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Histone Deacetylase Inhibitor Activates the WAF1/Cip1 Gene Promoter through the Sp1 Sites

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Treatment of cultured cells with trichostatin A (TSA), a specific histone deacetylase inhibitor, induces the histone hyperacetylation and modulates expression of some mammalian genes. We examined the effects of TSA on cell growth arrest, and its relation to expression of the WAF1/Cip1 gene, a potent inhibitor of cyclin-dependent kinases, in a p53-mutated human osteosarcoma cell line MG63. TSA at 500 ng/ml induced growth arrest at both G1 and G2/M phases, and the expressions of the WAF1/Cip1 mRNA and protein. We also examined the changes of acetylated isoforms of histone H4. Dose-response and kinetic analysis suggest a close correlation between the level of histone acetylation and the induction of the WAF1/Cip1 expressions. Using several mutant WAF1/Cip1 promoter fragments, we found that the TSA responsive elements are two Sp1 sites at −82 and −69 relative to the transcription start site. These findings indicate that TSA induces the WAF1/Cip1 promoter through the typical Sp1 sites, in a p53-independent fashion. Furthermore, the Sp1-luc plasmid, containing SV40 promoter-derived three consensus Sp1 binding sites, was markedly activated by TSA, compared to the mutant Sp1-luc plasmid. These results demonstrate that transcriptional activation through the Sp1 sites of the WAF1/Cip1 promoter by TSA coincides with induced hyperacetylation of histone H4.

WAF1/Cip1 protein potently inhibits the various cyclin-dependent kinases (1 - 3), which regulate cell cycle, thereby supposedly inducing the cell cycle arrest (1, 4). WAF1/Cip1 was first identified as a p53-inducible gene (5), but more recently its induction was shown to occur via p53-independent mechanisms in various cell lines stimulated for differentiation and growth arrest (6 - 14). We have recently shown that the pleitoropic agent sodium butyrate inhibited proliferation and induced WAF1/Cip1 expressions in p53-negative human cell lines WiDr and MG63, derived from colon cancer and osteosarcoma, respectively (6). Although butyrate is known to be pleitoropic, it has been suggested that it is a rather unspecific inhibitor of histone deacetylase, also affecting some other enzymes (15). In comparison, trichostatin A (TSA); which differs in chemical structure is antimycotic, and induces differentiation in Friend leukemia cells; was recently shown to be a specific inhibitor of histone deacetylase at much lower concentrations, *in vivo* and *in vitro* (16). To elucidate the role of histone hyperacetylation in the regulation of WAF1/Cip1 gene expression, we examined the effects of TSA on the expression of WAF1/Cip1 in MG63 cells.

Our results demonstrate that hyperacetylated histone H4 and expression of WAF1/Cip1 are induced upon TSA-treatment in MG63. When using a series of mutant WAF1/Cip1 promoter constructs, we found that two Sp1 sites at −82 and −69 relative to the transcription start site are involved in the activation of the WAF1/Cip1 promoter by TSA. Also, the Sp1-luc plasmid, containing SV40 promoter-derived three consensus Sp1 binding sites, was markedly activated by TSA compared to the mutant Sp1-luc plasmid, indicating that consensus Sp1 site is essential for activation by TSA.

MATERIALS AND METHODS

Cell culture, growth inhibition, and cell cycle analysis. MG63 cells were maintained in DMEM supplemented with 10% fetal calf serum and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

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For growth inhibition analysis, MG63 cells were seeded at a density of 1×10^5 cells/well in 6-well dishes. A solution containing 1 mg/ml of TSA (Wako) in ethanol was diluted to the various indicated concentrations in each dish one day after the seeding. The number of viable cells was counted until 5 days after seeding. This cell-growth study was carried out in triplicate.

For cell cycle analysis, TSA at 500 ng/ml was added to MG63 cells the following day of seeding, and cells were removed from culture dishes by trypsinization. After washing with PBS(−), cells were fixed by ethanol at 4°C. Fixed cells were treated with RNase A, and stained with propidium iodide. DNA contents in stained nuclei were analyzed by EPICS ELITE ESP (COULTER). Cell cycle was analyzed by using MultiCycle software (COULTER).

Protein extraction and Western blotting. MG63 cells were seeded at a density of 1×10^6 cell/dish in 10 cm culture dishes. One day afterwards, TSA was added to give the various TSA concentrations indicated for the dose-response analysis. Twenty-four hours after the TSA addition, the cells were harvested to prepare protein extracts. For the time-course analysis, TSA was added at 500 ng/ml, and cells were harvested at timed intervals. The protein extracts for western blotting was prepared as previously described (1). The protein extracts were heated and loaded onto precast 4-20% polyacrylamide gel (TEFCO), electrophoresed, and transferred to a PVDF membrane. A monoclonal antibody to WAF1/Cip1 (PM-15091A, Pharmingen) was used as the primary antibody. The signal was then developed with the enhanced chemiluminescence system (ECL, Amersham).

RNA isolation and Northern blot analysis. MG63 cells were cultured with either medium alone (−) or medium containing 500 ng/ml TSA (+). Cells were collected at indicated time points (0, 1, 3, 6 12 and 24 hours) after addition of TSA, and total RNA was isolated by using RNeasy, total RNA isolation kits (QIAGEN). Twenty μg of RNA per lane was separated on a 1% agarose/2M formaldehyde gel and blotted onto nylon membrane (hybond-N, Amersham). Hybridization was performed using expresshyb hybridization solution (Clontech). In brief, after UV crosslinking, the blot was hybridized with ^{32}P-labeled WAF1/Cip1 probe (1×10^6 cpm/ml), a full length human WAF1/Cip1 cDNA prepared by PCR, for 60 min at 65°C in expresshyb hybridization solution, and washed at 50°C for 40 min with $0.1 \times$ SSC containing 0.1% SDS. The mRNA level was visualized by bio-image analyzer BAS2000 (Fujix). The same blot was rehybridized with ^{32}P-labeled β-actin probe, 2 kb human β-actin cDNA (Clontech), to standardize the amount of loaded RNA.

