Butyrate Activates the WAF1/Cip1 Gene Promoter through Sp1 Sites in a p53-negative Human Colon Cancer Cell Line*

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Butyrate is a well known colonic luminal short chain fatty acid, which arrests cell growth and induces differentiation in various cell types. We examined the effect of butyrate on the expression of WAF1/Cip1, a potent inhibitor of cyclin-dependent kinases, and its relation to growth arrest in a p53-mutated human colon cancer cell line WiDr. Five millimolar butyrate completely inhibited the growth of WiDr and caused G1-phase arrest. WAF1/Cip1 mRNA was rapidly induced within 3 h by treatment with 5.0 mM butyrate, and drastic WAF1/Cip1 protein induction was detected. Using several mutant WAF1/Cip1 promoter fragments, we found that the butyrate-responsive elements are two Sp1 sites at −82 and −69 relative to the transcription start site. We also found that a TATA element at −46 and two overlapping consensus Sp1 sites at −60 and −55 are essential for the basal promoter activity of WAF1/Cip1. These findings suggest that butyrate arrests the growth of WiDr by activating the WAF1/Cip1 promoter through specific Sp1 sites in a p53-independent fashion.

Butyrate is one of the most abundant short chain fatty acids in the large intestine, generated by bacterial fermentation of dietary fibers (1). Butyrate shows potent effects on growth arrest and differentiation in vitro in various malignant tumor cell lines, such as breast cancer cells, hepatoma cells, and others (2–5). In colorectal cancer cells, butyrate inhibits cell growth and induces differentiation marker proteins such as alkaline phosphatase and carcinoembryonic antigen (6–9). Furthermore, butyrate arrests the cell cycle progression at the G1 phase (9) and decreases c-myc oncogene expression in human colon cancer cell lines (9, 10). However, the precise mechanism of growth suppression by butyrate in colon cancer cells has not been clarified.

WAF1/Cip1 protein potently inhibits the various G1 cyclin-dependent kinases activities (11–13) by suppressing the phosphorylation of retinoblastoma (RB) protein, thereby supposedly inhibiting the G1-S phase transition (11, 14). Besides its role as a kinase inhibitor, it has been reported recently that WAF1/Cip1 at low doses assembles kinase complexes and promotes a kinase activity (15). Furthermore, the transcription of the WAF1/Cip1 gene is directly activated by wild-type p53 protein (16). Thus, WAF1/Cip1 could play a key role as a downstream mediator of the p53-induced cell growth arrest.

Several studies have already shown the p53-independent induction of WAF1/Cip1 by serum, transforming growth factor β, and other differentiation-inducers (17–20). In addition, butyrate has been reported to induce WAF1/Cip1 mRNA independently of p53 during differentiation of hematopoietic cells, hepatoma cells, and colon cancer cells in vitro (18, 21). Butyrate can also dephosphorylate the retinoblastoma protein in mouse fibroblasts (22). To investigate the mechanism of butyrate-induced growth arrest, we used a human colon cancer cell line WiDr harboring a point mutation in p53 at codon 273 (23) and examined the effect of butyrate on the expression of the WAF1/Cip1 gene.

Our results demonstrate that WAF1/Cip1 mRNA is rapidly induced upon butyrate treatment, although WiDr lacks the wild-type p53 gene. We then found that butyrate markedly induces WAF1/Cip1 protein and causes G1-phase arrest. In addition, we observed that butyrate can strongly activate the WAF1/Cip1 promoter, and that the two p53-binding sites are not required for the transcriptional activation by butyrate. Using a series of mutant WAF1/Cip1 promoter constructs, we also found in p53-negative cell lines WiDr and human osteosarcoma cell line MG63 (24), 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 butyrate. Furthermore, the essential elements for the WAF1/Cip1 promoter activity have been shown to be two overlapping consensus Sp1 sites at −60 and −55 and TATA sequence at −46.

