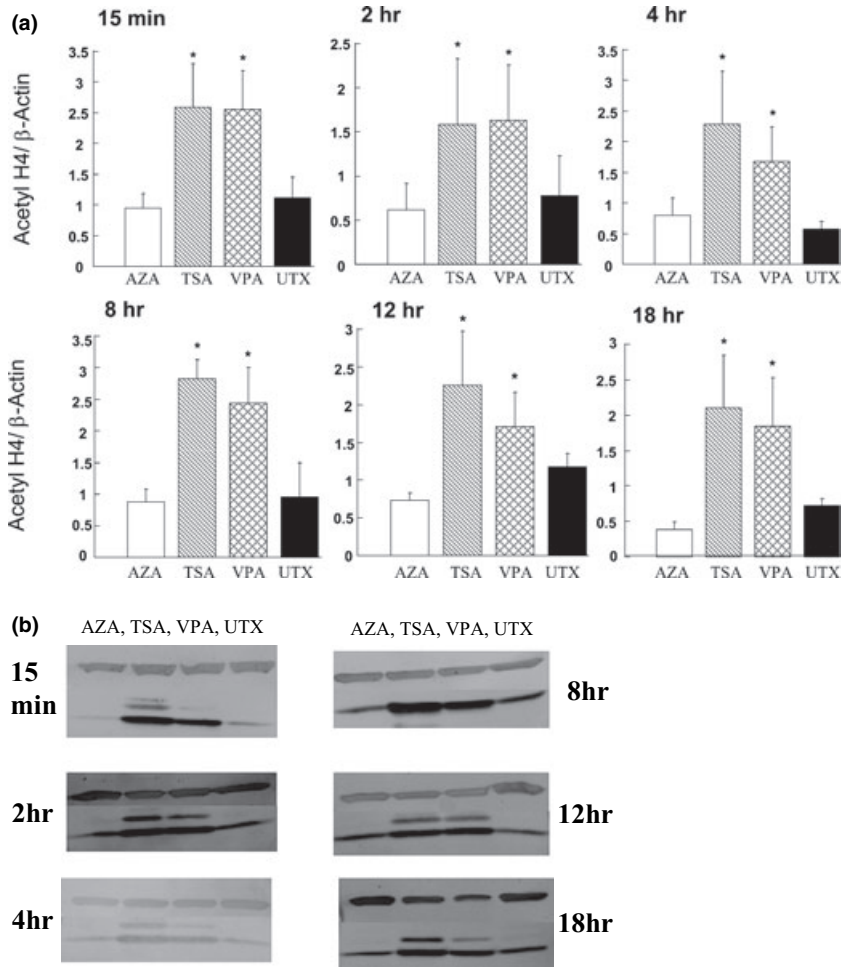

Fig. 4 Treatment of NT2 cells with TSA and VPA, but not AZA, rapidly increased acetylation of histone H4 relative to untreated cells at all timepoints. (a) Ratio of the area of the acetyl H4 band over the area of the β-actin band in protein samples harvested from NT2 cells following 15 min (upper left), 2 h (upper middle), 4 h (upper right), 8 h (bottom left), 12 h (bottom middle), or 18 h (bottom right) treatment with either AZA, TSA, or VPA, and UTX (each $n = 3$). Images were quantified with IMAGEQUANT software. *$p < 0.01$. (b) Representative western immunoblots of acetyl H4 (lower band) and β-actin (upper band) using protein samples harvested from NT2 cells following 15 min (upper left), 2 h (middle left), 4 h (bottom left), 8 h (upper right), 12 h (middle right), or 18 h (bottom right) treatment with either AZA, TSA, or VPA, and in untreated cells.

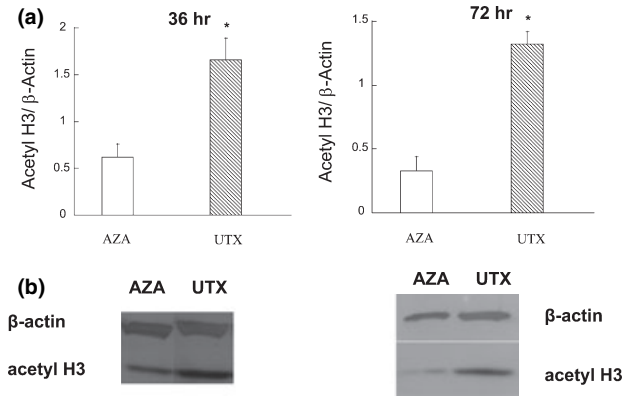

Fig. 5 Treatment of NT2 cells with AZA for extended durations decreased acetylation of histone H3 relative to untreated cells. (a) Ratio of the area of the acetyl H3 band over the area of the β-actin band in protein samples harvested from NT2 cells following 36 h (left) and 72 h (right) treatment with AZA and in untreated cells. *$p < 0.05$. (b) Representative western immunoblots of acetyl H3 (lower band) and β-actin (upper band) following 36 h (top) or 72 h (bottom) treatment with AZA and in untreated cells.