Pulse label with ^{14}C-acetate and isolation of histone. MG63 cells were cultured in DMEM at a density of 2×10^6 per 10 cm culture dishes. After removing the medium, fresh medium containing ^{14}C-acetate (50 μCi, NEN/DuPont) was added and incubated for 1 h. The cells were washed with the non-labeled medium and cultured in the absence or presence of various concentrations of TSA for 0.5, 1, 3, and 6 h. Histones were isolated at each of the time points (17). The scraped cells were washed with PBS containing 1 mM PMSF, and suspended in ice cold lysis buffer (10 mM Tris/HCl, 50 mM sodium bisulfate, 1% Triton X-100, 10 mM $MgCl_2$, 8.6% sucrose, 5 mM sodium butyrate, 1 mM PMSF, pH6.5). After Dounce homogenization (20 strokes, tight pestle), the nuclei were washed 3 times in the above buffer, and then once in wash buffer (10 mM Tris/HCl, 13 mM EDTA, 5 mM sodium butyrate, 1 mM PMSF, pH7.4). The pellet was suspended with ice cold water. After addition of 2 M H_2SO_4 to final concentration of 0.2 M, samples were rotated for 60 min at 4°C and then centrifuged for 10 min at 15,000 rpm. The supernatant was transferred to the new tube and 10 volume of ice-cold acetone were added. After precipitation at −20°C for 60 min, the pellet was collected by centrifugation 10 min at 15,000 rpm and dried under vacuum. This histone pellet was dissolved in water.

Analysis of histone by acid-urea-Triton (AUT) gel. The isolated histones were analyzed using an AUT gel (15% acrylamide, 0.1% bisacrylamide, 8 M urea, 8 mM Triton X-100) electrophoresis (18). Histone samples (10 μg) were incubated with equal volume of loading buffer (7.4 M urea, 1.4 M NH_4OH, 0.02% pyronin Y, 10 mM DTT) for 15 min at room temperature then electrophoresed in buffer (0.1 M glycine, 1 M acetic acid) at 10 mM for 7 h. Consequently, the gel was stained with 0.2% Coomassie Brilliant Blue R-250, and dried under vacuum. ^{14}C-acetate-incorporated histones were visualized by using BAS2000 Imageanalyzer (Fujix). The individual of histones (H1, H2a, H2b, H3, H4) was designated by comparing the stained gel with published migration patterns of histones (16).

Plasmid preparation. The human wild-type WAF1/Cip1 promoter-luciferase fusion plasmid, WWP-Luc, was kindly donated by Dr. T. Tokino and Dr. B. Vogelstein (5). The 2.4 kilobase-pair genomic fragment containing the transcription start site was subcloned into the Hind III site of the luciferase reporter vector, pGL3-Basic (Promega), to generate pWWP. A series of mutant WAF1/Cip1 promoters have been described previously (6).

The luciferase-reporter plasmid, Sp1-luc, which contains the sequence of 5′-CGCGTGGGCGGAACTGGGCGGAGTTAGGGGCGGGA-3′, consisting of three consensus Sp1 binding sites (GGGCGG) from the SV40 promoter, was generated to subclone the above fragment into pGL3-Basic. Mutant Sp1-luc, which contains the sequence of 5′-CGCGTG*TTTT*GAACTG*TTTT*GAGTTAGG*TTTT*GGA-3′, consisting of three mutant Sp1 binding sites was also generated as described above.

Transfection assay. MG63 cells were transfected by lipofection technique. MG63 cells were seeded at a density of 2×10^5 cells per 35 mm culture dishes. The next day, cells were transfected with 1 μg per dish of reporter plasmid DNA in lipofectamine (GIBCO BRL) for 5 h. Twenty-four hours after the transfection, the medium was changed for that with or without 500 ng/ml TSA, and 48 h afterwards, cell lysates were collected for the luciferase assay.

The luciferase activities of the cell lysates were measured according to the manufacture's recommendations (Promega). Luciferase activities were normalized for the amount of the protein in cell lysates. All the luciferase assays were carried out in triplicate. In Fig. 3B and 3D, the data are shown as means of three experiments and the fold induction represents the difference between the means of the samples with TSA and that of the control. In Fig. 3C, the data are shown as means ± SE (n = 3). Values were analysed statistically relative to the control using Student's *t* -test.

RESULTS

TSA Blocks Cell Proliferation at G1 and G2/M phases of MG63 Human Osteosarcoma Cells

We first examined the effect of TSA on proliferation of MG63 cells. Fig. 1A shows the growth-curve of MG63 cells in the absence or presence of various concentrations of TSA. A dose-dependent inhibition of the cell growth was observed. On day 5, the growth of cells was inhibited to 90, 87, 62, 5 or 2% of the control by TSA at 10, 50, 100, 500 or 1000 ng/ml, respectively. To identify the phase of cell cycle where MG63 cells were arrested by TSA, we examined changes in the distribition of cellular DNA contents after incubation in the presence or absence of TSA (500 ng/ml) using flowcytometry. As shown in Fig. 1B, There was a decrase in the percentage of cells in S phase from 49.1% in control cells to a nadir of 14.0%