MATERIALS AND METHODS

Cell Culture, Cell Growth Study, and Treatment of Butyrate—Human colon adenocarcinoma cell line WiDr was a kind gift from Dr. R. Takahashi of Kyoto University and human osteosarcoma cell line MG63 was kindly provided by Dr. Y. Yanase of Wakayama Medical College. WiDr harbors a point mutation at codon 273 of p53 (23), and MG63 contains rearrangements in the p53 gene (24). Both cell lines were maintained in DMEM supplemented with 10% fetal calf serum and were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. For the cell growth study, WiDr cells were inoculated at a density of 5 × 104 cells in 35-mm-diameter dishes. A solution of 500 mM butyrate was prepared by adjusting the concentration of n-butyrate (Sigma Chemical Co.) in DMEM and was diluted to its final concentration in each culture dish. Two days after the inoculation, butyrate was added at various concentrations. From the second day to the sixth day from the inoculation, the number of viable cells was counted by a trypan blue dye exclusion test. This cell growth study was carried out in duplicate and repeated at least three times.

Plasmid Preparation—The human wild-type WAF1/Cip1 promoter-
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luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein (16). The 2.4-kilobase pair genomic fragment containing the transcription start site was subcloned into the HindIII site of the luciferase reporter vector, pGL3-Basic (Promega), to generate pWWP. To generate pWWPdel-p53, pWWP was treated with an exonuclease III–based deletion. Kilo-sequence deletions were also constructed using synthesized oligonucleotides as follows (Fig. 8): pWP101 except for the sequence underlined (Fig. 8). To construct pWP101 containing four Sp1 sites termed Sp1-3, Sp1-4, and Sp1-5–6, were synthesized and cloned into the transcription start site (16) were synthesized, annealed, and cloned into the SacI and SmaI sites of pWPdel-Sma. Sequences of the oligonucleotides for pWP101 were sense, 5′-CGTGGGCCAGCCGGCTGCCTGCCCCTTGAGGCGGGCCC-3′ and antisense, 5′-GGGGCAGCTCAAGGGCGGCCACCGGCTGCCCCTTGAGGCGGGCCC-3′; pWP101-mtSp1–4: sense, 5′-CTCAGCTGGCGCAGCTCTGCGCGGCCCTGATATACAACCGCC-3′ and antisense, 5′-GGGGCAGCTCAAGGGCGGCCACCGGCTGCCCCTTGAGGCGGGCCC-3′; pWP101-mtSp1–3: sense, 5′-CTCAGCTGGCGCAGCTCTGCGCGGCCCTGATATACAACCGCC-3′ and antisense, 5′-GGGGCAGCTCAAGGGCGGCCACCGGCTGCCCCTTGAGGCGGGCCC-3′; pWP101-mtSp1–5–6: sense, 5′-CTCAGCTGGCGCAGCTCTGCGCGGCCCTGATATACAACCGCC-3′ and antisense, 5′-GGGGCAGCTCAAGGGCGGCCACCGGCTGCCCCTTGAGGCGGGCCC-3′; and pWP101-mtTATA: sense, 5′-GCACAGCGAGAGCGAGGCTCCGTCTGAGTCTAACTCATGCCAGAATTAGGATCTCGAGTTCGGCAATCGTCGAAAGAACGCTGAAGGTCTGCTAGTGCGGCTAGTAACTCTCATCTGAAGGGGACCTGGTCCCGTGGCCATGGCGGCTCTTGGGCTGAGTTAAGGACCCGTAACCGTGCCAGACCCCGTCGCCAGCAGGCTCCATCTGAGGTCTGCAGTGCACGATCAG-3′ and antisense, 5′-AGTTTGGGGTTGTTATATAACCGCGCCGAGTCCAGACGAGTCCGCTAGTGGTACACCAGTGATGCCTTCGAGAGGCTGCTGAGTTAAGGACCCGTAACCGTGCCAGACCCCGTCGCCAGCAGGCTCCATCTGAGGTCTGCAGTGCACGATCAG-3′. These sequences are identical to that of pWP101 except for the sequence underlined (Fig. 8). To construct pWP124, complementary oligonucleotides corresponding to the sequence between −124 and −61 of the WAF1/Cip1 promoter, containing all six Sp1 consensus binding sites (16, 25, 26), were synthesized and cloned into the KpnI and SmaI sites of pWPdel-Sma (Fig. 7). The extent of 5′ deletions was determined by sequencing or utilizing the restriction enzyme Sp1 sites. The luciferase-reporter plasmid, Sp1-luc, which contains the sequence of 5′-CCCGTGGGCGGAACTCGGGGGAGTTAGGGGGGCCG-3′, consisting of three consensus Sp1 binding sites underlined from the SV40 promoter, was a kind gift from Dr. Peggy J. Farnham (27). A vector containing pUC19-plasmid was purchased from Promega and used for control reporter plasmid.

