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ARTICLES

Both Sp1 and Sp3 Are Responsible for p21^{waf1} Promoter Activity Induced by Histone Deacetylase Inhibitor in NIH3T3 Cells

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Abstract Histone deacetylase inhibitor-induced expression of p21^{WAF1} is p53 independent. In the present study, we provide evidence that trichostatin A (TSA), a specific inhibitor of histone deacetylase, can elevate H3 and H4 acetylation and p21^{WAF1} expression in NIH3T3 cells at first. To identify the transcription factor which is responsible for histone deacetylase inhibitor-induced expression of p21^{WAF1} and understand the potential events occurred during this process, we analyze the response of the mouse p21^{WAF1} promoter to TSA in detail. The region responsive to TSA treatment in the p21 promoter is located −100 bp upstream from transcription initiation site and contains a GC-box. The mutation introduced into this GC-box decreases most of the basal and TSA-induced promoter activity. The results from gel-shift assay show that Sp1 and Sp3 bind to this GC-rich region. Cotransfection with Sp1 and/or Sp3 expression constructs elevate both basal and induced promoter activity, and this elevation is dependent on the present of the GC-box. By contrast, cotransfection with reverse oriented Sp1 or Sp3 cDNA decreased basal and induced-promoter activity, as well as GC-box dependency. These findings provide physical and functional evidence which strongly indicated that both Sp1 and Sp3 are responsible for TSA-induced transactivation of the murine p21^{WAF1} promoter in NIH3T3 cells. J. Cell. Biochem. 73:291–302, 1999.

Key words: Sp1, Sp3, p21^{WAF1}; promoter; histone acetylation

p21^{WAF1} (p21) was first cloned and characterized as an important effector that acts to inhibit cyclin-dependent kinase activity in p53-mediated cell cycle arrest induced by DNA damage [El-Deiry et al., 1993; Dulic et al., 1994; Gu et al., 1993]. It was found subsequently that the induction of p21 occurs in various differentiation systems independent of p53 [Halevy et al., 1995; Jiang et al., 1995; Macleod et al., 1995; Missero et al., 1995; Parker et al., 1995]. We have found that sodium butyrate induces senescence-like phenotype in NIH3T3 cells and enhances mouse p21 promoter activity in this cell line. We concluded that sodium butyrate-induced p21 promoter activity is p53 independent because mutants containing deletions of the p53 binding sites in this promoter also responded to butyrate treatment and the responsiveness of the p21 promoter to butyrate was not abolished in p53 deficient fibroblast cell line [Xiao et al., 1997]. Given that p21 is a marker gene for growth arrest and differentiation, an understanding of the details of its transcriptional regulation would be significant to studies involving in growth versus differentiation control.

It has been shown that a GC-rich region in the human p21 promoter, located upstream of the TATA box, acts as an important regulatory element responsive to various agents, such as phorbol myristate acetate (PMA)/okadaic acid, transforming growth factor-β (TGF-β), calcium, nerve growth factor (NGF), and butyrate [Biggs et al., 1996; Datto et al., 1995; Prowse et al., 1997; Yan et al., 1997; Nakano et al., 1997].

Abbreviations used: p21, p21^{WAF1}; EMSA, electrophoretic mobility shift assays; TSA, trichostatin A; RLU, relative luciferase unit; FCS, fetal calf serum.

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This GC-rich region and its in vivo binding factors, therefore, may play a critical role in p21 expression.

Sp1 is a sequence-specific transcription factor that recognizes GGGGCGGGG and closely related sequences, often referred to as GC boxes. The sequence specificity for DNA binding is conferred by its three zinc fingers, whereas a different region of Sp1 appears to regulate its affinity for DNA [Kadonaga et al., 1987]. In addition to Sp1, there are at least three homologous transcription factors in the Sp1 family: Sp2, Sp3, and Sp4 [Hagen et al., 1992; Kingsley et al., 1992]. Although all these factors can bind to the GC box and elevate RNA synthesis from the genes that contain the GC box according to in vitro experiments, they work in coordination or independently in various in vivo situations [Majello et al., 1994; Nielsen et al., 1998]. Moreover, some researchers state that at least one member of this family, Sp3, may function as a repressor of these family [Hagen et al., 1994; Denning et al., 1996]. Therefore, investigating the factors that function in vivo in special cellular contexts is becoming an essential step in the study of GC box containing promoters. There is no direct evidence that has defined which cellular transcription factor is responsible for histone deacetylase inhibitor-induced p21 activation up to now.