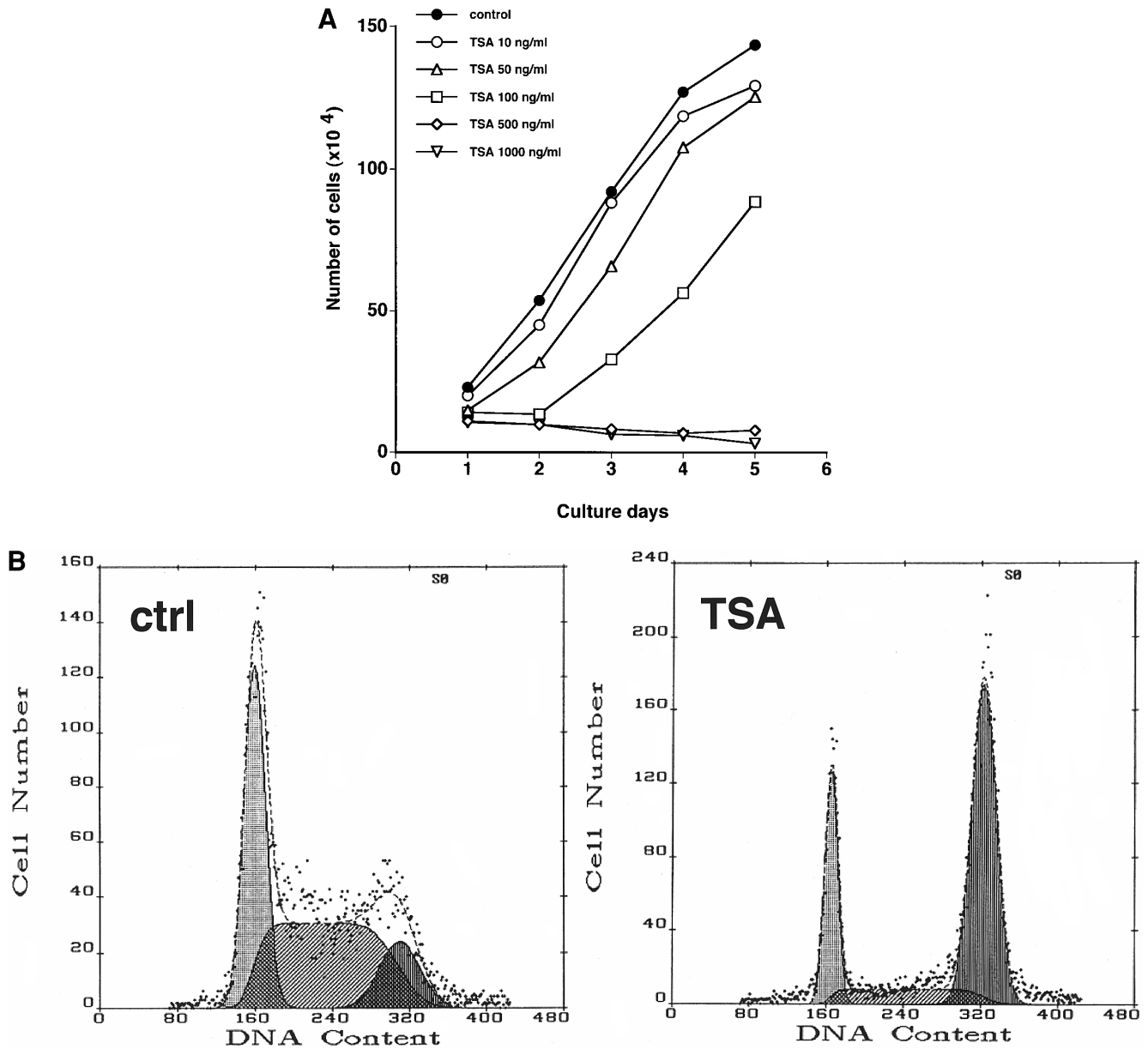

FIG. 1. (A) Effect of TSA on the growth of MG63 cells. One day after inoculation, TSA was added at 10 (○), 50 (△), 100 (□), 500 (◇) or 1000 (▽) ng/ml, and cell growth was compared with control culture (●). Data represent means of triplicate experiments. (B) Effects of TSA on distribution of cellular DNA contents in MG63 cells. After incubation with or without 500 ng/ml TSA for 24 hours, cells were collected and their isolated nuclei were analysed by a flowcytometry. Cell cycle was analyzed by using MultiCycle software. Ctrl: control (C) Dose response of TSA on the WAF1/Cip1 protein. Cells were exposed either to medium alone (0) or to medium containing 10, 50, 100, 500 and 1000 ng/ml TSA for 24 h, and then the expression of WAF1/Cip1 protein was examined. (D) Time course effects of TSA on the WAF1/Cip1 protein. Cells were exposed to medium containing 500 ng/ml TSA, and harvested at 2, 4, 6, 8, 10, 12 and 24 h after the start of the TSA treatment. For the control (ctrl), cells were exposed to medium without TSA, then harvested at 2 h after the medium change. (E) Time course effects of TSA on the WAF1/Cip1 mRNA. Cells were cultured with either medium alone (−) or medium containing 500 ng/ml TSA (+) and collected at the indicated time points (0, 1, 3, 6, 12 and 24 hours) after stimulation for total RNA isolation. Twenty μg of total RNA was analyzed by Northern blotting using ^{32}P-labeled full length WAF1/Cip1 cDNA probe (upper panel), as described in Materials and Methods. As an internal control, β-actin mRNA was analyzed using the same blot (lower panel).

in S phase in cells treated with TSA. A prominent accumulation of cells with 4N DNA content in the G2/M phase was observed in cells treated with TSA. These results suggest that MG63 cells are arrested by TSA at both G1 and G2/M phases, as was observed by sodium butyrate (6).

