Biochemical and Biophysical Research Communications **270,** 303–310 (2000)
doi:10.1006/bbrc.2000.2422, available online at http://www.idealibrary.com on IDEAL®

Transcriptional Repression of p21$^{(Waf1/Cip1/Sdi1)}$ Gene by c-jun through Sp1 Site

Chih-Hung Wang,*,†,‡ Yeou-Ping Tsao,* Huei-Jane Chen,* Hui-Ling Chen,* Hsing-Won Wang,‡ and Show-Li Chen*,[1]

**Department of Microbiology and Immunology and †Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan, Republic of China; and ‡Department of Otorhinolaryngology, Tri-Service General Hospital, Taipei, Taiwan, Republic of China*

Received February 28, 2000

Previously, we found that c-jun represses the tumor suppressor p21$^{(Waf1/Cip1/Sdi1)}$ (p21) gene expression. In this study, we further investigated the mechanism of the inhibitory effect of c-jun on p21. After analysis of a series of deletion and point mutants of p21 promoter, we found that Sp1-3 site (-77 and -83) relative to the transcription start site played an important role for c-jun-repressing-responsive element in the p21 promoter. Both Sp1 and Sp3 transcription factors were the key factors for this event. However, the data from electrophoretic mobility shift assay indicated that c-jun did not change the Sp1 DNA-binding affinity, suggesting that additional factors may be involved in the repression of p21 by c-jun. Furthermore, c-jun could inhibit butyrate-inducing p21 gene expression through Sp1, indicating at least one common pathway whereby p21 expression is affected by c-jun and butyrate in opposing actions. Moreover, the hyperphosphorylated retinoblastoma protein (Rb) increased in c-jun expressing cells, indicating that phosphorylated Rb may play a role in regulating Sp1 to repress p21 expression. This is the first demonstration of how housekeeping factors and oncogene product counteract the function of tumor suppressor genes to control cell cycle progression.

Key Words: **p21; c-jun; Sp1.**

The protein p21$^{(Waf1/Cip1/Sdi1)}$ [p21] is encoded by a recently cloned gene (Sdi) and is overexpressed in senescent fibroblast (1, 2). The same p21 protein was found to associate with different cyclin dependent kinase (CDK)-cyclin complexes and inhibits the kinase activity required for cell cycle progression (3, 4). Independent research showed that p53 causes induction of p21 gene and mediates the cell growth arrest induced by p53 (5).

Recent evidence has shown that p21 expression can be stimulated by a number of agents, including tumor growth factor β (TGF-β), phorbol esters, okadaic acid, butyrate, growth factor, interleukin 6, interferon-γ, retinoic acid, and vitamin D3 (6–14), it is possible that a number of elements in the p21 promoter function together to precisely regulate the level of p21 expression. For example, p21 is a retinoic acid (RA)-responsive target gene in U937 cell differentiation, and a functional RA response element in p21 promoter is through RA receptor/RA $\times$ receptor heterodimers (10). TGF-β markedly induces p21 protein and causes G1 growth arrest, and Sp1 is involved in the transcriptional activation of the p21 promoter in response to TGF-β (6). Similarily, Biggs *et al.* (1996) have reported that Sp1 is required for both the basal activity and the full activation of the p21 promoter by phorbol esters and okadaic acid. Also, Nakano *et al.* (1997) have identified Sp1 as the main butyrate-responsive element of the p21 promoter. In addition, nerve growth factor (NGF) which induces PC12 cell differentiation, activates the p21 promoter by stimulating the transactivation domain of Sp1 (12). Such multiple signals may be necessary to generate sufficient p21 expression for growth inhibition, since there are evidences demonstrating that the ratio of p21 protein to target molecules, such cyclin-dependent kinases, determines cell growth effect (7, 15, 16). These elements can be scattered throughout the promoter or may overlap to create potential combined or synergistic responses.

c-jun is an immediate early gene, whose expression is rapidly and transiently induced by extracellular stimuli. It acts as a nuclear messenger converting a cytoplasmic signal into alterations in gene expression (17–19). Previously, we had found that c-jun represses the tumor suppressor p21 gene expression (20). The interaction between the cellular oncogene c-jun and

[1] To whom correspondence should be addressed. Fax: 886-2-87923151. E-mail: yptsao@kimo.com.tw.

0006-291X/00 $35.00

tumor suppressor p21 resulting in the regulation cell cycle progression is intriguing. Hence, in this study we further investigated the mechanism of the c-jun repressing p21 by mutational analysis. Where we constructed a series of deletion and point mutants of p21 promoter to study the c-jun responsive element in p21 promoter. The results demonstrated that Sp1-3 site (-77 and -83) relative to the transcription start site played an important role for c-jun-repressing-response element in the p21 promoter, and Sp1 and Sp3 factors played an important role for c-jun-repressing p21 gene expression.

MATERIALS AND METHODS

Construction of 5′ deletion mutants of p21 promoter. A 2.456 kb HindIII fragment isolated from the full length p21 promoter plasmid-WWP/luc (kindly provided by Dr. Bert Vogelstein), was cloned into SK+ vector (Strategene) with HindIII digestion and alkaline phosphatase to remove 5′ end phosphate, and named pP21/SK. The plasmid pP21/SK was sequenced by dideoxynucleotide termination method. Based on sequence analysis, the restriction enzyme Sau3AI map in p21 promoter was position at nucleotide -2103, -955, -739, and -533 from the transcription start site (Fig. 2A). To generate a series of 5′ deletion mutants, first, we partially digested the plasmid (pP21/SK) with Sau3AI, 5′ deletion mutant fragments were isolated and introduced into the vector TTC which is derived from pGuP.PA.8 vector (kindly provided by Dr. Chen Jeou-Yuan) without a 58 bp fragment of heat shock promoter. Then, their sequence and orientation were confirmed by dideoxynucleotide termination sequencing and named pP21-2103, pP21-955, pP21-739, and pP21-533 respectively. The plasmid pP21-FL containing the full length p21 promoter fragment was constructed by inserting 2.456 kb HindIII fragment from pP21/SK into TTC vector. The orientation of the plasmid pP21-FL was also checked and confirmed by sequencing.

