Regulation of the Human \(O^6\)-Methylguanine-DNA Methyltransferase Gene by Transcriptional Coactivators cAMP Response Element-binding Protein-binding Protein and p300*

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\(O^6\)-Methylguanine-DNA methyltransferase (MGMT), a ubiquitous DNA repair protein, removes \(O^6\)-alkylguanine from DNA, including cytotoxic \(O^6\)-chloroethylguanine induced by chemotherapeutic \(N\)-alkyl \(N\)-nitrosourea-type drugs, e.g. 1,3-bis(2-chloroethyl)-1-nitrosourea. Treating the pancreatic carcinoma cell line MIA PaCa-2 with trichostatin A (TSA), a specific inhibitor of histone deacetylase, increased MGMT mRNA and protein levels by 2–3-fold. Surprisingly, TSA treatment increased MGMT promoter-dependent luciferase activity by some 40-fold in a transient reporter expression assay. Deletion and point mutation analysis showed that two AP-1 binding sites in the MGMT promoter are involved in activation by TSA. Ectopic expression of the transcriptional coactivators cAMP response element-binding protein-binding protein (CBP) and p300, which have intrinsic histone acetyltransferase activity, enhanced luciferase expression. Overexpression of adenovirus E1A, which binds CBP/p300, strongly inhibited both basal and TSA-inducible MGMT promoter activity, while a mutant E1A, defective in binding CBP/p300, did not. Chromatin immunoprecipitation assays revealed that TSA treatment increased histone acetylation in the endogenous MGMT promoter region, which also showed association with CBP/p300. Taken together, our results indicate that targeted histone acetylation results in the remodeling of chromatin by recruitment of the coactivator CBP/p300, and constitutes an important step in regulating MGMT expression.

Antitumor alkylating drugs of the 2-haloethyl-N-nitrosourea class, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), induce \(O^6\)-chloroethylguanine in DNA, which in a second reaction forms DNA cross-links, the ultimate cytotoxic lesion (1). \(O^6\)-Methylguanine-DNA methyltransferase (MGMT), a ubiquitous DNA repair protein, repairs the mutagenic, carcinogenic and cytotoxic \(O^6\)-alkylguanine adducts including the primary alkyl adducts induced by alkylnitrosoureas (2, 3). MGMT acts by transferring the \(O^6\)-alkyl group to a specific cysteine residue within its own sequence in a single step, stoichiometric reaction (4). This transfer irreversibly inactivates MGMT. Hence, MGMT is a major contributor to cellular protection from the mutagenic, carcinogenic, and cytotoxic effects of DNA-alkylating agents. MGMT expression is highly variable in normal tissues as well as in tumor cells (5, 6). A fraction of primary tumor cells, and 20% of human tumor cell lines, lack expression of MGMT (7, 8). These MGMT-defective (Mex−) cell lines are highly sensitive to alkylating agents and nitrosourea-type drugs (8). Conversely, some tumor cells express MGMT at a high level and are highly resistant to chemotherapy with BCNU (9). Thus, elucidating the molecular mechanisms controlling MGMT expression is of major clinical significance.

The MGMT gene encoding an mRNA of 950 nucleotides consists of five exons, and spans more than 170 kilobase pairs (10, 11). The 5′-regulatory sequence (including its promoter) has been cloned (12). The promoter is extremely GC-rich, and lacks both TATA and CAAT boxes. Several cis elements were identified, including six putative Sp1 sites within the CpG island, two glucocorticoid-responsive elements (GRE), and two each of putative AP-1 and AP-2 elements (12). The potential function of each of the GRE and AP-1 sites in activation of MGMT has been investigated previously (13, 14). However, the molecular basis for the lack of expression of MGMT in Mex− cell lines, in which no deletion or gross rearrangement in the gene was observed, is not understood (15). Reporter gene expression driven by the MGMT promoter indicates that Mex− cells do not lack necessary trans-acting factors (16). This suggests that gene silencing results from modification of cis elements, by mechanisms such as CpG methylation (17) and/or chromatin alteration (18).