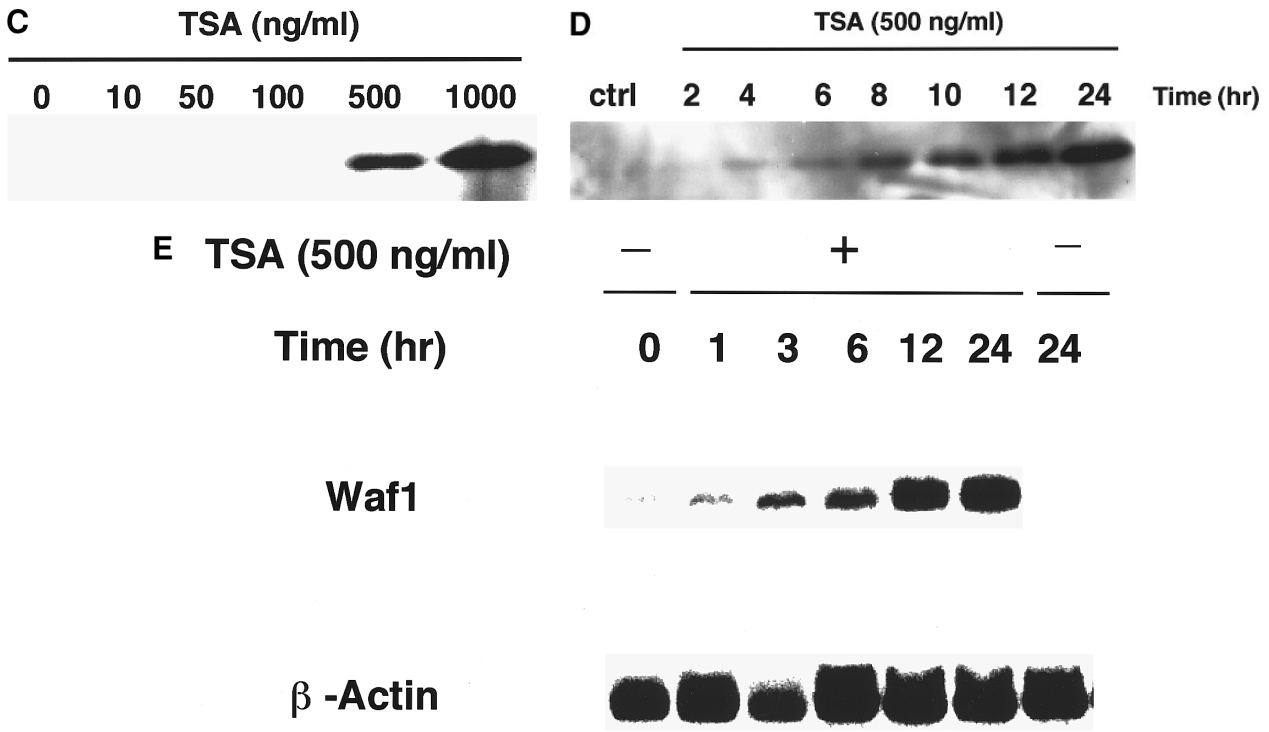

FIG. 1—*Continued*

TSA Increases the WAF1/Cip1 Protein and mRNA Levels in MG63 Cells

To investigate whether WAF1/Cip1 is involved in the TSA-induced growth arrest in MG63 cells, various doses of TSA-treated MG63 cells were assayed for WAF1/Cip1 protein expression by Western blotting. In the untreated control cells, WAF1/Cip1 expression was too weak to be detected, probably due to the lack of wild-type p53 gene in the cells (Fig. 1C, 0). However, twenty-four hours exposure to TSA caused marked WAF1/Cip1 induction at 500 ng/ml and 1000 ng/ml (Fig. 1C, 500 and 1000). This is consistent with the result that TSA completely inhibited the growth of MG63 at 500 and 1000 ng/ml. As p53 gene is deleted in MG63 cells (19), it is most likely that the induction of the WAF1/Cip1 protein is mediated through a p53-independent pathway. The time course study showed that the WAF1/Cip1 protein was induced 4 hours after the treatment with 500 ng/ml of TSA, and reached its peak at around 24 h after the treatment (Fig. 1D).

Next, we tried to elucidate whether the WAF1/Cip1 mRNA would also be induced by the treatment with 500 ng/ml of TSA. Northern blot analysis showed that WAF1/Cip1 mRNA was induced 3 hours after the treatment and reached its peak at around 12 hours after the treatment (Fig. 1E). In contrast, WAF1/Cip1 expression was hardly detected in control cells. Dose-response analysis indicated that twenty-four hours exposure to TSA caused marked WAF1/Cip1 mRNA induction at 500 ng/ml and 1000 ng/ml (data not shown). These results are also consistent with the results of TSA on the inhibition of cell growth and the induction of WAF1/Cip1 protein.

TSA Increases the Hyperacetylated Form of Histone H4

The level of histone acetylation was mainly controlled by equilibration between histone acetyltransferase and histone deacetylase activity. TSA was reported to inhibit histone deacetylase specifically *in vivo* and *in vitro* (16). To assess whether the pattern of WAF1/Cip1 induction caused by TSA corresponds to that of histone acetylation, histones were prepared from MG63 cells which were cultured in the medium containing TSA and analyzed by AUT gel electrophoresis. Each of the histone components (H1, H2a, H2b, H3, H4) was separated, and in particular four (mono-, di-, tri-, tetra-) acetylated forms of H4 were clearly resolved as slower migrated bands in the AUT gel.