Furthermore, histone deacetylase inhibitors have been shown to induce histone hyperacetylation in cultured cells [Schlake et al., 1994; Yamamoto et al., 1996], and the growth arrest and p21 expression induced by them would be the results of histone acetylation. This means that the p21 gene is one of the natural targets of histone deacetylase inhibitors. Herein, transactivation of p21 by a histone deacetylase inhibitor may provide a native model for investigating the molecular events concerned with histone acetylation, one of the most progressive research frontiers of recent times. Based on the above considerations, we focused our efforts on developing this line of research by (1) testing the effect of trichostatin A (TSA), a specific inhibitor of histone deacetylase [Yoshida et al., 1995], on NIH3T3 cells, with special emphasis on the state of histone acetylation and the expression of p21 in this cells; (2) defining the *cis*-element responsive to TSA in the p21 promoter by testing various mutations in the murine p21 promoter; and (3) identifying the transcriptional activators responsible for TSA-induced activation of the p21 promoter. We hope this work will contribute to an understanding of the transcriptional regulation of the p21 and provide a means of analyzing the interaction between histone acetylation and other signal transduction pathways.

MATERIALS AND METHODS

Cell Culture and Reagent

NIH3T3 cells from the American Type Culture Collection (ATCC, Rockville, MD) were maintained in a 37°C humidified atmosphere containing 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). TSA (Wako, Osaka, Japan) stock was dissolved in distilled phosphate-buffered saline (PBS) with 10 μg/ml of concentration and stored at 4°C.

Western Blotting

Cells were lysed with 2× loading buffer (60 mM Tris-base, pH6.8, 4% sodium dodecyl sulfate (SDS), 5.76 mM β-mercaptoethanol, 20% glycerol, and 0.2% bromophenol blue) and boiled for 10 min. A total of 30 μg of proteins for each lane were separated on 15% SDS-polyacryamide gel and electroblotted to a polyvinylidene difluoride (PVDF) membrane. The blots were blocked in TBS-Tween (0.05%) containing 5% ovalbumin for 1 h at room temperature and probed for 2 h with 1:200 diluted polyclonal antibody against acetylated histone H3 or polyclonal antibody against acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY). After washing three times with TBS-T for 5 min, the blots were incubated in 1:1,000 diluted peroxidase-conjugated secondary antibody (Chemicon, AP182P) for another 1 h. The blots were again washed as above and developed with an enhanced chemiluminescence system (ECL; Amersham, Piscataway, NJ).

Reverse Transcription Polymerase Chain Reaction

Total RNA from proliferating or TSA-treated NIH3T3 cells was isolated and used directly for the first-strand cDNA synthesis. Reverse transcription-polymerase chain reaction (RT-PCR) proceeded as described previously [Xiao et al., 1997]. The primers used for PCR are as follows: 5′-agatccacagcgatatccagac-3′ (p21sense), 5′-acacacagagagagggctaagg 3′ (p21antisense); 5′-tgaaggtcggtgtgaacggatttggc-3′ (G3PDH sense), 5′-catgtaggcc-atgaggtccaccac-3′(G3PDH antisense).