Recent studies have established that chromatin remodeling via histone modifying enzymes, namely histone acetyltransferase (HAT) and histone deacetylase (HDAC), is involved in transcriptional activation and repression, respectively (19, 20). It has been proposed that acetylation of the \(\varepsilon\)-amino group of lysine residues at the \(NH_2\)-terminal domain of histones promotes destabilization of histone-DNA interaction in the nucleosome, resulting in increased accessibility of the open chromatin to the transcriptional machinery (19), while histone deacetylase reverses this process by removing the acetyl groups, and represses transcription (20). In agreement with this hypothesis, several studies demonstrated enrichment of hyperacetylated histones within the transcriptionally active/competent chromatin in vivo, and hypoacetylated histones were shown to be concentrated in transcriptionally silenced domains (19, 21).

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1 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU); CBP, cAMP response element-binding protein-binding protein; CHIP, chromatin immunoprecipitation; HAT, histone acetyltransferase; HDAC, histone deacetylase; MGMT, \(O^6\)-methylguanine-DNA methyltransferase; ML, MGMT-luciferase; PCAF, p300/CBP-associated factor; TSA, trichostatin A; GRE, glucocorticoid-responsive element; PAGE, polyacrylamide gel electrophoresis.
Trichostatin A (TSA), a specific and potent inhibitor of histone deacetylase, modulates expression of only 2% of cellular genes, which implies that acetylation may be targeted to specific genes or chromosomal domains (22, 23). A simple but attractive hypothesis is that targeted histone acetylation is achieved by recruitment of acetyltransferase to the signal-responsive promoters. Strong support for this idea has been provided by the recent observations that transcription cofactors, including CBP, p300, P60, GCN5, ACTA, SRC-1, and TAFII 250 subunits of TFIIID have intrinsic HAT activity, and are recruited to the promoter region in a signal-dependent process (for review, see Ref. 24).

The transcriptional coactivators CBP and p300, originally identified as adenovirus E1A-binding proteins (25), have long been recognized as key molecules for gene regulation by communicating between transcription factors and the basal transcription machinery. CBP and p300 are functional homologues and henceforth referred to as CBP/p300 (26). CBP/p300 does not by itself interact with a specific DNA sequence; instead, it interacts with multiple transcription factors including AP-1 via dedicated domains, and forms multiprotein complexes, named “enhosomes” (27–29). The functional requirements for the HAT activities of CBP/p300 (30) and P60 have recently been examined, along with their roles in regulation of differentiation, transcription activation, and signaling pathways (21).

By examining the contribution of chromatin remodeling to transcriptional regulation of the human MGMT gene, we show in this report that histone hyperacetylation activates MGMT gene in MIA PaCa-2 cells, and transcriptional co-activator CBP/p300 is involved in both basal and TSA-induced, AP-1-mediated MGMT promoter activation. We also discuss the possible mechanism for the remodeling of chromatin structure of the MGMT gene that is needed to regulate its expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**MIA PaCa-2 (ATCC CRL-1420) cells were grown at 37 °C in high glucose Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 1 mM sodium pyruvate (Sigma), and penicillin (100 units/ml) and streptomycin (100 μg/ml). Trichostatin A (TSA) was purchased from Biomol (Plymouth Meeting, PA).

**Northern Analysis—**After extraction of total cellular RNA with RNAzol (Tel-Test, Inc.), 50 μg of RNA/lane was electrophoresed on a 1% agarose gel, and then transferred onto nitrocellulose membranes (Schleicher & Schuell) by capillary electrophoresis (31), and then hybridized with 32P-labeled MGMT cDNA or 18 S rRNA as probe. The MGMT cDNA probe was an EcoRI fragment of pRT100 (10). Both prehybridization and hybridization were carried out at 65 °C with QuickHyb hybridization solution (Stratagene), and the membrane was subsequently washed according to the manufacturer’s protocol. The signal intensity was quantified by ImageQuant (Molecular Dynamics).

**Immunoblot Analysis—**TSA-treated or untreated cells were lysed by incubating with lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 100 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Molecular Biochemicals), and sonicated 25 times for 10 s at 0 °C. After clarification of the lysates by centrifugation, 0.1 ml of supernatant containing solubilized chromatin was diluted 10-fold with dilution buffer containing 0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0. To reduce nonspecific background, the diluted chromatin solution was shaken for 90 min at 4 °C with 60 μl of protein A-agarose slurry as 50% suspension in 10 mM Tris-HCl, pH 8.0, 1.0 ml EDTA (TE), which was pretreated with salmon sperm DNA.