To firstly examine the effect of TSA on histone acetylation in MG63 cells, histones were pulse-labeled with ^{14}C-acetate and chased in the presence or absence of 500 ng/ml TSA for 0.5, 1, 3, and 6 h. After the isolation of histones from each time point, they were electrophoresed on an AUT gel, and ^{14}C-labeled histones were visualized by fluorography. Accumulation of tri- and

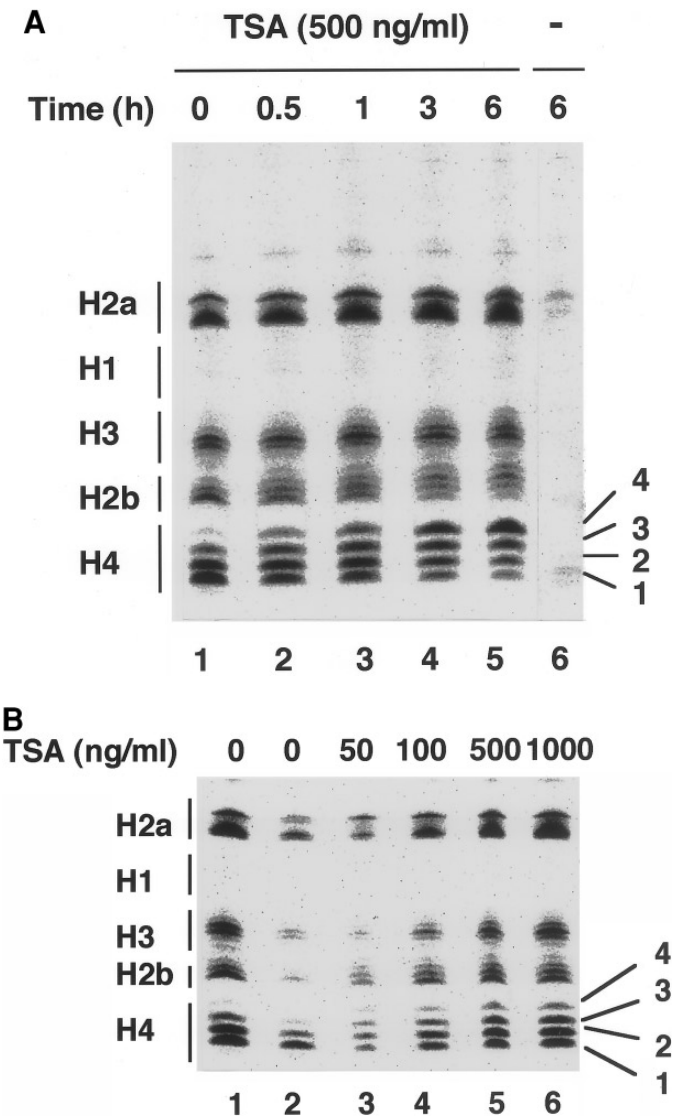

FIG. 2. (A) Time course effect of TSA on histone acetylation in MG63 cells. Cells were pulse-labeled with ^{14}C-acetate for 1 h. After removing the labeled medium, cells were cultured in the absence (lane 6) or presence (lanes 1-5) of 500 ng/ml TSA for 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 3 (lane 4), and 6 h (lane 5 and 6). Histones were isolated at the indicated time point and separated by AUT gel electrophoresis. ^{14}C-acetate incorporated histones were visualized by using BAS2000 Imageanalyzer (Fujix). Each of the individual histones (H1, H2a, H2b, H3, H4) is labelled on the left and acetylated H4 (mono-, di-, tri-, tetra-) on the right. (B) Dose response of TSA on histone acetylation in MG63 cells. Cells were pulse-labeled with ^{14}C-acetate for 1 h. After removing the labeled medium, cells were cultured with or without (lane 2) various concentrations of TSA (lanes 3-6) for 30 min, except lane 1, without the chase in nonlabeled medium. The extracted histones were analyzed on AUT gel electrophoresis. ^{14}C-acetate incorporated histones were visualized by using BAS2000 Imageanalyzer (Fujix).

tetra-acetylated forms of H4 was detected at 30 min after adding TSA (Fig. 2A, lane 2). The amounts of tri- and tetra-acetylated H4 were increased in the presence of TSA for 1, 3, and 6 h (lanes 3-5). In the absence of TSA, no bands of ^{14}C-labeled histones were apparent (lane 6). The turnover of previously incorporated ^{14}C-acetyl groups in histones were inhibited by TSA.

To test dose-response effects of TSA, MG63 cells were pulse-labeled with ^{14}C-acetate for 1 h and chased with the various concentrations of TSA for 30 min. The extracted histones were analyzed by using AUT gel electrophoresis. As shown in Fig. 2B, tri- and tetra-acetylated forms of histone H4 were detected at 50 ng/ml (lane 3) and their levels were increased in relation to the concentration of TSA (lanes 3-6). These results demonstrate that TSA inhibits the histone deacetylase activity in dose- and time-dependent manners, and suggest that the appearance of hyperacertylated form of histone and the induction of WAF1/Cip1 mRNA and protein are related events.

TSA Stimulates the WAF1/Cip1 Promoter Activity

Since the WAF1/Cip1 expression is markedly induced by TSA in MG63 cells, we subsequently investigated whether TSA can stimulate activity of the WAF1/Cip1 gene promoter. At first, the effect of TSA on the wild-type WAF1/Cip1 promoter-luciferase fusion plasmid, pWWP (Fig. 3A), was examined after transient transfection. Following a 24-h exposure to 500 ng/ml TSA, the luciferase activity from pWWP plasmid was increased 25-fold compared to the control (Fig. 3B, pWWP).