Analysis of Cell Cycle Progression—Cells were removed from culture dishes by trypsinization and collected by centrifugation. After washing with 90% ethanol, 10% 3 M KCl, and 200 mM sodium hydroxide solution, the cells were suspended in 100 mM-diameter dishes. The following day, butyrate was added, and cell lysates were collected at each indicated time.

Transient Transfection—WiDr cells were transfected by calcium phosphate coprecipitation technique. WiDr cells were inoculated at a density of 5 × 104 cells in 100-mm-diameter dishes. After 2 days, 8 μg of reporter plasmid DNA in calcium phosphate precipitates mixture were used for transfection for 8 h. Forty-eight h after the transfection, luciferase activity was measured. Twenty-four h after the transfection, the medium was changed, and 10.0 mM butyrate was added, and 48 h after the transfection, cell lysates were collected for the luciferase assay.

Luciferase Assay—the luciferase activities of the cell lysates were measured as described previously (30). Luciferase activities were normalized for the amount of the protein in cell lysates. All the luciferase assays were carried out at least in triplicate. Each experiment was repeated at least three times.

Electrophoretic Mobility Shift Assay (EMSA)1—Nuclear extracts were prepared according to the method of Dignam et al. (31). In brief, MG63 cells were scraped and incubated in 10 mM Hepes-KOH buffer, pH 7.9, containing 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 5 mM sodium fluoride, 5 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride on ice for 10 min. Cells were disrupted by Dounce homogenizer. After centrifugation, nuclei were resuspended in 20 mM Hepes-KOH buffer, pH 7.9, containing 0.05 mM NaCl, 1.5 mM MgCl2, 25% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM sodium fluoride, 5 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride and incubated at 4 °C for 30 min. The mixture was centrifuged at 35,000 rpm for 30 min at 4 °C, and the supernatant was recovered as nuclear extracts. Nuclear extracts were dialyzed against 20 mM Hepes-KOH buffer, pH 7.9, containing 0.05 mM NaCl, 1.5 mM MgCl2, 25% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM sodium fluoride, 5 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride.

Annealed oligonucleotides containing the sequence between −87 and −72 of the WAF1/Cip1 promoter (AGCTCGGTTGCGCCCTTCTTCTGCGGAGACCAGCCGAGCCG-3′, which were labeled with [α-32P]dCTP using the Klenow fragment of Escherichia coli DNA polymerase I and were used as a probe, termed wt −87 to −72. The reaction mixture for the EMSA contained 8 μg Tris-HCl, pH 7.9, 24 μM Hepes-KCl, pH 7.9, 120 mM KCl, 24% (v/v) glycerol, 2 μM EDTA, 2 mM dithiothreitol, 1 μg of poly(dIdC) (Pharmacia Biotech, Inc.) and 8 μg of nuclear extract. After preincubation for 5 min, 32P-labeled probe DNA was added to the mixture, and the binding reaction was allowed to proceed at room temperature for 20 min. The reaction mixture was further incubated for 20 min in the presence or absence of anti-Sp1 or Sp3 antibody (Santa Cruz Biotechnology). The product was then resolved by electrophoresis on a 6% polyacrylamide gel.