For immunoprecipitation, the treated chromatin solution was incubated overnight at 4 °C with 5 μg of anti-acetylated histone H4 antibody. The immunocomplex was then purified by binding to 60 μl of protein A-agarose slurry as before. After incubation for 1 h at 4 °C, the agarose beads were collected by centrifugation, sequentially washed twice with dilution buffer, once with dilution buffer containing 500 mM NaCl, and once with a buffer containing 0.25% LcI, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1. Finally the beads were washed with TE, and the complexes eluted with two 250-μl aliquots of elution buffer (1% SDS, 0.1 mM NaHCO3) at room temperature for 3 h. The pooled eluates were heated to 65 °C for 15 min to reverse the formaldehyde cross-links and then treated with proteinase K for 3 h. The DNA was subsequently extracted with phenol/chloroform, precipitated with ethanol, and the precipitate dissolved in 20 μl of TE.

PCR amplification of DNA was carried out with diluted aliquots, using oligonucleotides 5′-GCTCCAGGGAAAGTCTTCCTGCCTCC and 5′-GGCTCTGGGTTGCGGAGTTCGCCAG as 5′ and 3′ primers, respectively, encompassing the two AP-1 sites in the MGMT promoter region. To ensure that PCR amplification was in the linear range, the PCR products with different dilutions of input DNA were quantitated. The PCR products were separated by agarose gel electrophoresis and their sequences confirmed directly. In some experiments, cells were transfected with MGMT promoter-reporter (p−954/+24ML) and the CHIP assay was performed as before. In this case the 5′ primer for PCR, 5′−GCCCTTGTTGCGGTCCGTCGTC, was degenerated at 9′ to 3′ of the MGMT promoter and the 5′ primer 5′−TGTATCTTATGGTACTGTAA−CTG to a sequence in the pGL2 basic vector. For PCR for non-coding region of the pGL2 basic vector, the 5′ primer was 5′−GGTAAATCGCGTTCCACAGAAT and the 3′ primer was 5′−GGTACAGTTGGCGTGCAGTGCAGTGGC.

**Transient Transfection and Plasmids—**Exponentially growing MIA PaCa-2 cells (5 × 106/dish) were suspended in 300 ml of PBS, electroporated at 960 microfarads and 220 V using a Gene Pulser (Bio-Rad), and transferred back to dishes containing culture medium. The medium was replaced with fresh medium containing TSA or ethanol, at 24 h after transfection. After TSA treatment for another 24 h, the cells were harvested and lysed with Reporter Lysis Buffer (Promega). The luciferase activity was measured in a luminometer using the luciferase assay kit (Promega). The luciferase activity was normalized with respect to the protein concentration of the lysate. In some experiments, 2 μg of β-galactosidase expression plasmid pCMVβ (CLONTECH) was included in the transfection procedure, so that β-gal activity could be used to correct for variation in transfection efficiency. The following plasmids were used in the transfection assays. All the deletion constructs of MGMT-luciferase (p−725/+24ML, p−575/+24ML, p−954/+24ML, and p−3500/+24ML) cloned in pGL2 basic vector (Promega) have been described previously (13). Site-directed mutation of two AP-1 sites in p−954/+24ML reporter plasmid was described by Boldogh et al. (14). pCdNA3 (Invitrogen), pG2-3L control vector (Promega), and pCMVβ (CLONTECH) were used as controls. pCMV-E1A encoding adenovirus E1A 12 S protein and mutant Δ2–36 E1A were kind gifts of Dr. P. K. Roychoudhury (University of Illinois, Chicago, IL). pCR/RSV mCBP encoding the full-length mouse CBP was generously provided by Dr. R. H. Goodman (Oregon Health Science University, Portland, OR). pCMV-p300 was obtained from Dr. S. Grossman (Dana Farber Cancer Research Institute, Boston, MA). pCI-PCAF encoding the human PCAF and pCAF antibody were kind gifts of Dr. Y. Nakatani (National Institutes of Health, Bethesda, MD).