Constructs

p21 promoter-containing reporter constructs were made as follows: pGL3b-4542 was constructed via insertion of a 4.6-kb fragment of the murine p21 promoter region, from −4542 to +132 bp relative to TATA box, into a firefly luciferase reporter vector, pGL3-basic, at *Kpn*I and *Xho*I sites as desribed previously [Xiao et al., 1997]. 5′ deletion mutants such as pGL3b-60 (−60 bp upstream from TATA box) were obtained by making deletions in pGL3b-4542 from the *Kpn*I site, using an exonuclease III-based system (Nippon Gene, deletion kit). The extent of the 5′ deletion was determined by sequence analysis. To create a panel of site-directed mutants, mutagenesis of pGL3b-60 was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The oligonucleotides used in these mutagenesis reactions are as follows: pGL3b-#1, 5′-ccgggatcggtactagtgagggttggtctgcctc-3′ and 5′-aggcaggaccaaccctcactagtaccgat-cccgggg-3′; pGL3b-#2, 5′-gtgaaggagtgactagtcaatgcctctgagggg-3′ and 5′-ccccta-gaggcattgactagtcactccttcac-3′; pGL3b-#3, 5′-gggttggtcctaactagttgggggcggggcc-3′ and 5′-ggccccgccccccaactagttaggaccaaccc-3′; pGL3b-#4, 5′-gcctctgagggactagtgc-ctgggccgag-3′ and 5′-ctcggcccaggcactagtccctcagaggc-3′; pGL3b-#5, 5′-ggcg-gggccagactagtgctataaggaggcagctcgac-3′ and 5′-gctgcctccttatagcactagtctggcccc-gcccccc-3′; pGL3b-#6, 5′-gggccgagccactagttggcagctcgacgccaactg-3′ and 5′-ggc-gtcgagctgccaactagtggctcggcccaggccc-3′. As these constructs are not suitable for cotransfected with Sp1 and Sp3 (see Results and Discussion for details), the inserts of some of these constructs were reintroduced into pRL-null vector (Promega, Madison, WI), which carries *Renilla* luciferase cDNA instead of firefly luciferase cDNA, at the *Sac*I and *Xho*I sites. For example, the insert of pRL-60 was from that of pGL3b-60, the one of pRL-#4 from that of pGL3-#4.

The Sp1 cDNA expression construct (pCGN-Sp1) was the kind gift of Dr. T. Shenk, which was constructed by inserting the blunt-ended *Eco*RI fragment of the human Sp1 cDNA sequence from pAct-Sp1 into the blunt-ended *Xba*I site of the pCGN vector to generate an epitope-tagged Sp1 variant for expression in eukaryotic cells [Parks et al., 1996]. Sp3 cDNA expression construct (pCGN-Sp3) was created as follows: PCR driven with pfu polymerase was used to generate a full-length human Sp3 cDNA with a *Spe*I site added at the 5′ terminus and a *Bam*HI site at the 3′ terminus. Primer sequences were 5′-agactagtaattc-cgggccatcgcc-3′ (sense) and 5′-tcggatccatttactccattgtctcatttccag-3′(antisense). pCMV4-Sp3flu (kindly provided by J.M. Horovitz)[Udavadia et al., 1995] was used as a template. After being treated with *Spe*I and *Bam*HI, the PCR product was ligated at the *Xba*I/*Bam*HI sites of the pCGN vector. To avoid the possible mismatch synthesis that occurs during PCR, the *Not*I and *Eco*RV fragment of this construct, which contains most of the cDNA, was exchanged with the *Not*I and *Eco*RV fragment of pCMV4-Sp3flu.

Sp1 antisense expression plasmid (pcDNA3.1-αSp1) was generated by introducing the *Xba*I and *Sma*l fragment of pERV2/Sp1 (a gift from G. Suske) into a CMV promoter containing expression vector pcDNA 3.1/His C (Invitrogen, Carlsbad, CA), the resulting plasmid contains the human Sp1 cDNA sequence in the opposite direction. The *Eco*RI and *Bam*HI fragment of the human Sp3 cDNA sequence from pCMV4-Sp3flu was used for construction of the Sp3 antisense expression plasmid (pcDNA3.1-αSp3). The fragment was inserted in reverse orientation into pcDNA3.1/His C at the *Eco*RI and *Bam*HI sites.

Stable Transfectants

A total of 6 μg of p21 promoter containing luciferase expression plasmids pGL3b-4542 and 2 μg of pSV2neo vector was cotransfected into NIH3T3 cells in a 10-cm dish culture with calcium phosphate procedure. The transfected colonies were selected by 400 μg/ml of G418 (GIBCO-BRL, Gaithersburg, MD) for 3 weeks. More than 50 colonies were pooled. Luciferase activity of these transfectants was normalized by protein concentration.