Furthermore, the plasmid-pP21-2275 was constructed by digesting the plasmid pP21/SK with SacI and HindIII; and the plasmid-pP21-1263 by digesting with DraI and HindIII; the plasmid-pP21-215 by digesting with PstI and HindIII (Fig. 2A). Each restriction fragment was then separately isolated and subcloned into TTC vector. Moreover, the 215 bp regions from the transcription start site to -215 included three SmaI sites located at positions -127, -112, and -63, from the transcription start site (Fig. 2B). Likewise, we partially digested the plasmid pP21-215 with SmaI, 5′ deletion mutant fragments were isolated, introduced into the vector TTC, then confirmed their sequence and orientation by dideoxynucleotide termination method, generating pP21-127, pP21-112, pP21-63, pP21-54, pP21-47 and pP21-43 mutants. All 5′ end deletion mutants contained TATA box of p21 promoter and their downstream were fused with the luciferase reporter gene.

Northern blot hybridization. Total cytoplasmic RNA was prepared from cell lines and analyzed by Northern blot hybridization. Filters were washed to remove nonspecifically bound probe, then air-dried, and exposed to Kodak XAR film with Dupont Lightning-plus intensification screens.

Transfections and luciferase assay. Human embryonic epithelial cells-293 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum (Life Technologies, Inc.) and antibiotics. 293 cells were seeded at a density of 500,000 cells per 100-mm dish 24 h prior to transfection. Transfections were carried out by calcium phosphate precipitation method with 5 μg of plasmid pC-jun containing c-jun gene (20) and 5 μg of each luciferase reporter plasmid. As an internal control for the variation in transfection efficiency, 2 μg of pCH110, a plasmid containing the *Escherichia coli* lacZ that was used to monitor β-galactosidase activity was cotransfected with the test plasmids to normalize the luciferase activity. Transfected cells were cultured for 48 h, then lysed with 100 μl of 1 $\times$ cell culture lysis reagent (Promega) for 10 min at room temperature. After spinning 5 s to pellet large debris, 20 μl of supernatant was added to 100 μl of luciferase assay reagent (Promega). Light emission was detected in a luminometer (BioOrbit, Finland).

Immunoblots. Cellular proteins were extracted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 1% SDS, 1 mM phenyl methylsulphonyl fluoride (PMSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin; 600 μl buffer per 100 mm dish) on ice for 30 min. After being boiled for 10 min, about 100 μg of each crude protein lysate was separated by SDS-PAGE, transferred to a nitrocellulose filter, reacted with appropriate dilution of primary antibody to Sp1 (mouse monoclonal serum, Santa Cruz Biotech, CA), Sp3 (rabbit polyclonal serum, Santa Cruz Biotech, CA), or retinoblastoma protein (Rb, mouse monoclonal serum, Santa Cruz Biotech, CA) and visualized by the enhanced chemoluminacence system (Amersham) using procedures recommended by the manufacturer.

Nuclear protein extraction and electrophoretic mobility shift assay (EMSA). Nuclear protein extracts from monolayer culture of cells were prepared as described previously (21). Sequence-specific DNA binding activity in cell nuclear extracts was assayed by EMSA. Assays for binding activity in nuclear extracts from cells generally contained in a 15 μl volume, 5 μg of nuclear protein, 2 μg of poly (dI-dC) carrier (Pharmacia Biotech), 25 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.9), 10% glycerol, 50 mM KCl, 5 mM $MgCl_2$, 2 mM spermidine, 0.1 mM EDTA, and 0.5 mM dithiothreitol. After a short preincubation on ice, the radioactive end-labeled Sp1 DNA probe was added and incubated for 30 min on ice, then the reaction mixtures were loaded onto 5% polyacrylamide gel. The Sp1 DNA probe was performed by annealing oligonucleotides containing Sp1 sequence (5′-ATTCGATCGGGGCGGGGCGAG-3′ and 5′-CTCGCCCCGCCCCGATCGAAT-3′) and labeling with [γ-^{32}P]ATP using the T4 polynucleotide kinase. In competition experiments, the unlabeled DNA, present in 25-fold molar excess, was added to the preincubation mixture. Competitors were as follows, Sp1 is a double-stranded synthetic oligonucleotides (5′-ATTCGATCGGGGCGGGGCGAG-3′) carrying an optimal binding site for the Sp1 homologue. The other competitors, one is a Sp1 mutant competitor (5′-ATTCGATCGGTTCGGGGCGAG-3′) carrying a destructive binding site for the Sp1 homologue; the other is nonspecific oligonucleotides (5′-AATCCGTCGAGCAGAGTT-3′). The reaction mixture was further incubated at 4°C for 1 h in the presence of anti-Sp1 or anti-Sp3 antibody. Complexes and unbound DNA were resolved by electrophoresis at 15 to 20 mA for 5 to 6 h. Gels were dried prior to autoradiography.