**RESULTS**

**Activation of MGMT by TSA—**To investigate whether chromatin remodeling via histone acetylation plays a regulatory role in MGMT expression, we examined the effect of TSA, a...
specific histone deacetylase inhibitor, on MGMT expression. Northern blot analysis showed that the level of MGMT mRNA, when normalized with respect to the level of 18S rRNA, was approximately 3-fold higher at 24 h after treatment with 100 ng/ml TSA (Fig. 1A). Concomitantly, we observed approximately a 3.5-fold increase in the MGMT protein level by Western blot analysis (Fig. 1B). Time course studies on the MGMT protein level showed that the increased level of the MGMT protein could be detected after 16 h of TSA treatment, with the protein level reaching the maximum by 24 h (Fig. 1C). These results raised the possibility that inhibition of histone deacetylation was responsible for activating the MGMT gene. Because TSA was shown earlier to arrest cell cycle progression in some cell lines in the G1 or G2/M phase (33), we used fluorescence-activated cell sorting to investigate the effects of various amounts of TSA on cell cycle progression of MIA PaCa-2 cells. TSA at 100 ng/ml had a negligible effect on cell cycle progression in this cell line (Table I). Consequently, we used 100 ng/ml TSA for MGMT activation in all subsequent studies.

**Activation of the MGMT Promoter by TSA**—Because earlier studies indicated that the primary effect of inhibiting histone deacetylation is to modulate transcription of a subset of genes (23), we determined whether TSA-mediated activation of MGMT occurred at the promoter level. An MGMT promoter-luciferase reporter construct (p−954/+24ML), containing the MGMT promoter sequence from −954 to +24 base pairs (13), was used for transient expression of luciferase reporter in transfected MIA PaCa-2 cells as described under “Experimental Procedures.” SV40 promoter-dependent luciferase expression plasmid was used as the control. TSA (100 ng/ml) activated MGMT promoter-driven luciferase expression by some 40-fold. Some promoter activation (−4-fold) could be detected with TSA concentration as low as 20 ng/ml. We also observed a ∼5-fold increase in luciferase activity driven by the SV40 promoter, consistent with an earlier observation (34). A time-course study on reporter expression with 100 ng/ml TSA showed that activation of luciferase could be detected after 4 h of TSA treatment, and the enzyme activity reached the maximum after approximately 24 h.

**Two AP-1 Binding Elements Are Necessary for Promoter Activation by TSA**—To identify the factors that are responsible for TSA-induced activation of the MGMT promoter, we carried out promoter deletion and mutagenesis analysis. MIA PaCa-2 cells were transiently transfected with a series of 5’ promoter deletion constructs, treated with TSA, and the luciferase activity then measured with cell-free extracts. A 10-fold activation of the promoter activity by TSA was observed with the minimal promoter reporter construct (p−72/+24ML), whereas the maximum induction (∼45-fold) was observed with the (p−954/+24ML) promoter reporter (Table II). Interestingly, only a ∼25-fold increase was observed after TSA treatment with the longer, p−3500/+24ML, reporter construct. This result suggests the presence of a negative regulatory element in the upstream (−3500 to −954 bp) region of the MGMT promoter. The presence of a similar negative regulatory element was also observed in TSA-mediated activation of p21/WAF1 promoter (35). Deletion of the promoter sequence from −954 to −575 base pairs decreased TSA-mediated induction by 2.5-fold. Further deletion of the sequence to position −72 decreased TSA-mediated induction by 4-fold. In any case, we conclude from these data that the cis elements required for promoter activation by TSA are present within the −72 bp region and between −575 and −954 bp upstream of the transcription start site.

Examination of the sequence within the −72 bp region revealed the presence of three Sp1-binding sites. Earlier in vivo footprinting studies showed DNA protein interaction at six Sp1 sites, including two sites, in an MGMT-expressing cell line (18). Examination of the sequence between −954 and −575 bp revealed several transcription factor-binding sites (12), including two AP-1 sites and two GREs. The AP-1 sites and GREs were previously shown to be involved in activation of MGMT with phorbol ester and dexamethasone, respectively (13, 14).

To determine which, if any, of these two AP-1-binding elements are required for TSA-mediated induction, cells were transfected with MGMT promoter-luciferase constructs in which both AP-1 sites were mutated, individually or simultaneously. Mutation of both AP-1 sites reduced the basal activity by 2–3-fold (data not shown). However, as shown in Table II, mutation of either AP-1 site reduced TSA-mediated activation from ∼45-fold to ∼9-fold. Mutation of both AP-1 sites caused no additional reduction of activation over that observed with single mutations. Deletion of the two GREs had no effect on TSA-mediated induction (data not shown). These data indicate that two AP-1 sites but not GREs are involved in MGMT promoter activation by TSA.