Transient Transfection and Reporter Gene Assay

NIH3T3 cells were plated onto 48-well plates at a density of 20,000 cells/well. Cells were transfected with 0.2 μg/well of plasmid by the standard calcium phosphate procedure. When cotransfected with the cDNA expression plasmid or antisense expression plasmid, Superfect reagent (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions. TSA treatments were started 32 h after transfection and generally continued for 16 h, except where noted in the figure legends. Two days after transfection, cells were lysed, and lucifer-

ase activity in the lysates was measured by luminometer. The transfection efficiency was estimated via cotransfection with thymidine kinase promoter-driven *Renilla* luciferase reportor vector (pRL-TK plasmid, dual luciferase assay system, Promega, Madison, WI). When the promoter was constructed in pRL-null vector and cotransfected with Sp1 or Sp3 expression plasmid, *Renilla* luciferase activity was normalized by protein concentration for the reseason that we failed to find a control reporter construct that is not responsive to overexpression of Sp1 or Sp3 (see also Results and Discussion).

Electrophoretic Mobility Shift Assays

Nuclear extracts from either proliferating or TSA-treated NIH3T3 cells were prepared as described [Hasegawa et al., 1997]. A total of 5 μg of the nuclear extract was incubated with 5 fmol of ^{32}P end-labeled double-stranded oligonucleotides with a sequence corresponding to the region from −40 to −10 bp from TATA box in the p21 promoter (GC-probe). Incubation was carried out in 1 vol of 10 μl at room temperature for 20 min. All binding reactions contained 10 mM Tris-HCl (pH 7.5), 4% glycerol, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, and 0.5 mg/ml of poly(dA-dT). For competition, 1 pmol of unlabeled oligonucleotides were included in the reactions. In supershift experiments, 1 μl of antibody was added 10 min before the labeled probe. The electrophoretic mobility shift assay (EMSA) products were separated on a 4% polyacrylamide 0.5× Tris-borate EDTA gel at room temperature at 150 V for 1.5 h. The gel was dried and subjected to autoradiography. The oligonucleotides used in these experiments were as follows: GC-probe: 5′-ggttggtcctgcctctgaggggcggggcctgggccgag-3′; mutated GC-probe: 5′-ggttggtcctgcctctgagggttcggggcctgggccgag-3′; consensus Sp1 binding oligo: 5′-attcgatcggggcggggcgagc-3′; mutated Sp1 binding oligo: 5′-attcgatcggttcggggcgagc-3′. The polyclonal antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

Increased Histone Acetylation and p21 Expression After TSA Treatment

The effects of TSA on cellular function of NIH3T3 cells were investigated first. TSA-induced histone hyperacetylation was confirmed by immunoblot probed with antibodies specific to acetylated histone H3 and acetylated histone H4 (Fig. 1). The acetylation levels of both histone H3 and histone H4 in cells were increased markedly and in a dose-dependent manner after treatment with 10–400 ng/ml TSA for 16 h. In accordance with these changes, the results from RT-PCR showed that p21 mRNA expression was elevated gradually from 3 to 24 h after treatment with 100 ng/ml TSA (Fig. 2). These results suggest that TSA is an effective agent for inducing histone hyperacetylation and p21 expression in NIH3T3 cells.

Induction of p21 Promoter Activity by TSA

To understand the mechanism of TSA-induced p21 expression, analysis of the p21 pro-

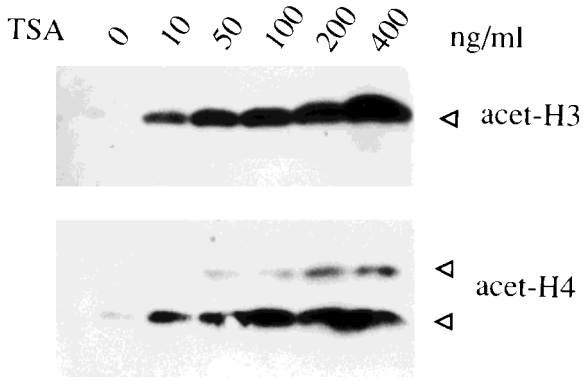


Fig. 1. Western blot analysis of core histone acetylation. NIH3T3 cells were cultured in 6-cm dishes with different amounts of trichostatin A (TSA). After overnight culture, cells were washed with phosphate-buffered saline (PBS), centrifuged, and the pellets lysed. A total of 30 μg of lysate was loaded in each lane on a 15% separating gel. Blotted membranes were probed with anti-acetylated H3 or anti-acetylated H4 antibody as described under Materials and Methods.