RESULTS

p21 Is Repressed by c-jun Genes

We previously found that the concentration of p21 protein was lower in c-jun transfected keratinocytes than that in the parental keratinocytes (20). In the present study, we investigated the effect of c-jun on p21 concentration in human embryonic epithelial cells (293 cells). Figure 1 reveals that concentration of p21 RNA decreased in c-jun transfected cells (c-jun/293, lane 1) as compared with the 293 cells containing vector only (V/293, lane 2). The decreased concentration of p21 RNA by c-jun could be due to repression of p21 promoter activity. The promoter activity of p21 gene was therefore investigated by transient transfection as-

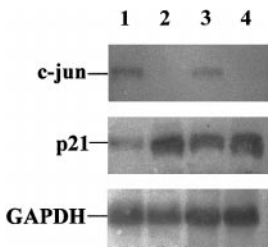

FIG. 1. c-jun repress p21 RNA expression by Northern blot. RNA (20 μg) was fractionated by agarose gel electrophoresis, blotted, and hybridized with p^{32}-labeled p21, c-jun, and GAPDH cDNA (glyceraldehyde-3-phosphate dehydrogenase) separately. A GAPDH probe was used to ensure equal RNA loading. Lane 1: c-jun/293: 293 cells containing c-jun gene; lane 2: V/293: 293 cells containing the vector alone (pCEP4); lane 3: c-jun/293 cells treated with 1.0 mM sodium butyrate for two days; lane 4: V/293 cells treated with 1.0 mM sodium butyrate for two days.

says. We cotransfected the p21 promoter luciferase reporter plasmid (WWP-Luc, 5 μg) and c-jun plasmid (5 μg) into 293 cells. Luciferase reporter analysis shows that c-jun repressed p21 promoter activity in 293 cells (Fig. 2).

Analysis of c-jun-Responsive Elements in the p21 Promoter

Next, we tried to determine what regions of the p21 promoter were responsive to c-jun repression. For this purpose, a series of 5′ deletion constructs of the p21 promoter were generated as described under Materials and Methods. Five μg of each of the 5′ deletion mutants, containing 2456 bp (pP21-FL), 2275 bp (pP21-2275), 2103 bp (pP21-2103), 1263 bp (pP21-1263), 955 bp (pP21-955), 739 bp (pP21-739), 533 bp (pP21-533), 215 bp (pP21-215), 127 bp (pP21-127), 112 bp (pP21-112), 63 bp (pP21-63), 54 bp (pP21-54), 47 bp (pP21-47), or 43 bp (pP21-43) of 5′ flanking sequences which were cloned upstream of the luciferase reporter gene (Fig. 2A and 2B) and cotransfected either with 5 μg pC-jun or 5 μg pCEP4 into 293 cells using the calcium phosphate precipitation method. After 48 h, the cells were harvested and assayed for luciferase activity. The plasmid pC-jun is a c-jun expressing vector, and pCEP4 acts as a control plasmid vector. We included an internal control plasmid pCH110 (2 μg) in every transfection to allow normalization for transfection efficiency in the subsequent analysis. As shown in Fig. 2A, the repression activities of pP21-FL, pP21-2275, pP21-2103, pP21-1263, pP21-955, pP21-739, pP21-533, and pP21-215 by c-jun in 293 cells were 4.94, 4.70, 4.81, 3.90, 3.96, 4.01, 4.26, and 3.86 fold of those of pCEP4 vector from three independent experiments. Furthermore, Fig. 2B shows that the repression activities of pP21-215, pP21-127, pP21-112, pP21-63, pP21-54, pP21-47, and pP21-43 by c-jun in 293 cells were 3.86, 3.78, 3.76, 1.44, 1.26, 1.39, and 1.10 fold of those of pCEP4 vector from three independent experiments. Taken together, these results indicate that Sp1 containing region between -112 and -43 bp is responsible for the repression of p21 promoter by c-jun, indicating that the Sp1 site of the p21 promoter can be repressed by the c-jun gene.

This 70-bp region between -112 and -43 bp harbors two independent and two overlapping nearly consensus binding sites for transcription factor Sp1 (6, 8). Previously, they are termed Sp1-3, Sp1-4, Sp1-5 and Sp1-6 from the upstream. To accurately determine whether these Sp1 binding sites are involved in the repression by c-jun. A series of point mutation of p21 promoter including p21P 93-S, p21P 93-S mut#1, p21P 93-S mut#2, p21P 93-S mut#3, and p21P 93-S mut#4 were kindly provided by Dr. Xiao Fang Wang (6), and were used to assay the luciferase activity. These plas-