Treatment–Duration interaction approached significance ($F_{3,24} = 1.3$, $p > 0.05$).

Promoter specific histone acetylation changes accompany treatment of NT2 cells with TSA and VPA

Treatment of the neural progenitor cultures with either 5 mM VPA or 0.3 μM TSA for 24 h rapidly increased the extent of histone acetylation. Moreover, these changes also occurred in the vicinity of the *reelin* upstream sequences that have been defined previously to possess promoter activity (Chen *et al*. 2002). Chromosomal DNA was cross-linked to chromatin proteins with formaldehyde and the DNA was sheared using sonication. The cell lysates were treated independently with antibodies against the acetyl H3 and H4 histone proteins to immunoprecipitate chromatin and associated DNA. The cross-links were disrupted and the amount of *reelin* promoter fragments was detected using a nested PCR reaction. As shown in Fig. 8, VPA (5 mM, 24 h) increased the amount of acetyl H3 and acetyl H4 associated with the *reelin* promoter. TSA (0.3 μM, 24 h) produced a comparable increase in the amount of *reelin* promoter

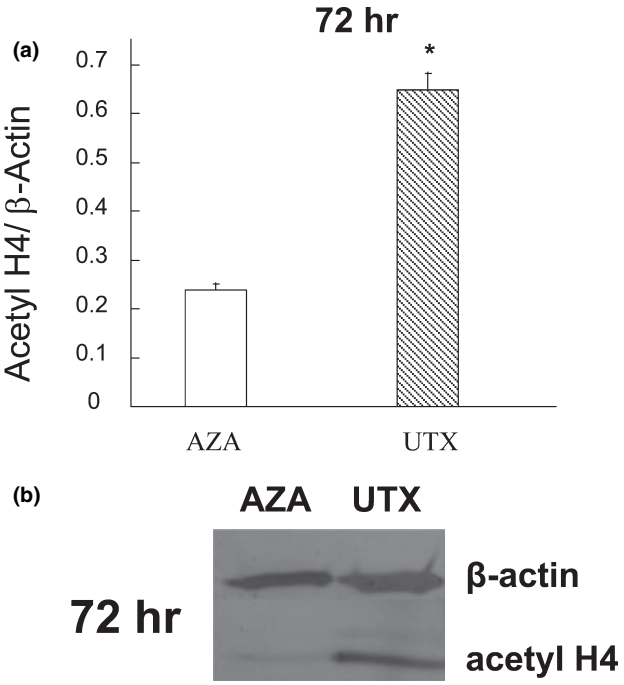

Fig. 6 Treatment of NT2 cells with AZA for 72 h decreased acetylation of histone H4 relative to untreated cells. (a) Ratio of the area of the acetyl H4 band over the area of the β-actin band in protein samples harvested from NT2 cells 72 h treatment with AZA and in untreated cells. *$p < 0.05$. (b) Representative western immunoblot of acetyl H4 (lower band) and β-actin (upper band) following 36 h treatment with AZA and in untreated cells.

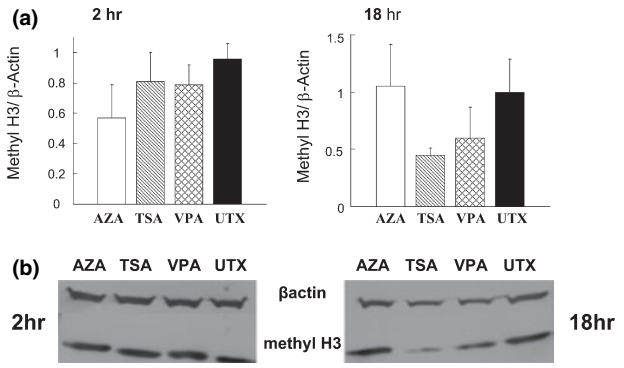

Fig. 7 Treatment of NT2 cells with TSA and VPA, but not AZA, produced a delayed decrease in the methylation of histone H3 relative to untreated cells. (a) Ratio of the area of the methyl H3 band over the area of the β-actin band in protein samples harvested from NT2 cells following 2 h (left) or 18 h (right) treatment with either AZA, TSA, or VPA, and in untreated cells (each $n = 3$). *$p < 0.01$. (b) Representative western immunoblots of methyl H3 (lower band) and β-actin (upper band) using protein samples harvested from NT2 cells following 2 h (left) or 18 h (right) treatment with either AZA, TSA, or VPA, and UTX.