RESULTS

Butyrate Inhibits the Proliferation of WiDr Human Colon Cancer Cells—We first examined the effect of butyrate on the proliferation of WiDr cells. Fig. 1 shows the growth of WiDr cells in the presence of various concentrations of butyrate. Butyrate showed little effect on the viability of the cells as assessed by trypan blue dye exclusion test up to 5.0 mM. However, 10.0 mM butyrate was slightly cytotoxic. A dose-dependent inhibition of the cell growth was observed at concentrations of 0.625 mM or more. On day 6, the growth of cells was inhibited 1 The abbreviations used are: EMSA, electrophoretic mobility shift assay; CHX, cycloheximide; RB, retinoblastoma; bp, base pair.
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Butyrate Increases WAF1/Cip1 mRNA and Protein Levels in WiDr Cells—To investigate whether WAF1/Cip1 is involved in the butyrate-induced growth arrest in WiDr cells, butyrate-treated or untreated WiDr cells were assayed for WAF1/Cip1 mRNA expression by Northern blot analysis. 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. 2A, lane 1). However, 24-h exposure to butyrate caused distinct WAF1/Cip1 induction in a dose-dependent fashion from 0.625 mM up to 5.0 mM (Fig. 2A, lanes 2–5). It is consistent with the result that butyrate has inhibited the growth of WiDr dose-dependently at 0.625 mM or more. As p53 is mutated in WiDr cells (23), it is most likely that the induction of WAF1/Cip1 mRNA is mediated through a p53-independent pathway. The time course study showed that WAF1/Cip1 mRNA was induced 3 h after the treatment with 5.0 mM butyrate and reached its peak around 12 h after the treatment, when mRNA levels were induced approximately 13-fold compared with the control. The induction remained at least for 24 h (Fig. 2B). On the other hand, butyrate could not activate other major cyclin-dependent kinase inhibitors such as p27Kip1 (32, 33) and p16INK4A (34) at a mRNA level (data not shown). Thus, we conclude that butyrate specifically induces WAF1/Cip1 at a mRNA level.

Next, we tried to elucidate whether the WAF1/Cip1 protein would also be induced by treatment with butyrate in WiDr cells. Western blot analysis showed that WAF1/Cip1 protein expression was hardly detected in the untreated control cells (Fig. 3A, lane 1), as expected from the result of Northern blot analysis. In contrast, treatment with 5.0 mM butyrate for 24 h induced expression of WAF1/Cip1 protein (Fig. 3A, lane 2).

Taken together, these results indicate that butyrate specifically induces WAF1/Cip1 mRNA and consequently increases WAF1/Cip1 protein levels in WiDr cells through a p53-independent pathway. To confirm these results, another p53-negative osteosarcoma cell line MG63 (24) was used. Similarly in MG63 cells, butyrate effectively suppressed the cell growth (data not shown) and specifically induced WAF1/Cip1 mRNA in the absence or presence of cycloheximide (CHX) (Fig. 2C) but not that of p27Kip1 and p16INK4A (data not shown). Furthermore, 24 h after the treatment with butyrate, the WAF1/Cip1 protein level was drastically increased (Fig. 3B, upper panel), and subsequently, the majority of the RB protein was converted into a hypophosphorylated form (Fig. 3B, lower panel).

Butyrate arrests WiDr Cells at the G1 Phase in Cell Cycle Progression—To investigate the effect of butyrate on cell cycle progression of WiDr cells, the DNA content of nuclei of WiDr cells was measured by flow cytometric analysis. As shown in Fig. 4A, fluorescence analysis revealed that a 24 h exposure to 2.5 or 5.0 mM butyrate apparently decreased the population of S-phase cells in a dose-dependent manner. S-fit analysis of the DNA histograms also revealed that 5.0 mM butyrate caused the accumulation of cells in the G1 phase from 42 to 71% (Fig. 4B). A time course study showed that cells started to accumulate in G1 phase at least 16 h after the addition of 5.0 mM butyrate, and the effect reached its maximum at 24 h after the treatment (data not shown). Furthermore in MG63 cells, 24 h treatment of butyrate also caused G1 phase arrest with weak G2-M arrest (data not shown).

Butyrate Stimulates WAF1/Cip1 Promoter Activity—Having demonstrated that WAF1/Cip1 mRNA expression is drastically induced by butyrate in WiDr cells lacking wild-type p53, we subsequently investigated whether butyrate can stimulate activity of the promoter of the WAF1/Cip1 gene. The effect of butyrate on the wild-type WAF1/Cip1 promoter-luciferase fusion plasmid, WWP-Luc, was examined by transient transfection. Following a 24-h exposure to 5.0 mM butyrate, the luciferase activity from the WWP-Luc plasmid was increased 55-fold compared with the untreated control (Fig. 5A). For further examination, we prepared a WiDr cell line stably transfected with the WWP-Luc plasmid. As shown in Fig. 5B, the luciferase activity was increased in a dose-dependent manner up to 8-fold by the treatment with 5.0 mM butyrate. Time course study indicated that treatment with 5.0 mM butyrate for 1 or 3 h caused a slight increase of the WAF1/Cip1 promoter activity.