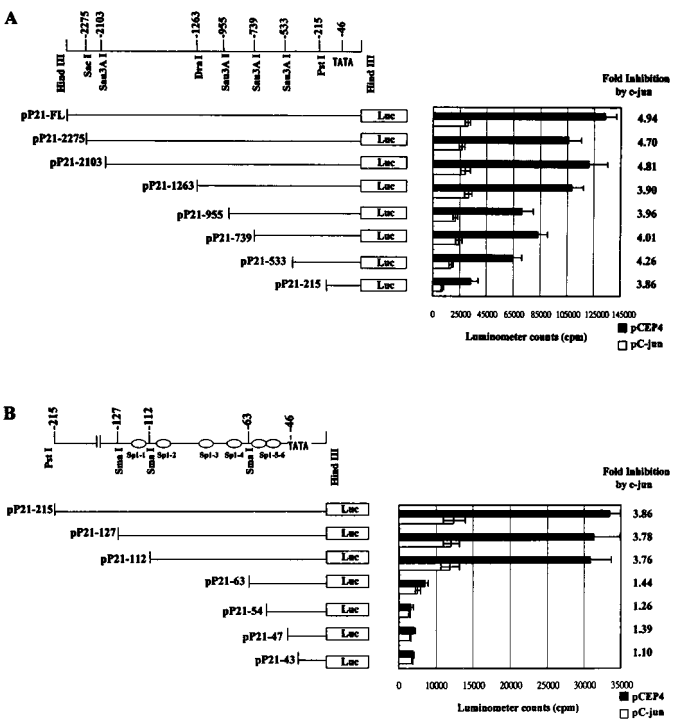

FIG. 2. Sp1 is the c-jun responsive element in p21 promoter by a series of 5′ deletion mutants of p21 promoter. (A) 5 μg of each 5′ deletion mutants of p21 promoter, including pP21-FL, pP21-2275, pP21-2103, pP21-1263, pP21-955, pP21-739, pP21-533, and pP21-215, cotransfected with either 5 μg pC-jun or 5 μg pCEP4 into 293 cells using the calcium phosphate precipitation method. After 48 h, the cells were harvested and assayed for luciferase activity. The plasmid pC-jun is a c-jun expressing vector, and pCEP4 acts as a control plasmid vector. The fold repression is the ratio of luciferase activity cotransfection experiments with the c-jun expression vectors to the activity obtained with the control vector plasmid, and is the average of three different experiments. (B) The same experiments were performed as described in A; the test promoters include pP21-215, pP21-127, pP21-112, pP21-63, pP21-54, pP21-47, and pP21-43. Results are shown as means ± S.D. from three independent experiments. Significant difference of fold inhibition by c-jun between pCEP4 and pC-jun at $P < 0.05$ by Student's t test.

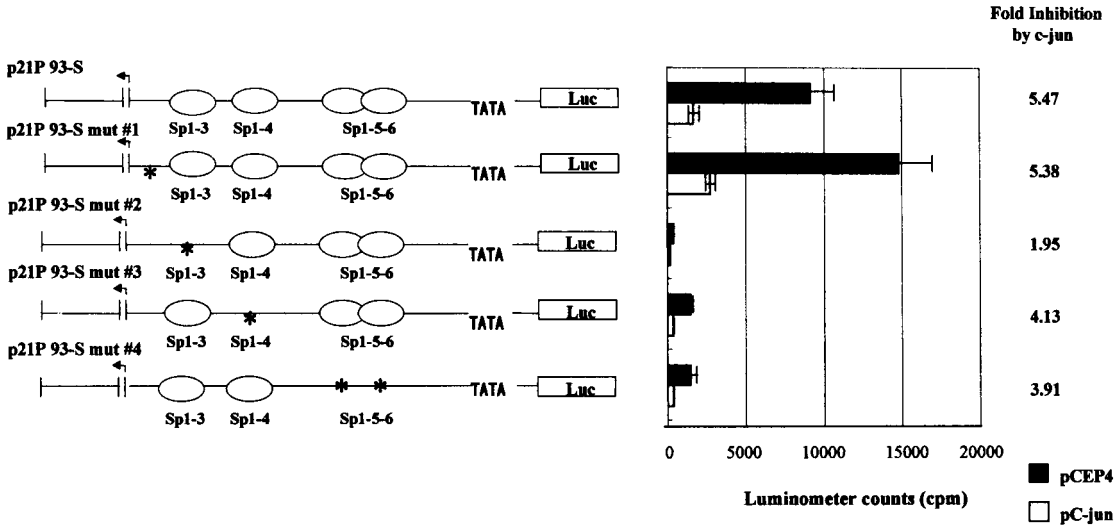

FIG. 3. Sp1-3 site is c-jun responsive element in the p21 promoter by point mutants of p21 promoter. 5 μg of each Sp1 point mutants of p21 promoter, including p21P 93-S, p21P 93-S mut#1, p21P 93-S mut#2, p21P 93-S mut#3, and p21P 93-S mut#4 were cotransfected with either 5 μg or pC-jun or 5 μg pCEP4 into 293 cells using the calcium phosphate precipitation method. The fold of repressing p21 gene expression by c-jun was measured as described in Fig. 2. An asterisk indicates the position of each Sp1 point mutation. Results are shown as means ± SD from three independent experiments. Significant difference of fold inhibition by c-jun between pCEP4 and pC-jun at $P < 0.05$ by Student's t test.

mids were also co-transfected with control vector or vector expressing c-jun. Figure 3 presents the data from three separate experiments and demonstrates that Sp1-3 binding site played an important role for repressing p21 promoter activity by c-jun.

Identification of Proteins Interacting with the Main c-jun Responsive Element

To determine if Sp1 or other proteins can interact with the main c-jun-responsive element, EMSAs were performed using the oligonucleotides containing the wild-type Sp1 site. Nuclear extracts were purified from either c-jun-transfected (c-jun/293) or vector-transfected cells (V/293). As shown in Fig. 4, two major DNA-protein complexes were detected, which were competed away by an excess of unlabeled homologous Sp1 oligonucleotides (lanes 3 and 10), but not by the Sp1 mutant competitor (lanes 4 and 11) both in V/293 and c-jun/293 cells, respectively. 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 6 and 13), and the lower complex was diminished in the presence of Sp3 antibody (lanes 7 and 14) both in V/293 and c-jun/293 cells respectively. However, both the mobility pattern and intensity were not different between V/293 and c-jun/293 cells.

The electrophoretic mobility shift experiments confirmed that c-jun did not induce Sp1 and Sp3 to Sp1 site binding affinity. We also detected and compared the protein levels of Sp1 and Sp3 between V/293 cells and c-jun/293 cells by immunoblot analysis. As shown in Fig. 5, the results show that there was no difference between the expression of Sp1 (A) and Sp3 (B) in these two cells, indicating that there may be some pre-existing factors modulating p21 gene expression by c-jun.