Next, we tried to determine what regions of the WAF1/Cip1 promoter are responsive to TSA activation. For this purpose, 5′ deletion construct of the WAF1/Cip1 promoter was generated (Fig. 3A). The resulting plasmid was transiently transfected into MG63 cells and luciferase activities following TSA treatment were measured. Approximately 109-fold activation of the 133 bp promoter fragment from pWPdel-BstX I by TSA was still observed (Fig. 3B, pWPdel-BstX I). This suggests that TSA-responsive elements exist within 133 base pair region relative to the start site of transcription as in the case of sodium butyrate (6). Furthermore, it is considered that the two p53 binding sites, at −2.3 kb and −1.3 kb, are not required for the transcriptional activation by TSA, since the promoter region of pWPdel-BstX I plasmid lacks these sites.

A dose-response study using pWPdel-BstX I indicated that 500 ng/ml and 1000 ng/ml TSA treatment dramatically increased the WAF1/Cip1 promoter activity (124-, 159-fold over control) (Fig. 3C). These data are also consistent with the results that TSA has inhibited the growth of MG63, induced WAF1/Cip1 protein expression, and increased the hyperacetylated forms of histone H4 at the same doses.

Analysis of the TSA Responsive Elements in the WAF1/Cip1 Promoter

We also tried to determine which sites of the promoter region of pWPdel-BstXI are responsive to TSA

activation. This 133 bp region harbors four independent and two overlapping nearly consensus binding sites for transcription factor Sp1 (8, 20). From upstream, we termed these: Sp1-1, Sp1-2, Sp1-3, Sp1-4 and Sp1-5-6 (Fig. 3A). To determine whether these binding sites are involved in activation by TSA, the following reporter plasmids were constructed and assayed for luciferase activity in the absence or presence of TSA: pWPdel-BstX I, containing all the six Sp1-binding sites; pWP101, lacking Sp1-1 and Sp1-2; and, pWPdel-SmaI, lacking Sp1-1 to Sp1-4 sites. As shown in Fig. 3B, the luciferase activity of pWP101 as well as pWPdel-BstX I (109-fold over control) was increased to approximately 144-fold over control by 500 ng/ml TSA, a level higher than that of the activation by pWWP, the full-size promoter (25-fold over control). In contrast, the activation by TSA of pWPdel-SmaI was approximately 23-fold over control. Furthermore, the basal promoter activity of pWPdel-SmaI was significantly decreased to 0.5% of pWWP. We then generated a series of mutants of pWP101 having mutations in the respective Sp1 sites, and we termed these pWP101-mtSp1-3, pWP101-mtSp1-4, pWP101-mtSp1-5-6, respectively (Fig. 3A). These plasmids were transiently transfected into MG63 cells and their luciferase activities were measured in the absence or presence 500 ng/ml TSA. The activation by TSA in pWP101-mtSp1-3 decreased from 144-fold in pWP101 to only 10-fold over control. Similarly the activation by TSA of pWP101-mtSp1-4 was decreased to 68-fold over control (Fig. 3B). In contrast, the basal activity of pWP101-mtSp1-5-6 was reduced to background levels, approximately 0.05% of pWP101, and the activation by TSA was entirely abolished in these constructs. Consequently, we conclude that: the Sp1-3 site, located between −82 and −77 relative to the transcription start site, is the main TSA responsive element; the Sp1-4 site, between −69 and −64, is also partially involved in the activation; the Sp1-5-6 site, between −60 and −50, is the essential region for the WAF1/Cip1 gene expression.

Using WAF1/Cip1 mutant constructs, we found that TSA activates the WAF1/Cip1 promoter through the effect at the Sp1 sites. In order to confirm that Sp1 elements are indeed activated by TSA, the reporter plasmid Sp1-luc, containing SV40 promoter-derived three consensus Sp1 binding sites but no TATA box, and mtSp1-luc, containing three mutant Sp1 binding sites were constructed (Fig. 3A). These plasmids were transfected into MG63 cells individually, and activation of the promoter by TSA was analyzed. As shown in Fig. 3D, TSA markedly activated the Sp1-luc plasmid to approximately 392-fold over control, compared to mtSp1-luc where activity was low (13-fold over control).

DISCUSSION

In the present study, we have shown that the treatment with TSA on MG63 cells activates the promoter activity of WAF1/Cip1 and induces the WAF1/Cip1 mRNA and protein, resulting in the arrest of the cell growth at both G1 and G2/M phases in a p53-independent manner. Although WAF1/Cip1 has been commonly associated with the G1 checkpoint, it has been shown that during cell cycle of normal human fibroblast, the levels of WAF1/Cip1 mRNA peak twice, during G1 and G2 (21), which is suggestive of a potentional role of WAF1/Cip1 in G2/M phase. In addition, there is also a precedent for induction of G2/M arrest by TSA in T24 cells (22).

Furthermore, high levels of histone acetylation were also detected by TSA treatment. Since these effects of TSA were shown at doses of 500 and 1000 ng/ml, a coincidence between the induction of WAF1/Cip1 mRNA and protein, and the appearance of hyperacetylated form of histone H4 seems to exist.

A series of mutation analyses of the WAF1/Cip1 promoters have revealed that the main TSA responsive element is the Sp1 site, located between −82 and −77 relative to the transcription start site (Sp1-3 site in the present paper); the Sp1 site, between −69 and −64 (Sp1-4 site), is also partially involved in this activation. In addition, we found that TSA is capable of activating transcription from the luciferase reporter plasmid which only has three Sp1 sites. These results strongly suggest that Sp1 is involved in the transcriptional activation of the WAF1/Cip1 promoter in response to TSA. EMSA using MG63 cells revealed that Sp1 and Sp3 can specifically interact with this main TSA responsive element, the Sp1-3 site (data not shown). However, the intensity and mobility pattern of the retarded bands were not changed by TSA, which means that TSA activation of WAF1/Cip1 promoter does not appear to be due to increasing the binding of Sp1 or Sp3 itself (data not shown).