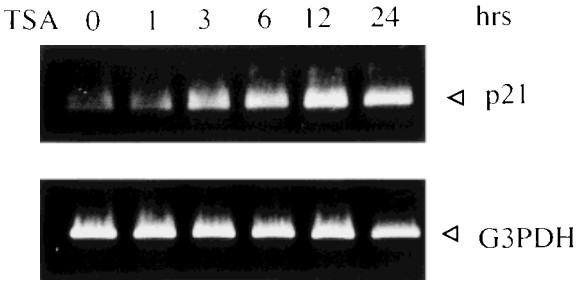


Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of p21 expression. NIH3T3 cells were treated with 100 ng/ml trichostain A (TSA). At the indicated time, cells were collected and used for RNA isolation. RT-PCR was performed with the primers described under Materials and Methods. G3PDH was used as control.

moter is indispensable. As the first step, we investigated the dose dependency and time course of p21 promoter activity using cells stablly transfected with pGL3b-4542, which is driven by our longest (4.6-kb) murine p21 promoter and contains the luciferase reporter gene (for details, see Materials and Methods and Fig. 4). In parallel with TSA-induced p21 mRNA expression (Fig. 2), the activity of the p21 promoter-luciferase gene construct was increased in a dose- dependent manner and almost plateaued at 100 ng/ml of TSA (Fig. 3A). The activity was increased with time from 3 to 24 h (Fig. 3B). These results showed that TSA upregulates p21 promoter activity in NIH3T3 cells and provided the experimental conditions used in the following experiments.

Mapping of TSA-Responsive Element in p21 Promoter

In order to identify the TSA-responsive regions in the murine p21 promoter, NIH3T3 cells were transiently transfected with a series of 5′ terminus-truncated mutants of the p21 promoter linked to the luciferase reporter gene. The transfected cells were either kept in DMEM medium or treated with TSA for 16 h before luciferase assay. As shown in Figure 4, with the exception of the three longest constructs, pGL3b-4542, pGL3b-3038, and pGL3b-1981, the basal activity of mutant promoters remained at the same level until truncation up to −60 (100-bp fragment, from −60 to +40 relative to TATA box; pGL3b-60). Meanwhile, the basal activity of constructs shorter than pGL3b-60 dramatically decreased. Similarly, TSA-induced activity remained at a high level in the longer mutants but decreased to the same level as the empty vector in mutants shorter than pGL3b-60 (Fig. 4).

From these observations, we defined a 100-bp fragment (pGL3b-60) as the p21 minimal promoter and believe that TSA-responsive element is located in this region. According to database analysis, we noticed that there are several GC clusters but no other known consensus protein binding elements in this region (data not shown). To examine the role of these GC clusters in p21 promoter activity more precisely, site-directed mutations were introduced into the p21 minimal promoter (Fig. 5). While other mutations did not result in significantly changed TSA responsiveness, a mutation in a typical GC-box (#4 in Fig. 5) decreased both basal and TSA-induced transcription. A mutation in the TATA box (#6 in Fig. 5) decreased basal but not