The c-jun-Repressing p21 Gene Is the Same Pathway but the Opposite Direction of Butyrate-Activating p21

Previous reports demonstrated that sodium butyrate can induce the p21 gene expression via Sp1-3 binding site in p21 promoter; also the EMSA assay shows that the mobility and intensity patterns of Sp1 and Sp3 factors are not changed in butyrate-treated cells (8). Similarly, we find that Sp1-3 binding site of p21 promoter is important for c-jun to repress p21 expression. Hence, we investigated whether the process of c-jun repression of p21 occurs along the same pathway as butyrate activation of p21, but in the opposite direction. First, we performed the experiments by introducing c-jun gene and p21 promoter reporter plasmid into 293 cells; 24 h after the transfection, different dose of sodium butyrate was added. Twenty-four hours later, cells were harvested for luciferase activity assay. Figure 6A shows that c-jun could inhibit butyrate-induced full-length p21 promoter activity when the concentration of butyrate was 0.3, 0.6, and 1.0 mM respectively. Likewise, Fig. 6B demonstrates that c-jun also inhib-

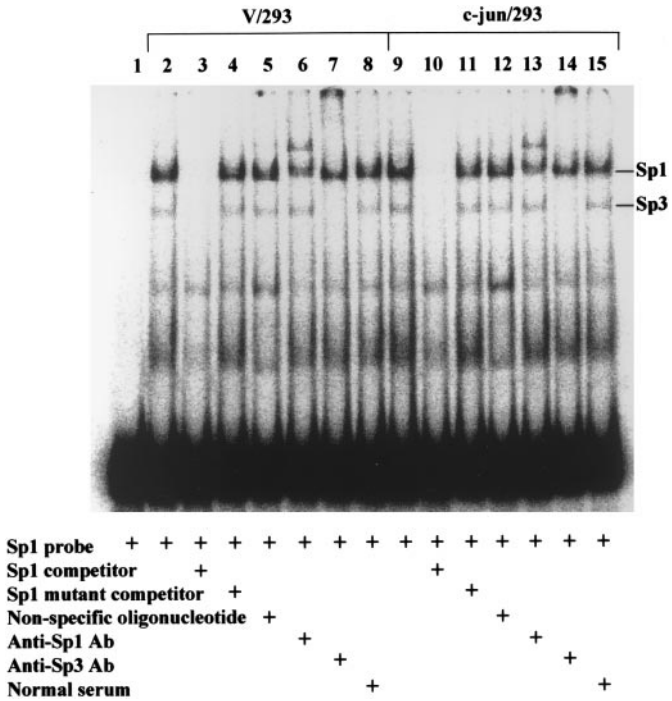

FIG. 4. Electrophoretic mobility shift assay of Sp1 and Sp3 in cells. EMSAs were performed as described in Materials and Methods. Lane 1: labeled Sp1 containing probe without nuclear extracts; lanes 2 to 8: labeled Sp1 probe was incubated with nuclear extracts of V/293 cells; lanes 9 to 15: labeled Sp1 probe was incubated with nuclear extracts of c-jun/293 cells. In experiments depicted in lanes 3 and 10, 25-fold molar excess of non-radioactive synthetic oligonucleotides of Sp1 were included in the incubation reaction; lanes 4 and 11, 25-fold molar excess of nonradioactive synthetic oligonucleotides of mutant Sp1; lanes 5 and 12, 25-fold molar excess of nonradioactive and nonrelated synthetic oligonucleotides were included in the incubation reaction. In experiments depicted in lanes 6 and 13, cell extracts were preincubated with 4 μg of anti-Sp1 antibody for 1 h at 4°C before probes were added. In experiments depicted in lanes 7 and 14, cell extracts were preincubated with 4 μg of anti-Sp3 antibody; lanes 8 and 15, cellular extracts were preincubated with 4 μg of normal serum.

ited butyrate-induced promoter activity in pP21-215, which only contains six Sp1 binding sites. Additionally, as shown in Fig. 1, c-jun/293 and V/293 cells were treated with 1.0 mM sodium butyrate for two days. Comparing with V/293 cells (lane 4), c-jun in butyrate-treated c-jun/293 cells could decrease p21 gene expression (lane 3). Taken together, c-jun can inhibit butyrate-inducing p21 gene expression through Sp1, indicating at least one common pathway whereby p21 expression is affected by c-jun and butyrate in opposing actions.

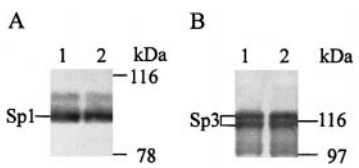

FIG. 5. Immunoblot analysis of Sp1 and Sp3 protein. Cellular extracts were isolated by antiphosphatase buffer treatment as described under Materials and Methods. Sp1 and Sp3 proteins were measured by immunoblot analysis with Sp1 antibody (panel A) and Sp3 antibody (panel B). Lane 1: c-jun/293: 293 cells containing c-jun gene; lane 2: V/293: 293 cells containing the vector alone (pCEP4).