Since our present study fully corroborates our previously data that sodium butyrate arrests the growth of WiDr cells by activating the WAF1/Cip1 promoter through exactly the same Sp1 sites (6), we suggest that this previous data reflects the consequence of the inhibitory action upon histone deacetylase of sodium butyrate. This suggests the importance of histone acetylation upon the regulation mechanisms of the WAF1/Cip1 promoter through the Sp1 site(s).

Recently, strong interest in the roles of core histone acetylation during transcriptinal regulation has been evident. There is a general correlation between core histone acetylation and gene activity, and the notion that core histone acetylation facilitates gene expression has gained further support as transcription factors such as Gcn5, CBP/p300, and $TAF_{II}250$ have been found to possess histone acetyltransferase activity (reviewed in Ref. 23). Conversely, several recent papers have shown that histone deacetylase can function to repress transcription (24 - 30). Noticeably, those papers

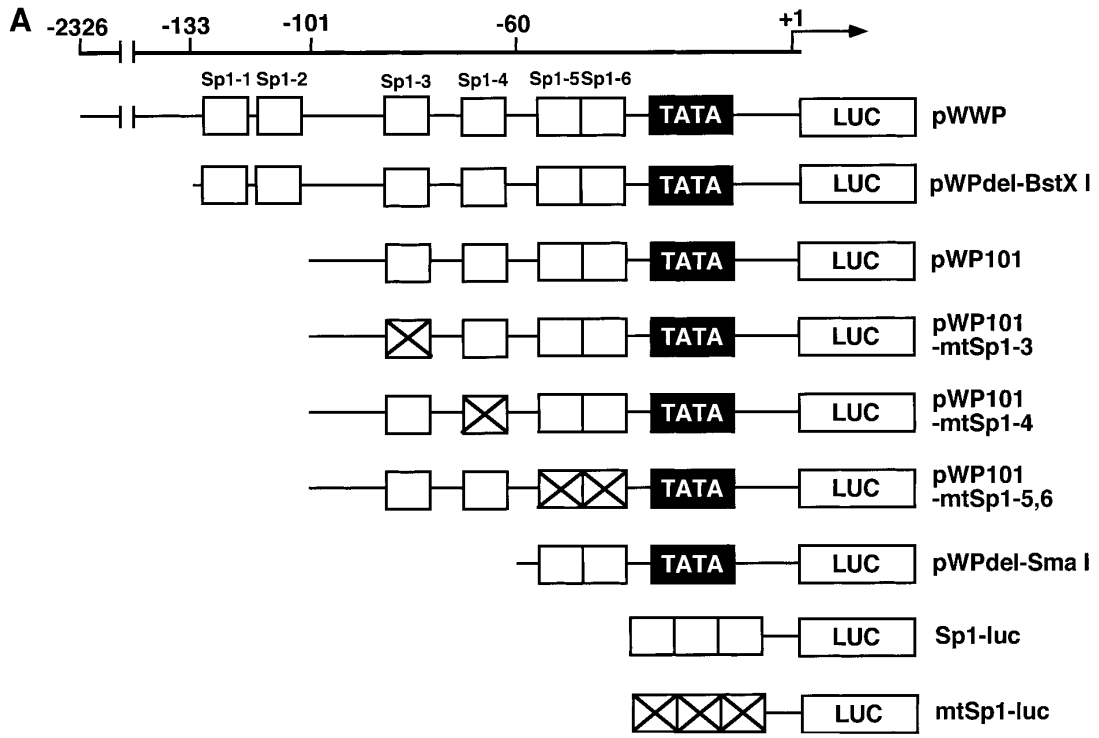

FIG. 3. (A) Schematic representation of: full-size (pWWP), deletion (pWPdel-BstX I, pWP101, pWPdel-SmaI), and mutant (pWP101-mtSp1-3, pWP101-mtSp1-4, pWP101-mtSp1-5,6) WAF1/Cip1 promoter-luciferase plasmids, and three tandem repeats of consensus Sp1 sites (Sp1-luc) and mutant Sp1 sites (mtSp1-luc) driving luciferase plasmids used in transfection assays. The open boxes show the locations of the Sp1 elements of the promoter. The crossed boxes show the locations of the mutant Sp1 elements of the promoter. The 5′ end of the deletion mutants is indicated with vertical bars and numbered relative to the start site of transcription. (B) Deletion and mutation analysis of the activation of the WAF1/Cip1 promoter by TSA in MG63 cells. The constructed plasmids pWWP, pWPdel-BstX I, pWP101, pWP101-mtSp1-3, pWP101-mtSp1-4, pWP101-mtSp1-5,6, pWPdel-SmaI were transiently transfected into MG63 cells and luciferase activities were measured after 24-h treatment with (closed bar) or without (open bar) 500 ng/ml TSA for 24 h. Data are shown as means (n = 3). Fold induction by TSA is calculated to compare with the activities of the control (without TSA treatment). (C) Dose response of TSA on the WAF1/Cip1 promoter activity. Cells were transiently transfected with the pWPdel-BstX I reporter plasmid, and luciferase activity was measured after the treatment either with medium alone or a various concentrations of TSA for 24 h. Data are shown as means ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$. (D) Activation of the promoter activity of Sp1-luc in MG63 cells. Cells were transiently transfected with either the Sp1-luc reporter plasmid or mutant reporter plasmid mtSp1-luc and luciferase activities were measured after 24-h treatment with (closed bar) or without (open bar) 500 ng/ml TSA for 24 h. Fold induction by TSA is calculated to compare with the activities of the control (without TSA treatment).

present the data that the transcriptional repression by a sequence-specific DNA-binding factor can be mediated by the recruitment of a deacetylase (Rpd3/HDAC1/HDAC2) to the promoter region. These concepts, that a linkage between some sequence-specific DNA-binding factors and the deacetylase, would explain our results; the inhibition of histone deacetylase by TSA induced the WAF1/Cip1 expression dependent on Sp1 sites of the WAF1/Cip1 promoter. We now investigate whether the Sp1 site sequence-specific DNA binding factors form a complex with deacetylase, which would act as transcriptional repressor.