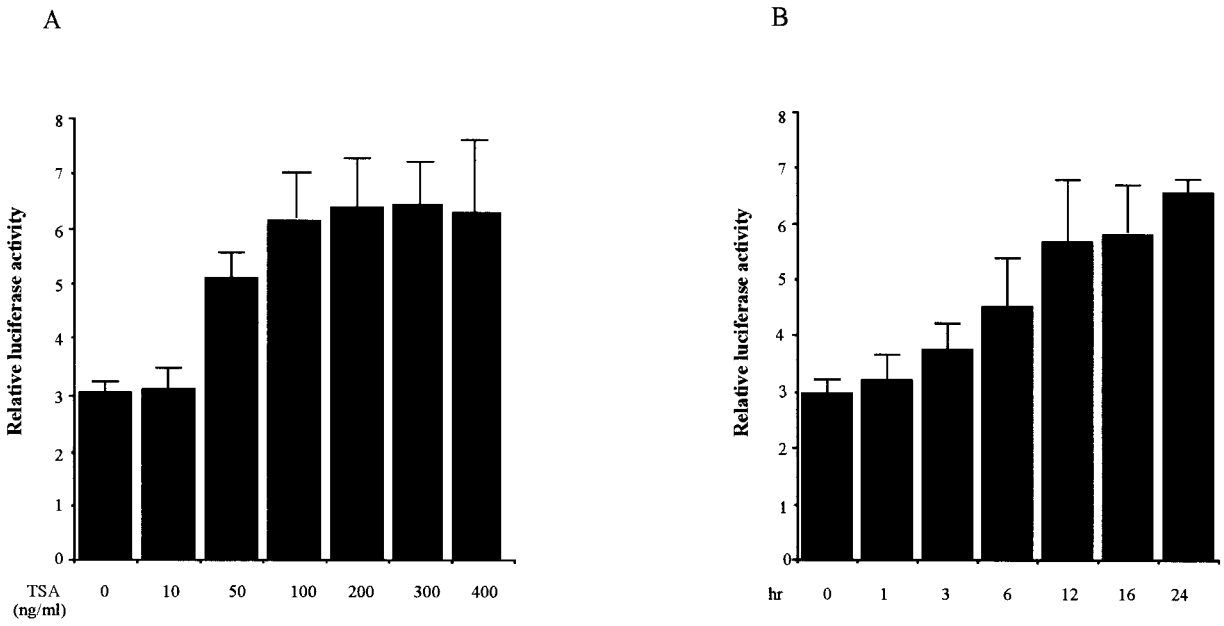


Fig. 3. Dose- and time-dependent changes of trichostatin A (TSA)-induced p21 promoter activities. NIH3T3 cells stably transfected with a 4.6-kb murine p21 promoter fragment inserted into a luciferase reporter plasmid (pGL3b-4542) were treated with TSA. Luciferase activity of the cell lysates was measured. **A:** Various amounts of TSA were added to the cells for 24 h. **B:** 100 ng/ml TSA was added at various times before termination of the experiment. Luciferase activity was normalized by protein concentration and expressed as relative luciferase activity. Duplicate transfections were performed for each experiment. The results are the mean and standard deviation of three separate experiments (n = 6).