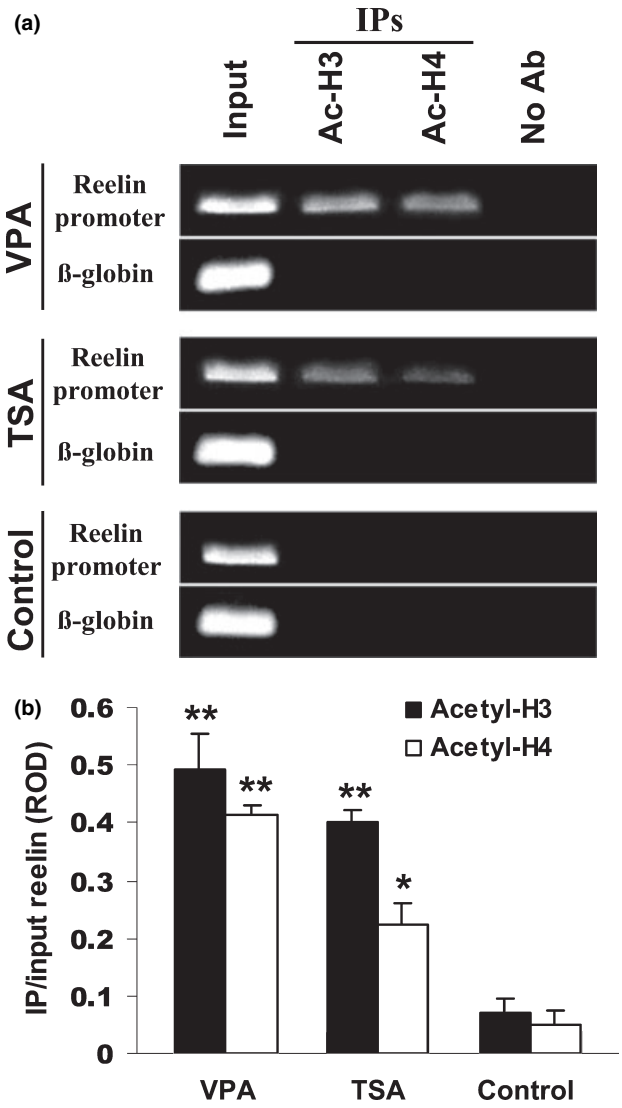

Fig. 8 Valproic acid and TSA induce acetylation of H3 and H4 histones located in the *reelin* promoter region. (a) Representative gels of the amplified *reelin* promoter region (507 bp band) and *β-globin* gene fragment (216 bp band) from non-immunoprecipitated input, samples immunoprecipitated with anti-acetyl histone H3 antibody (Ac-H3 IP) or anti-acetyl histone H4 antibody (Ac-H4 IP) and negative control (No antibody). (b) Bars show levels of *reelin* amplificaiton product immuneprecipitated using anti-acetylated histone H3 and H4 in 5 mM VPA treated-, 0.3 μM TSA treated- and vehicle treated (control) samples normalized to the corresponding DNA input levels. Data are presented as a ratio of relative optical densities (RODs) of the bands within the immunoprecipitated sample (IP) and input lanes derived from the ethidium bromide stained gels, mean ± SEM; *$p < 0.005$, **$p < 0.001$. Fold increase induced by each treatment expressed as a ratio was, for acetyl H3: (VPA/control, 7.1; TSA/control, 5.8), and for acetyl H4 (VPA/control, 8.6; TSA/control, 4.6).

associated with acetylated H3. In contrast, the effect of TSA on acetyl H3 was more robust as compared to that associated with acetylated histone H4. We used the *β-globin* locus control region as a negative control for changes in H3 and H4 acetylation status as this gene is not activated in NT2 cells in response to the inhibition of histone deacetlyation. Acetyl

H3 and acetyl H4 pull down assays with this promoter showed no increases associated with either treatment paradigm as would be predicted.

Discussion

This study demonstrates that the HDAC inhibitors, VPA and TSA, and the non-methylatable analog of cytosine, AZA, robustly decrease *reelin* promoter methylation in NT2 cells following treatment regimens that have been demonstrated to increase *reelin* mRNA expression in these cells (Chen *et al*. 2002). The lack of specificity of *reelin* promoter hypomethylation, i.e. the lack of changes in the methylation status of specific cytosines or transcription factor binding sites was somewhat surprising. However, we did observe a decrease in cytosine methylation within the functionally defined enhancer region (Chen *et al*. 2002) with all three treatments, a finding that provides support to the view that methylation may be a dominant mechanism regulating *reelin* expression *in vitro*. While it remains unclear as to whether an overall change in methylation of this region is sufficient to account for the observed results, we are now in the process of defining more precisely the location of binding sites for several transcription factors, including Tbr-1, Sp1 and Pax6, to examine whether methylation directly impacts transcription factor binding. A plausible alternative is that the overall methylation status of the *reelin* promoter determines local chromatin compaction which in turn affects transcription factor accessibility within the region. As anticipated, we also showed that treatment of NT2 cells with the HDAC inhibitors, TSA and VPA, produced marked increases in levels of acetylated histones H3 and H4. The observed changes occurred quite rapidly, with maximal increases in acetyl H3 and acetyl H4 occurring after 15 min of treatment. TSA and VPA also exhibited an insignificant tendency to decrease levels of methylated H3 after 18 h of treatment; this tendency was not apparent for the treatment lasting 2 h. Levels of acetyl H3, acetyl H4, and methyl H3 were not altered up to 18 h after treatment with AZA. In fact, longer treatment durations actually decreased acetyl H3 and acetyl H4. So, while AZA positively impacts *reelin* expression as we have shown previously (Chen *et al*. 2002) and produces a change in promoter methylation status, the mechanism may be somewhat different from TSA and VPA as these effects occur without comparable changes in the levels of the acetylated histones H3 and H4.