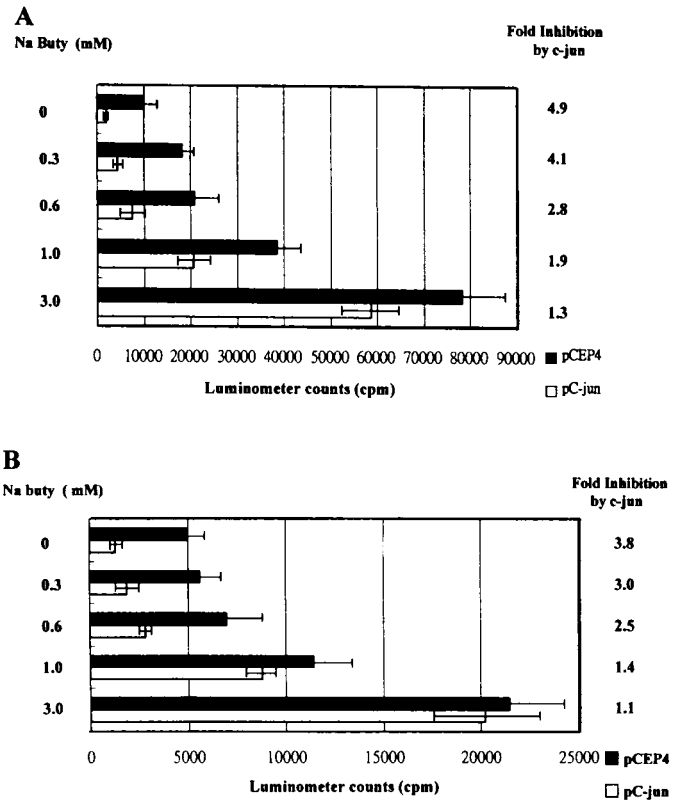

FIG. 6. The pathway of c-jun repressing-p21 is the same but the opposite direction of butyrate-activating-p21. The full length p21 promoter pP21-FL (A) or pP21-215 containing six Sp1 binding sites (B) was co-transfected with c-jun expression vector into 293 cells in the presence or absence of different concentration of sodium butyrate, separately. Luciferase activities were measured from three independent transfection experiments as described in Fig. 2. Results are shown as means ± S.D. from three independent experiments. Significant difference of fold inhibition by c-jun between pCEP4 and pC-jun at $P < 0.05$ by Student's t test.

c-jun Repressing p21 Gene Expression Can Affect Rb Phosphorylation

Several groups have shown that Rb is able to regulate transcription of c-fos, c-myc, and TGF-β1 promoters (22–24) by transient assays in either a positive or negative manner, dependent on the cell type. The Rb control elements (RCEs) in these promoters have been defined and find to interact with Sp1 (22–24), and Sp1 also plays an important role for TGF-β-activating p21 gene expression (6). In this study, we demonstrate that Sp1 is involved in c-jun-repressing p21 gene expression. In order to determine the relationship between

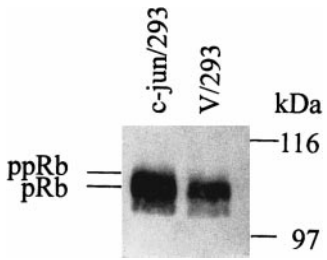

FIG. 7. c-jun repressing p21 gene expression affects pRb phosphorylation. Cellular proteins were extracted from antiphosphatase treated cells as described under Materials and Methods. Rb protein was measured by immunoblot analysis with mouse monoclonal anti-Rb antibody. c-jun/293: 293 cells containing c-jun gene; V/293: 293 cells containing the vector alone (pCEP4).

Rb and p21 in c-jun expressing cells, we investigated the level and phosphorylation status of Rb. As shown in Fig. 7, c-jun would increase the level and phosphorylation of Rb protein, indicating that hyperphosphorylated Rb induction by c-jun may either release E2F to transactivate the cell growth related genes or decrease p21 gene expression to allow cells enter cell cycle.

DISCUSSION

Using several mutant p21 promoter fragments, our results find that Sp1-3 (-77 and -83) site relative to the transcription start site played an important role for c-jun-repressing-responsive element in the p21 promoter. Recently, many evidences showed that p21 expression can be stimulated by a number of agents, including butyrate, TGF-β, phorbol esters, and okadaic acid through Sp1 binding sites of p21 promoter (6–8). In the p21 promoter, there are four independent Sp1 binding sites and two overlapping Sp1 binding sites, and they are termed Sp1-1, Sp1-2, Sp1-3, Sp1-4, Sp1-5, and Sp1-6 from the upstream. A series of mutation analyses of the p21 promoters have revealed that the main butyrate-responsive element is the Sp1-3 site between -83 and -77 relative to the transcription start site. Datto *et al.* (1995) also identified Sp1-3 site as the main TGF-β-responsive element of the p21 promoter. Biggs *et al.* (1996) have reported that the region between -122 and -61 from the transcription start site, including Sp1-1 to Sp1-4 sites is required for both the basal activity and the full activation of the p21 promoter by phorbol esters and okadaic acid. In sum, it is of interest that the main butyrate, TGF-β, and c-jun response element are in the same area of the genome (Sp1-3).

In this study, we demonstrate that Sp1 and Sp3 could interact Sp1 binding site and played a role for c-jun-repressing p21 gene expression. Sp1 is a ubiquitously expressed nuclear protein that was initially identified as a protein that binds and stimulates transcription of the SV40 early promoter (25, 26). Sp3 belongs to the same family of Sp1 related transcription factor, and is also a ubiquitously expressed protein (27). Previous studies indicate that Sp1 and Sp3 appear to bind similar, if not identical, nucleotide sequences with similar affinity, indicating Sp1 and Sp3 may compete for their cognate DNA-binding sites in mammalian cells (28). Sp1 and Sp3 have shown to play a central role in trans-activation of a multitude of cellular promoters such as p21 promoter (6, 8). Consequently Sp1 and Sp3 have fallen into the stereotype of being a "house-keeping" transcription factor, whose activities are necessary solely for the basal transcription of many genes. In contrast to previous reports of Sp1 and Sp3 being a positive role for inducing p21 gene expression by butyrate (8) and TGF-β (6, 29–31), our study shows that Sp1 and Sp3 negatively regulates p21 gene expression by c-jun.

Our results reveal that Sp1 and Sp3 could specifically interact with Sp1 site of p21 promoter, however, the intensity and mobility pattern of the retarded bands were not changed by c-jun (Fig. 4), and c-jun did not affect the phosphorylation and level patterns of the Sp1 and Sp3 proteins (Fig. 5). It implies that activation of the p21 promoter by c-jun is not due to the increase in the binding of Sp1 or Sp3. Consistent results of the intensity and mobility patterns of Sp1 and Sp3 also occur in butyrate and TGF-β inducing p21 gene expression experiments (6, 8). Therefore, there may be some shared and pre-existing factors responsible for the induction of p21 by butyrate and TGF-β or the repression of p21 by c-jun. These factors may become phosphorylated or interact with proteins that are modified or phosphorylated upon these treatments to activate or repress transcription. Very little is presently known of how the Sp1 and Sp3 modifying cellular factors affect transcription. Thus, further studies will be required to elucidate the mechanism of how c-jun modulates the potent transcriptional function of Sp1 and Sp3.