![Butyrate Increases WAF1/Cip1 mRNA and Protein Levels in WiDr Cells](image1.png)

**Fig. 1. Effect of butyrate on the growth of WiDr cells.** Two days after inoculation, butyrate at 0.625 (■), 1.25 (○), 2.5 (●), 5.0 (▲), or 10.0 (▲) mM was added, and cell growth was compared with control culture (□). Data represent means of duplicate experiments.

![Northern blot analysis of WAF1/Cip1 mRNA in p53-negative cell lines, WiDr and MG63.](image2.png)

**Fig. 2. Northern blot analysis of WAF1/Cip1 mRNA in p53-negative cell lines, WiDr and MG63.** In A, WiDr cells were treated with various concentrations of butyrate. Lane 1, untreated cells; lane 2, 0.625 mM; lane 3, 1.25 mM; lane 4, 2.5 mM; lane 5, 5.0 mM. The expression of mRNA was examined after 24-h exposure to butyrate. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. In B, WiDr cells were exposed either to medium alone (−) or to 5.0 mM butyrate (+), and total RNA was extracted at the indicated times after stimulation. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, MG63 cells were exposed for 24 h to medium alone (lanes 1), 0.1 μg/ml CHX (lanes 2), 0.4 μg/ml CHX (lanes 3), 5.0 mM butyrate (lanes 4), 5.0 mM butyrate with 0.1 μg/ml CHX (lanes 5), and 5.0 mM butyrate with 0.4 μg/ml CHX (lanes 6). The same blot was hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to normalize the amount of loaded RNA.
compared with the controls. Six-h treatment with 5.0 mM butyrate significantly increased the WAF1/Cip1 promoter activity, and 24-h treatment strongly stimulated the promoter activity up to 9.2-fold (Fig. 5C).

**Analysis of the Butyrate-responsive Elements in the WAF1/Cip1 Promoter**—Next, we tried to determine what regions of the WAF1/Cip1 promoter are responsive to butyrate activation. For this purpose, a series of 5′ deletion constructs of the WAF1/Cip1 promoter were generated (see “Materials and Methods”). The resulting plasmids were transiently transfected into WiDr cells, and luciferase activities following butyrate treatment were measured relative to full-size promoter (pWWP). The pWPdel-p53 plasmid, including a 1219-bp promoter fragment lacking two p53 binding sites, was consistently fully activated by butyrate up to 20–25-fold, whereas that of the full-length promoter (pWWP) in WiDr cells (data not shown), suggesting that the two p53 binding sites are not required for the transcriptional activation by butyrate. Furthermore, about 20-fold activation by butyrate was still observed in the 210-bp promoter fragment from pWPdel-PstI (data not shown; the location of the PstI site is shown in Fig. 6). These results suggest that butyrate-responsive elements exist within the 210-bp region relative to the start site of transcription (Fig. 6).

This 210-bp region harbors four independent and two overlapping nearly consensus binding sites for transcription factor Sp1 (16, 25, 26). We termed them Sp1-1, Sp1-2, Sp1-3, Sp1-4, and Sp1-5-6 from the upstream (Fig. 6). To determine whether these Sp1 binding sites are involved in activation by butyrate, a series of 5′ deletion plasmids, pWP124, containing all of the six Sp1 binding sites, pWP101, lacking Sp1-1 and Sp1-2, and pWPdel-SmaI, lacking Sp1-1 to Sp1-4 sites, were constructed and assayed for luciferase activity in the absence or presence of butyrate (Figs. 6 and 7). As shown in Fig. 7, luciferase activity of pWP101 as well as pWP124 was activated about 26–31-fold by 5.0 mM butyrate, a level similar to that of the activation by the full-size promoter (pWWP). On the other hand, in pWPdel-SmaI the activation by 5.0 mM butyrate was only 3.9-fold. Furthermore, the basal promoter activity of pWPdel-SmaI significantly decreased to 13.4% of pWWP, whereas that of pWP124 or pWP101 did not significantly decrease. We then generated a series of mutants of pWP101 having mutations in the various Sp1 sites or TATA element, and we termed them pWP101-mtSp1-1, pWP101-mtSp1-3, pWP101-mtSp1-4, pWP101-mtSp1-5-6, and pWP101-mtTATA, respectively (Fig. 8). These constructs were transiently transfected into WiDr cells, and their luciferase activities were assayed in the absence or presence of 5.0 mM butyrate. As shown in Fig. 8A, the basal activity of pWP101-mtSp1-3 was reduced to 2.5% of that of pWP101, and the activation by butyrate in pWP101-mtSp1-3 decreased to only 2.1-fold from 33.3-fold activation in wild-type pWP101. Similarly, the basal activity of pWP101-mtSp1-4 was reduced to 7.1% of pWP101, and the activation by butyrate decreased to 11.0-fold from 33.3-fold activation in wild-type pWP101. On the other hand, the basal activity of pWP101-mtSp1-5-6 was reduced to 0.4% of pWP101, and the activation by butyrate was entirely abolished in these constructs (Fig. 8A).