**Inhibition of MGMT Promoter Activity by Adenovirus E1A**—Because E1A (27, 28) and possesses HAT activity (30), we considered the possibility that TSA-induced MGMT promoter activation resulted from interaction between the AP-1 transcription factor and histone acetyltransferase or deacetylase. In the course of our earlier observation that CBP/p300 interacts with AP-1 proteins in vivo (27, 28) and possesses HAT activity (30), it appeared that CBP/p300 could act as a coactivator in MGMT promoter expression. We tested this by investigating the effect

![Fig. 1. TSA-mediated activation of MGMT mRNA and protein in MIA PaCa-2 cells.](image-url)
TABLE II
Deletion and mutational analysis of MGMT promoter in reporter assay for TSA response

| MGMT-promoter reporter* | Increase of luciferase activity ± S.D. after treatment with TSA |
|-------------------------|------------------------------------------------------------|
| p-72/+24ML              | 10.3 ± 1.5                                                  |
| p-575/+24ML             | 19.0 ± 2.6                                                  |
| p-954/+24ML             | 45.0 ± 6.2                                                  |
| p-3500/+24ML            | 25.5 ± 3.5                                                  |
| p-954/+24ML (AP-1(1)mut)| 9.0 ± 1.1                                                   |
| p-954/+24ML (AP-1(2)mut)| 7.9 ± 1.0                                                   |
| p-954/+24ML (AP-1(1,2)mut)| 12.2 ± 1.3                                               |

*The plasmid constructs were described earlier (13, 14). The numbers in the plasmid names refer to the position of the first and last nucleotides of the MGMT promoter relative to the transcription start site.

of the adenovirus E1A protein, which binds to the CH3 domain of CBP/p300 and abolishes its coactivator function (28, 36). Cotransfection of cells with a fixed amount of MGMT promoter-luciferase reporter plasmid and varying amounts of an E1A expression plasmid showed strong inhibition of luciferase expression in an E1A dose-dependent manner, and a maximum of 10-fold reduction was observed with 5 μg of E1A plasmid (Fig. 2A). Because E1A binds to multiple regulatory proteins, including the retinoblastoma gene product Rb (37), the possibility remained that E1A inhibited MGMT promoter activity by binding to other factors needed for MGMT activation, in addition to CBP/p300. To test this, we cotransfected cells with an MGMT promoter-reporter construct and an expression vector for amino-terminal deletion, Δ2–36 E1A, which was shown to be defective in CBP/p300 binding but capable of binding to members of the Rb protein family (38). As shown in Fig. 2B, mutant Δ2–36 E1A did not inhibit MGMT promoter-luciferase expression; rather, an increase in promoter activity was reproducibly observed. Taken together, these results suggest that transcriptional coactivator CBP/p300 is involved in MGMT promoter expression.

E1A Inhibits TSA-mediated MGMT Activation—Recent reports indicate that E1A directly represses the histone acetyltransferase activity of both CBP/p300 and its associated factor PCAF in vitro and during p300-dependent transcription in vivo (39). We therefore asked whether the HAT activity associated with CBP/p300 or the CBP/p300-PCAF complex was responsible for TSA-mediated activation of the MGMT promoter. We cotransfected cells with 15 μg of the (p-954/+24ML) MGMT promoter construct and different amounts of the E1A expression vector. Twenty-four h after transfection, the cells were treated with TSA or ethanol for another 24 h. As shown in Fig. 3, E1A inhibited TSA-mediated induction of the MGMT promoter in a dose-dependent fashion. These data provide the first in vivo evidence for TSA-mediated activation of a natural promoter in a reporter plasmid, which could be inhibited by E1A.

Overexpression of p300 and CBP Enhanced MGMT Promoter Activity and Potentiated Transactivation with TSA—To provide direct evidence for involvement of CBP/p300 in MGMT promoter activation, we examined the effect of overexpression of p300 and/or CBP on reporter activity. Ectopic expression of full-length human p300 increased MGMT promoter-driven luciferase activity by 5-fold (Fig. 4A). Similarly, overexpression of full-length mouse CBP enhanced MGMT promoter-driven luciferase activity by 2-fold (Fig. 4B). Furthermore, ectopic expression of p300 or CBP had a synergistic effect with TSA (Fig. 4, A and B).