In this study, we demonstrate that Sp1 binding site was a target for c-jun repressing p21 expression (Fig. 3), and hyperphosphorylated Rb increased in c-jun expressing cells (Fig. 7), indicating that phosphorylated Rb may play the role for regulating Sp1 to repress p21 expression. Previous evidences indicate that Rb can function as a positive or negative regulator of transcription and that Sp1 is one potential target, directly or indirectly, for transcriptional regulation by Rb (22–24). For example, overexpression of Rb can lead to activation of TGF-β through Sp1 factors interacting with Rb control element (22–24), and TGF-β can cause growth arrest by inducing p21 gene expression through Sp1 (6, 29–31). Hence, it is proposed that by maintaining Rb in an hypophosphorylated state, TGF-β may exert its effects on the p21 promoter through Sp1. Induction of p21 would in turn lead to a further increase in the hypophosphorylated form of Rb, thus establishing a positive feedback. Recent evidence also

indicates that the Sp1/Sp3 trans-activation domains are bound by cellular proteins that may negatively regulate their activity in vivo (28, 32, 33). The overexpression of Rb can superactivate Sp1/Sp3 by liberating these factors from negative regulation. These negatively-regulating factors have been identified, one is a 20-kDa inhibitor of Sp1 (Sp1-I) that interacts between Sp1 and Rb. When Rb binds and inactivates Sp1-I, leading to transcriptional activation by Sp1 (32). The other is a 74-kDa protein that binds to the transactivation domain of Sp1 and inhibits Sp1-mediated transactivation (33). These reports suggest that there are multiple inhibitors of Sp1, which could inhibit interaction of Sp1 with the transcription factors. In the future, it would be interesting to investigate how c-jun regulates inhibitors of Sp1, and interaction between Rb and Sp1 to regulate p21 gene expression.

Our results show that c-jun could inhibit butyrate-inducing p21 gene expression through Sp1, indicating that at least one common pathway whereby p21 expression is affected by c-jun and butyrate in opposing actions. Previous report demonstrated that sodium butyrate can induce the p21 gene expression via Sp1-3 binding site, also the EMSA assay shows that the mobility and intensity patterns of Sp1 and Sp3 factors are not changed by butyrate-treated cells (8). Similarly, our results find that Sp1-3 binding site is important for c-jun repressing p21 expression. Hence, we further investigated whether the pathway of repressing-p21 gene expression by c-jun was the same but the opposite direction of activating-p21 gene expression by butyrate. Figure 6 shows that c-jun could inhibit butyrate-inducing the p21 promoter activity in different concentration of sodium butyrate.

In this study, we are the first demonstration that Sp1-3 site (-77 and -83) relative to the transcription start site play an important role for c-jun-repressing-responsive element in the p21 promoter, and both Sp1 and Sp3 transcription factors are the key factors for this event. Moreover, the hyperphosphorylated Rb increased in c-jun expressing cells, indicating that phosphorylated Rb may play the role for regulating Sp1 to repressing p21 expression. These provide an example of the interaction between oncogene and tumor suppressor gene and give clues how housekeeping factors and oncogene product counteract the function of tumor suppressor genes to control cell cycle progression.

ACKNOWLEDGMENTS

We thank Dr. Xiao-Fan Wang for providing Sp1 point mutant plasmids of p21 promoter, Dr. Bert Vogelstein for providing p21 promoter, Dr. Jeou-Yuan Chen for providing the plasmid pGuP.PA, Dr. Jer-Tsong Hsieh for critical discussion, and Mr. John Wu for editing the English for the manuscript. This research was supported by National Science Council (NSC 87-2314-B016-045), Taipei, Taiwan, Republic of China.

REFERENCES

1. Noda, A., Ning, Y., Venable, S. F., Pereira, S. O., and Smith, J. R. (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* **211,** 90–98.
2. Tsao, Y. P., Kuo, S. W., Li, S. F., Liu, J. C., Lin, S. Z., Chen, K. Y., and Chen, S. L. (1995) Differential regulation of cyclin A, cyclin B, and p21 concentrations in a growth-restricted human fibroblasts cell line. *Biochem. J.* **312,** 693–698.
3. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature* **366,** 701–704.
4. Xiong, Y., Zhang, H., and Beach, D. (1993) Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. *Genes Dev.* **7,** 1572–1583.
5. El-Deiry, W. S., Tokino, S. T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* **75,** 817–825.
6. Datto, M. B., Yu, Y., and Wang, X. F. (1995) Functional analysis of the transforming growth factor β responsive elements in the WAF1/Cip1/p21 promoter. *J. Biol. Chem.* **270,** 28623–28628.
7. Biggs, J. R., Kudlow, J. E., and Kraft, A. S. (1996) The role of the transcription factor Sp1 in regulating the expression of the WAF1/CIP1 gene in U937 leukemic cells. *J. Biol. Chem.* **271,** 901–906.
8. Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H., and Sakai, T. (1997) Butyrate activates the WAF1/Cip1 gene promoter through sp1 sites in a p53-negative human colon cancer cell line. *J. Biol. Chem.* **272,** 22199–22206.
9. Liu, M., Lee, M. H., Cohen, M., Bommakanti, M., and Freedman, L. P. (1995) Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* **10,** 142–153.
10. Liu, M., Iavarone, A., and Freedman, L. P. (1996) Transcriptional activation of the human $p21^{WAF1/CIP1}$ gene by retinoic acid receptor. *J. Biol. Chem.* **271,** 31723–31728.
11. Duttaroy, A., Qian, J. F., Smith, J. S., and Wang, E. (1997) Up-regulated $p21^{cip1}$ expression is part of the regulation quantitatively controlling serum deprivation-induced apoptosis. *J. Cell Biochem.* **64,** 434–446.
12. Yan, G. Z., and Ziff, E. B. (1997) Nerve growth factor induces transcription of the p21 WAF1/CIP1 and cyclin D1 genes in PC12 cells by activating the Sp1 transcription factor. *J. Neurosci.* **17,** 6122–6132.
13. Xiao, H., Hasegawa, T., Miyaishi, O., Ohkusu, K., and Isobe, K. I. (1997) Sodium butyrate induces NIH3T3 cells to senescence-like state and enhances promoter activity of $p21^{WAF/CIP1}$ in p53-independent manner. *Biochem. Biophys. Res. Commun.* **237,** 457–460.
14. Bellido, T., O'Brien, C. A., Roberson, P. K., and Manolagas, S. C. (1998) Transcriptional activation of the $p21^{WAF1, CIP1, SDI1}$ gene by interleukin-6 type cytokines. *J. Biol. Chem.* **273,** 21137–21144.
15. Sherr, C. (1994) G1 phase progression: Cycling on cell. *Cell* **79,** 551–555.
16. Zhang, H., Hannon, G. J., and Beach, D. (1994) P21-containing cyclin kinases exist in both active and inactive states. *Genes Dev.* **8,** 1750–1758.
17. Ryder, K., and Nathans, D. (1988) Induction of protooncogene c-jun by serum growth-factors. *Proc. Natl. Acad. Sci. USA* **85,** 8464–8467.
18. Wisdom, R., Johnson, R. S., and Moore, C. (1999) c-jun regulates