As shown in Fig. 8B, in MG63, similar to the results in WiDr, the activation by butyrate in pWP101-mtSp1-3 was 3.6-fold, and that in pWP101-mtSp1-4 was 15.1-fold, whereas 70.0-fold activation was detected in pWP101. Taken together, we conclude that the Sp1-3 site located between −82 and −77 relative to the transcription start site is the main butyrate-responsive element, and that the Sp1-4 site between −69 and −64 is also
partially involved in the activation (Fig. 8). We also show that the Sp1-5-6 site and TATA element are the most important core promoter elements indispensable for the basal promoter activity of the \textit{WAF1/Cip1} promoter (Fig. 8).

Using \textit{WAF1/Cip1} mutant constructs, we found that butyrate activates the \textit{WAF1/Cip1} promoter through the effect at the Sp1 sites. To confirm that Sp1 elements are indeed activated by butyrate, the reporter plasmid Sp1-luc, containing SV40 promoter-derived three consensus Sp1 binding sites but no TATA box (see “Materials and Methods”), was transfected into WiDr cells, and activation of the promoter by butyrate was analyzed. As shown in Fig. 9, butyrate significantly activated the Sp1-luc plasmid about 37-fold, whereas vacant vector pGL2-Basic, lacking Sp1 sites, was not activated (Fig. 9).

Identification of Proteins Interacting with the Main Butyrate Responsive Element—To determine if Sp1 or other proteins can interact with the main butyrate-responsive element, EMSAs were performed using the oligonucleotide containing the wild-type Sp1–3 site, between \(2^{87}\) and \(2^{72}\) from the transcription start site. Nuclear extracts were purified from either butyrate-treated or untreated MG63 cells. As shown in Fig. 10, two major DNA-protein complexes were detected, which were competed away by an excess of unlabeled homologous oligonucleotide (data not shown). To elucidate whether the retarded bands represent the binding of Sp1 or Sp3 (a member of Sp1 family), EMSA was performed with the nuclear extracts preincubated with Sp1- or Sp3-antibody. In the presence of Sp1-antibody, the upper complex was supershifted (lanes 2 and 5), and the lower complex was diminished in the presence of Sp3-antibody (lanes 3 and 6). However, both the mobility pattern and intensity were not changed by butyrate treatment.

**DISCUSSION**

Mounting evidence indicates that mutations in p53 are among the most common genetic events in the development of human cancer (35, 36). On the other hand, \textit{WAF1/Cip1} is well known to be induced by wild-type p53 (16). Hence, it might be plausible that little or no expression of \textit{WAF1/Cip1} is also a common event in cancer cells. Therefore, it would be of great value to identify the p53-independent pathway of \textit{WAF1/Cip1} induction, which could lead to an alternative pathway to suppress the oncogenic progression.