Co-expression of CBP and its associated factor PCAF, which also possesses HAT activity, did not show a significant additional synergistic effect with TSA (data not shown). To show more directly that the E1A-mediated inhibition of MGMT promoter activity was due to squelching of CBP/p300, we asked whether overexpression of CBP/p300 could restore the promoter activity in the presence of E1A. Ectopic expression of
p300 completely reversed the inhibition of MGMT promoter activity with E1A, suggesting that this inhibition was indeed due to titration of a limiting amount of endogenous CBP/p300 (Fig. 4C). These data provide further support for involvement of CBP/p300 in MGMT promoter activation.

**Inhibition of the Minimal MGMT Promoter by E1A—TSA-mediated enhancement of MGMT minimal promoter-driven luciferase expression (Table II) raised the possibility that CBP/p300 is involved in the function of the MGMT minimal promoter as well. We tested this by cotransfecting cells with a fixed amount of (p−72/+24ML) promoter reporter plasmid and increasing amount of wild type or mutant E1A expression plasmid. Fig. 5A shows that wild type E1A strongly inhibited MGMT promoter activity, while mutant E1A did not. In fact, the mutant protein had a stimulatory effect on the promoter activity as was also observed with the longer promoter (p−954/+24ML) (Fig. 2B). Moreover, ectopic expression of p300 enhanced the minimal promoter-dependent luciferase activity (Fig. 5B). The −72 bp minimal promoter region is highly GC-rich, and three Sp1-binding sites in this segment were previously shown to be functional in vivo (18). Thus, it appears that the transcriptional co-activator CBP/p300 is also required for the minimal basal promoter activity of the MGMT gene, which acts presumably by recruiting Sp1 transcription factors, or by directly interacting with the basal transcription machinery.

**TSA Caused Accumulation of Acetylated Histones in MIA PaCa-2 Cells—TSA was shown to cause accumulation of acetylated histone species in various mammalian cell lines, which could be separated by AUT gel electrophoresis (22). We investigated the effect of TSA (100 ng/ml) on histone acetylation in MIA PaCa-2 cells by analyzing histones from TSA-treated or untreated cells. Fig. 6A shows that higher levels of tri- and tetra-acetylated forms of H4 and H2B histones were present in the TSA-treated cells relative to the control. Hyperacetylated histone H4 plays a critical role in enhancing the binding of
transcription factors to nucleosomal DNA in vitro (40). Therefore, to determine the abundance of hyperacetylated histone H4 after TSA treatment, immunoblot analysis was carried out using anti-acetylated histone H4 antibody which recognizes tri- and tetra-acetylated isoform of histone H4. As shown in Fig. 6B, TSA treatment caused a significant increase in the level of hyperacetylated histone H4 in these cells.

**Effect of TSA Treatment on the Abundance of Hyperacetylated Histone H4 Bound to the MGMT Promoter in Vivo**—The observation that TSA modulates expression of only 2% of cellular genes implies that histone acetylation is targeted to specific genes or chromosomal domains (23), and that such targeting is achieved by recruitment of HAT to the signal-responsive promoters. In order to show that TSA specifically increased acetylation of histones bound to the MGMT promoter, we utilized chromatin immunoprecipitation (CHIP) assay as outlined in Fig. 7A. MIA PaCa-2 cells were treated with TSA for 9 h, and then the cells on the dish were treated with formaldehyde and the fragmented chromatin was isolated and immunoprecipitated with anti-acetylated histone H4 antibody. PCR amplification of an MGMT promoter sequence was carried out with DNA extracted from the immunocomplex. Fig. 7B shows that the MGMT promoter sequence was significantly enriched (3–4-fold) in the immunocomplex containing hyperacetylated H4 histone from TSA-treated cells (lane 3) compared with that from the control cells (lane 6). Appropriate controls used in this experiment provided further support for this observation. Thus, little or no MGMT promoter sequence was detected by the PCR assay in the absence of anti-acetylated histone H4 antibody (lane 7). Similarly, enrichment of the MGMT promoter sequence was not observed when a nonspecific antibody was used (lane 4). Furthermore, as expected, no PCR product was observed when the formaldehyde cross-linking step was omitted (lane 5). In order to establish that TSA treatment enhanced the level of hyperacetylated histones selectively bound to the MGMT promoter rather than to the MGMT gene as a whole, we used the PCR assay to determine the relative amounts of the MGMT promoter sequence and exon 2 sequence in the hyperacetylated histone H4 immunocomplex. No difference was found in the abundance of hyperacetylated histone H4 in the MGMT exon 2 region before and after TSA treatment (lanes 8 and 9). We extended this study to analyze the acetylation status of nucleosomes formed at the MGMT promoter sequence of the transfected plasmid molecules. Chromatin immunoprecipitation was carried out using MIA PaCa-2 cells transfected with MGMT promoter-reporter (p–954/+24ML). The MGMT promoter was again found to be selectively associated with acetylated histone H4 (Fig. 7C, lane 4), and TSA treatment caused a significant increase in the amount of acetylated histone H4 associated with this promoter (Fig. 7C, lane 6). No significant change in the level of acetylated histone H4 in the non-coding region of MGMT promoter-reporter was observed as a result of TSA treatment (lanes 13 and 14). These results indicate that the transfected MGMT promoter was preferentially associated with hyperacetylated histone H4 in the presence of TSA.