cell cycle progression and apoptosis by distinct mechanisms. *EMBO J.* **18,** 188–197.

19. Treisman, R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.* **8,** 205–215.
20. Tsao, Y. P., Li, L. Y., Tsai, T. C., and Chen, S. L. (1996) Human papillomavirus type 11 and 16 E5 represses $p21^{Waf1/SdiI/CipI}$ gene expression in fibroblasts and keratinocytes. *J. Virol.* **70,** 7535–7539.
21. Chen, S. L., Lin, Y. K., Li, L. Y., Tsao, Y. P., Lo, H. Y., Wang, W. B., and Tsai, T. C. (1996) E5 proteins of HPV-11 and HPV-16 transactivate the c-fos promoter through NF1 binding element. *J. Virol.* **70,** 8558–8563.
22. Kim, S. J., Lee, H. D., Robbins, P. D., Busam, K., Sporn, M. B., and Roberts, A. B. (1991) Regulation of transforming growth factor β1 gene expression by the product of the retinoblastoma-susceptibility gene. *Proc. Natl. Acad. Sci. USA* **88,** 3052–3056.
23. Kim, S. J., Onwuta, U. S., Lee, Y. I., Li, R., Botchan, M. R., and Robbins, P. D. (1992) The rebinoblastoma gene product regulates Sp1-mediated transcription. *Mol. Cell. Biol.* **12,** 2455–2463.
24. Udvadia, A. J., Rogers, K. T., Higgins, P. D. R., Murata, Y., Martin, K. H., Humphrey, P. A., and Horowitz, J. M. (1993) SP-1 binds promoter elements regulated by the RB protein and Sp-1-mediated transcription is stimulated by RB coexpression. *Proc. Natl. Acad. Sci. USA* **90,** 3265–3269.
25. Dynan, W. S., and Tjian, R. (1983) Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. *Cell* **32,** 669–680.
26. Dynan, W. S., and Tjian, R. (1983) The promoter-specifei transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* **35,** 79–87.
27. Kennett, S. B., Udvadia, A. J., and Horowitz, J. M. (1997) Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription. *Nucleic Acids Res.* **25,** 3110–3117.
28. Udvadia, A. J., Templeton, D. J., and Horowitz, J. M. (1995) Functional interactions between the retinoblastoma (Rb) protein and Sp-family members: Superactivation by Rb requires amino acids necessary for growth suppression. *Proc. Natl. Acad. Sci. USA* **92,** 3953–3957.
29. Yingling, J. M., Datto, M. B., Wong, C., Frederick, J. P., Liberati, N. T., and Wang, X. F. (1997) Tumor suppressor Smad4 is a transforming growth factor β-inducible DNA binding protein. *Mol. Cell. Biol.* **17,** 7019–7028.
30. Moustakas, A., and Kardassis, D. (1998) Regulation of the human p21/WAF1/Cip1 promoter in hepatic cell by functional interactions between Sp1 and Smad family members. *Proc. Natl. Acad. Sci. USA* **95,** 6733–6738.
31. Hunt, K. K., Fleming, J. B., Abramian, A., Zhang, L., Evans, D. B., and Chiao, P. J. (1998) Overexpression of the tumor suppressor gene Smad4/DPC4 induces $p21^{waf1}$ expression and growth inhibition in human carcinoma cells. *Cancer Res.* **58,** 5656–5661.
32. Chen, L. I., Nishinaka, T., Kwan, K., Kitabayashi, I., Yokoyama, K., Fu, Y. F., Grunwald, S., and Chiu, R. (1994) The retinoblastoma gene product RB stimulates Sp1-mediated transcription by liberating Sp1 from a negative regulator. *Mol. Cell. Biol.* **14,** 4380–4389.
33. Murata, Y., Kim, H. G., Rogers, K. T., Udvadia, A. J., and Horowitz, J. M. (1994) Negative regulation of Sp1 trans-activation is correlated with the binding of cellular proteins to the amino terminus of the Sp1 trans-activation domain. *J. Biol. Chem.* **269,** 20674–20681.