In summary, our results suggest that the TSA-induced growth arrest in MG63 cells is due to the p53-independent activation of WAF1/Cip1 promoter mediated through the specific Sp1 sites in the promoter region. Recently, we proposed a novel approach for chemotherapy or chemoprevention against cancer, which we termed "gene-regulating chemotherapy or chemoprevention" (31). Our strategy is to increase the potent function of growth-inhibitory genes, which are activating targets of p53. WAF1/Cip1 gene is one of our candidates, because WAF1/Cip1 appears to be rarely mutated in human common tumors (32, 33), whereas the converse is true for the p53 gene (34, 35). Therefore, in future, clarification of the p53-independent activating pathway of WAF1/Cip1 might contribute to the therapy or the prevention of cancer when p53 is mutated. Furthermore, TSA is not only a drug to inhibit the cell proliferation by p53-independent pathway, but also a key compound to elucidate the roles of the acetylation status of core histone during transcriptinal regulation.

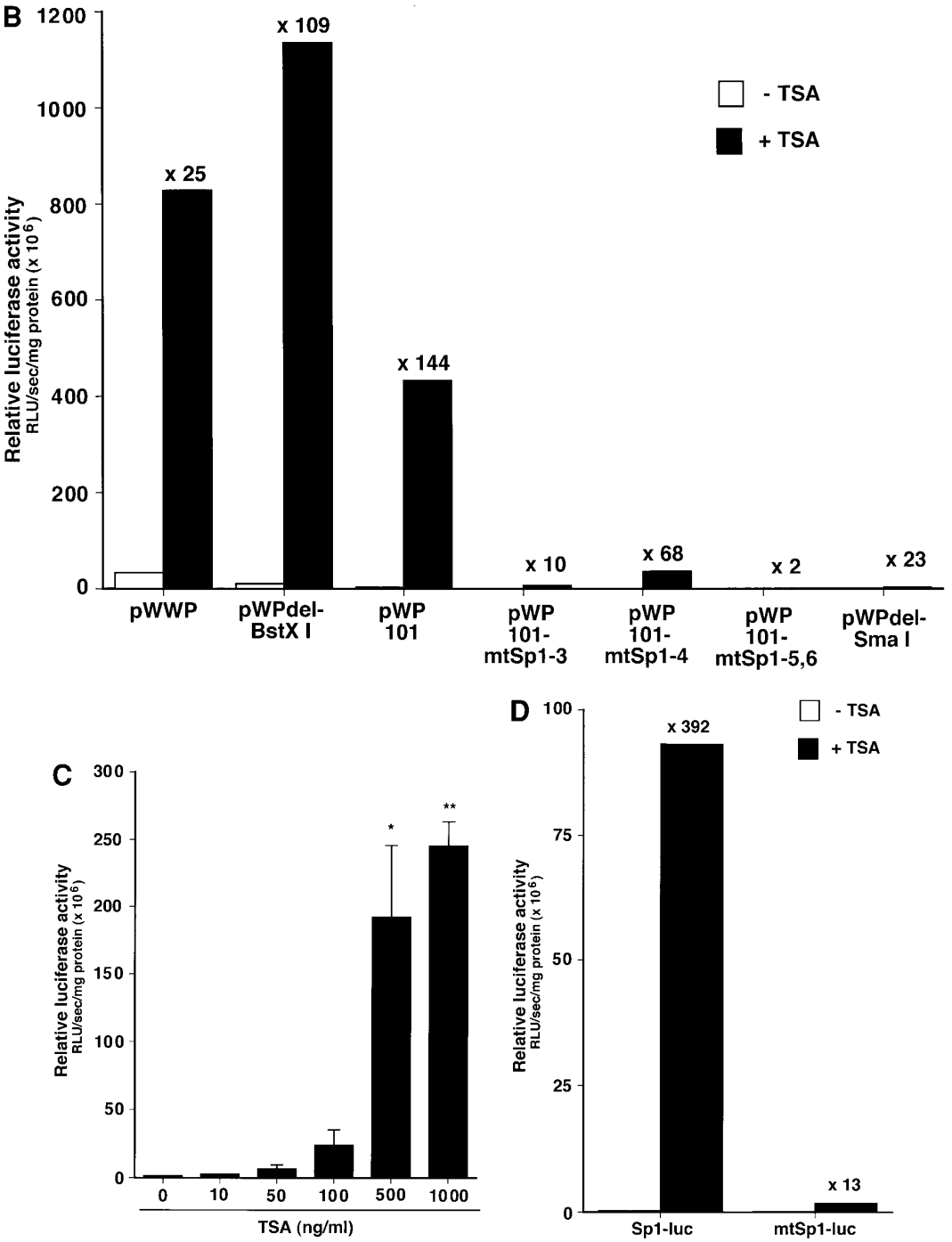

FIG. 3—*Continued*

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