In the present study, we have shown that treatment of either WiDr or MG63 cells with butyrate specifically induces \textit{WAF1/Cip1} mRNA and protein, resulting in G1 arrest of the cell cycle progression in a p53-independent manner. A series of mutation analyses of the \textit{WAF1/Cip1} promoters have revealed that the main butyrate-responsive element is the Sp1 site between \(-82\) and \(-77\) relative to the transcription start site (the Sp1-3 site in this report), and the Sp1 site between \(-69\) and \(-64\) (Sp1-4...
site) is also partially involved in this activation. In addition, we found that butyrate is capable of activating transcription from the luciferase reporter plasmid including only three Sp1 sites. These results strongly suggest that Sp1 is involved in the transcriptional activation of the WAF1/Cip1 promoter in response to butyrate; in fact, EMSA using MG63 cells revealed that Sp1 and Sp3 can specifically interact with this main butyrate-responsive element, the Sp1-3 site. However, the intensity and mobility pattern of the retarded bands were not changed by butyrate, which means that activation of the WAF1/Cip1 promoter by butyrate does not appear to be due to increasing the binding of Sp1 or Sp3. Additionally, butyrate could not affect the phosphorylation pattern of the Sp1 protein (data not shown), and CHX did not block the WAF1/Cip1 mRNA induction by butyrate in MG63 cells (Fig. 2C, lanes 5 and 6). Hence, there is a possibility that Sp1-related or other unknown factors pre-exist and will be subject to modulation, such as phosphorylation, and involved in the activation of
WAF1/Cip1 promoter in response to butyrate. On the other hand, very little is presently known of how the Sp1 modification affects transcription except for phosphorylation or glycosylation (37). Thus, further studies will be required to elucidate the mechanism of how butyrate modulates the potent transcriptional function of Sp1.

Recently, several studies have reported the p53-independent induction of WAF1/Cip1 (17–20). The promoter analysis of the p53-independent pathways has also been documented (25, 26, 38, 39). Biggs et al. (26) have reported that the region between −122 and −61 from the transcription start site, including Sp1-1 to Sp1-4 sites in our paper, is required for both the basal activity and the full activation of the WAF1/Cip1 promoter by phorbol esters and okadaic acid and suggested that Sp1 is involved in this activity by using gel mobility shift assays (26). Furthermore, Datto et al. (25) have identified the main transforming growth factor β-responsive element of the WAF1/Cip1 promoter, termed TβRE, as an element including Sp1 site between −82 and −77 (Sp1-3 site), by using a series of deleted or mutated constructs. It is of interest that TβRE in the WAF1/Cip1 promoter corresponds to the main butyrate-responsive element including the Sp1 site between −82 and −77 (Sp1-3 site). Sp1 protein is a ubiquitously expressed transcription factor that regulates a large number of constitutive and induced mammalian genes by interacting with specific GC-rich elements (GC boxes) (40, 41). It would thus be of great interest to clarify the mechanism by which butyrate and other WAF1/Cip1-inducing factors such as TGF-β act on the Sp1 transcription factor.

In addition, we clearly showed that two overlapping Sp1 sites between −60 and −51 (Sp1-5-6 site) and TATA box are the most essential for the WAF1/Cip1 promoter activity. This discrepancy with the results of Biggs et al. (26) or Datto et al. (25) may be explained by the different cell lines or by small differences in the sequences of generated plasmids (25, 26).

In summary, our results suggest that butyrate-induced growth arrest in WiDr cells is due to the p53-independent activation of WAF1/Cip1 promoter mediated through 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” (42). Our strategy is to activate the potent function of growth-inhibitory genes, which are activating targets of p53. The WAF1/Cip1 gene is one of the good candidates, because WAF1/Cip1 appears to be rarely mutated in human common tumors (43, 44), whereas the p53 gene is frequently mutated (35, 36). Therefore, in the future, clarification of the p53-independent activating pathway of the WAF1/Cip1 gene might contribute to the therapy or the prevention of cancer when p53 is mutated.

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**Fig. 9.** Activation of the promoter activity of Sp1-luc in WiDr cells. WiDr cells were transiently transfected with either the Sp1-luc reporter plasmid or control reporter plasmid pGL2-Basic (Promega), and luciferase activities were measured after incubation with (+) or without (−) 5.0 mM butyrate for 24 h. Data are shown as means (bars, SD) (n = 3). *, p < 0.02.

**Fig. 10.** Sp1 and Sp3 can interact with the main butyrate-responsive element. EMSA was carried out with nuclear extracts prepared from butyrate-treated (lanes 4–6) or untreated (lanes 1–3) M063 cells. End-labeled oligonucleotide containing the sequence of the main butyrate-responsive element between −87 and −72 from the transcription start site was used as a probe. Anti-Sp1 antibody (lanes 2 and 5) or anti-Sp3 antibody (lanes 3 and 6) were used as indicated. The positions of Sp1 and Sp3 are indicated on the left.
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