To provide evidence that TSA-mediated activation of MGMT promoter involved the AP-1 sites, we carried out CHIP assay with MIA PaCa-2 cells transfected with MGMT promoter-reporter (p–954/+24ML) containing mutated AP-1 sites. As shown in Fig. 7C, TSA treatment did not increase significantly the level of acetylated histone H4 when the two AP-1 elements were mutated (lane 10). We, therefore, propose that AP-1 binding elements are essential for TSA-induced association of acetylated histones with the MGMT promoter.

**Association of p300 with MGMT Promoter**—The results of the CHIP assay are consistent with the scenario that TSA increased the level of hyperacetylated histones bound to the MGMT promoter in both endogenous and episomal states, and that such acetylation was mediated by the HAT activity of CBP/p300. It was thus important to establish that CBP/p300 is indeed associated with the MGMT promoter sequence in vivo. We transfected MIA PaCa-2 cells with the (p–954/+24ML) reporter plasmid. After formaldehyde cross-linking followed by chromatin isolation, we isolated immunocomplex by adding anti-human p300 antisera. After reversal of cross-links and DNA extraction from the immunocomplex, a 460-bp segment of the MGMT promoter was amplified by PCR. Fig. 7D shows that the MGMT promoter sequence was selectively enriched by treatment with anti-p300 antibody and not with nonspecific
sera. These results suggest that p300 is normally associated with the MGMT promoter, at least in the episomal state.

**DISCUSSION**

This report provides the first evidence that histone acetylation plays a role in MGMT expression, because both endogenous MGMT gene expression and MGMT promoter-driven reporter expression were enhanced by TSA, a histone deacetylase inhibitor. However, it was somewhat unexpected that TSA activated the MGMT promoter in the episomal state to a much greater extent than the promoter of the chromosomal MGMT gene. It was previously shown that transfected plasmids interact with histones to form nucleosome-like structures so that inhibiting histone deacetylase activity could activate transcription from such plasmids (35, 41). A possible explanation for large activation of the episomal MGMT promoter by TSA inhibitor is that even though the nucleosome structure of transfected genes may not be normal (42), histone association is still required for transcription from their promoters. Furthermore, acetylation of histones could result in a more open chromatin structure in the episomal gene promoters than in the chromosomal promoters. The large magnitude of activation due to the inhibition of histone acetylation may also reflect a gene dosage effect, due to the large copy number of episomal promoters compared with two copies in the chromosomal DNA. It is also possible that many transfected plasmid molecules form complexes with histones in a closed form not suitable for transcription until the histones are acetylated.

Our earlier studies showed that two AP-1 sites in the MGMT promoter are involved in its activation by phorbol esters (14). The present study showing that these AP-1 sites are also involved in TSA-mediated activation of the MGMT promoter suggests that the cofactors recruited at these sites possess HAT activity. This would result in targeted acetylation of histones, leading to loosening of the nucleosome structure in the promoter region which, in turn would facilitate binding of AP-1 and Sp1 transcription factors. This possibility is supported by the observation that ectopic expression of the transcriptional coactivator CBP/p300 at which other key regulatory proteins interact, the help of Drs. T. Biswas, T. K. Hazra, and I. Boldogh in this study, and we thank Wanda Smith for secretarial assistance and Dr. David Konkel for critically reading the manuscript.

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