Interestingly, the VPA- and TSA-induced reduction of *reelin* promoter cytosine methylation following the treatment paradigms, which also increased *reelin* mRNA expression, suggest that inhibiting HDAC activity may, in effect, induce demethylation. From the studies reported here, it remains uncertain whether this effect is direct or indirect. However, consistent with this concept, others have suggested that the activity of DNA demethylases, such as MBD2, may be potentiated by histone deacetylase inhibition (Cervoni and Szyf 2001; Szyf 2001; Detich *et al*. 2002). It seems probable that there is a link between acetylated histone levels and cytosine methylation that might occur through changes in local chromatin conformation. VPA has been shown previously to decrease the methylation of an artificially methylated and transfected template in HEK293 cells with a comparable time frame as to what we have shown (Detich *et al*. 2003). Based on their studies, these authors concluded that VPA induced demethylation through the action of MBD2 as the decreased methylation was attenuated by an antisense inhibitor of MBD2. One main difference between that study and ours is that we have extended their findings to show that histone deacetylase inhibitors can alter the methylation status of an endogenous genomic promoter and that these changes are accompanied by increases in H3 and H4 histone acetylation which also map proximal to the functionally defined *reelin* promoter. Moreover, we had established previously that the treatment correlates with the increased expression of the corresponding mRNA (Chen *et al*. 2002).

We hypothesized previously that the increased *reelin* mRNA expression produced by AZA (Chen *et al*. 2002) probably occurs through decreasing promoter methylation, thereby reducing the recruitment of HDACs by methyl binding proteins. However, the finding that AZA reduced *reelin* promoter cytosine methylation following a treatment paradigm which, in parallel, increased *reelin* mRNA expression (Chen *et al*. 2002), and that AZA did not increase histone acetylation, suggests that the reduction in promoter cytosine methylation is sufficient to increase mRNA expression without altering histone acetylation. It remains a possibility that the decrease in methylation levels within the promoter/enhancer region causes a decrease in the association of methyl CpG binding proteins in the promoter and opens the chromatin to transcription factors in the absence of histone deacetylation per se. The decrease in histone acetylation that we observed with longer treatments of AZA was unexpected but may reflect a compensatory mechanism to control gene expression. Alternatively, this may reflect a subtle, untoward effect of AZA which was not observed at the cellular level.

Taken together, the current finding, along with our previous data, suggests that *reelin* mRNA expression is regulated by an interplay between cytosine methylation within the promoter region and changes in chromatin configuration modulated by histone acetylation, and that these mechanisms can be influenced therapeutically. Superimposed upon this regulation is the interaction of various transcription factors such as Tbr-1, Pax 6 and Sp1 which interact to positively modulate *reelin* transcription (Y. Chen, unpublished data). The extent to which methylation within these binding regions interrupts transcription factor recognition is being assessed, although an initial inspection of the maps (Fig. 2) suggests that a more global change in

methylation may be the dominant effect. Consistent with this possibility, we have demonstrated that treatment of mice with methionine concomitantly decreases *reelin* mRNA expression and increases methylation of *reelin* promoter cytosines in the frontal cortex of treated animals (Tremolizzo *et al*. 2002). Administration of VPA increases cortical expression of *reelin* mRNA, antagonizes down-regulation of *reelin* mRNA and cytosine hypermethylation within the *reelin* promoter induced by methionine, and increases histone H3 acetylation in frontal cortex (Royaux *et al*. 1997; Tremolizzo *et al*., unpublished observation). However, the observed changes in methylation do not appear to be site specific as originally anticipated. Additionally, the increase in *reelin* mRNA levels produced by differentiation of NT2 cells to hNT neurons by retinoic acid is associated with a decrease in cytosine methylation within the *reelin* promoter and an increase in DNAse I hypersensitive sites, suggesting that the opening of the chromatin in the vicinity of the promoter may be rate limiting (Chen *et al*. 2002).

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