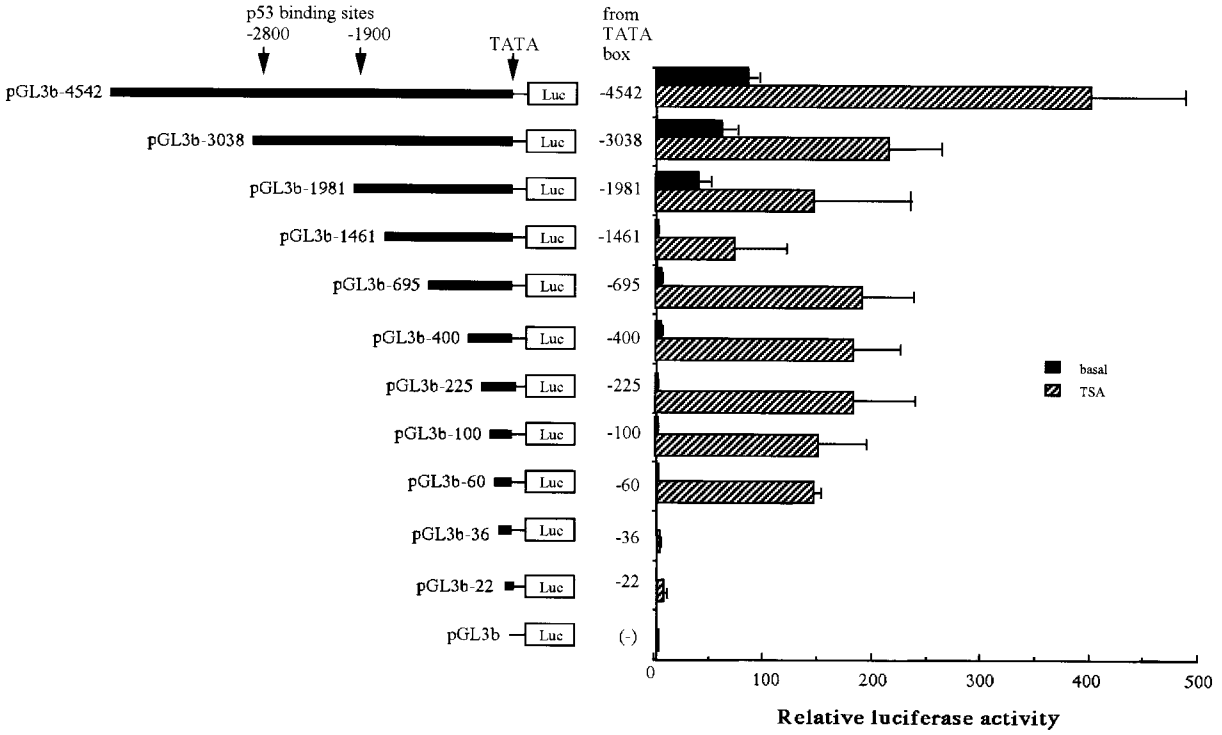


Fig. 4. Deletion analysis of p21 promoter. 0.25 μg DNA of wild-type or 5′ terminus-truncated mutant p21 promoter-firefly luciferase reporter constructs was transiently transfected into NIH3T3 cells. At 32 h after transfection, cells were either kept in DMEM medium or treated with 100 ng/ml TSA for 16 h. Results were correlated with renilla luciferase activity that came from cotransfected pRL-TK and expressed as relative luciferase activity. The results are the mean and standard deviation of six transfections from three separate experiments.

TSA-induced promoter activity. These results strongly suggest that the GC box located in the minimal promoter is the *cis*-responsive element for basal and TSA-induced transcription of p21.

Identification of the Nuclear Factors Involved in TSA Response

In order to identify the proteins that bind to this GC-rich region, EMSA was performed with ^{32}P-labeled oligonucleotides corresponding to this GC-rich region (GC-probe) and nuclear extracts from cultured NIH3T3 cells. Several DNA-protein complexes were observed (Fig. 6). The specificity of these complexes was confirmed by competition, using an excess amount of unlabeled GC-probe, which almost eliminated detection of complex formation, while competition with mutated GC-probe did not (Fig. 6A). The same result was obtained by using a consensus Sp1-binding oligo versus a mutated consensus Sp1 binding oligo, the sequences of which were from Santa Cruz Biotechnology. Given that these complexes appeared similar to those observed in previous reports [Biggs et al., 1996; Datto et al., 1995; Prowse et al., 1997], we investigated whether the complexes consisted of Sp1 or Sp3. The addition of Sp1 or Sp3 antiserum to the binding reaction caused the major complexes to supershifted (Fig. 6B,a,b,c to a′,b′,c′). Anti-Sp1 shifted complex a to complex a′, and anti-Sp3 shifted complexes b and c to complex b′ and c′, respectively. The pattern and intensity of these bands were not obviously changed when the nuclear extracts from TSA-treated cells were used (data not shown). This data provided in vitro evidence that both Sp1 and Sp3 can bind to the GC-box in the p21 minimal promoter.

Functional Involvement of Sp1 and Sp3 Transcription Factors in p21 Promoter Activity

In order to determine whether Sp1 or Sp3 were involved in p21 transcription in vivo, we cotransfected Sp1 and Sp3 expression vectors together with the p21 minimal promoter-firefly luciferase expression plasmid (pGL3b-60). Although both Sp1 and Sp3 enhanced the basal and TSA-induced activity of the p21 promoter

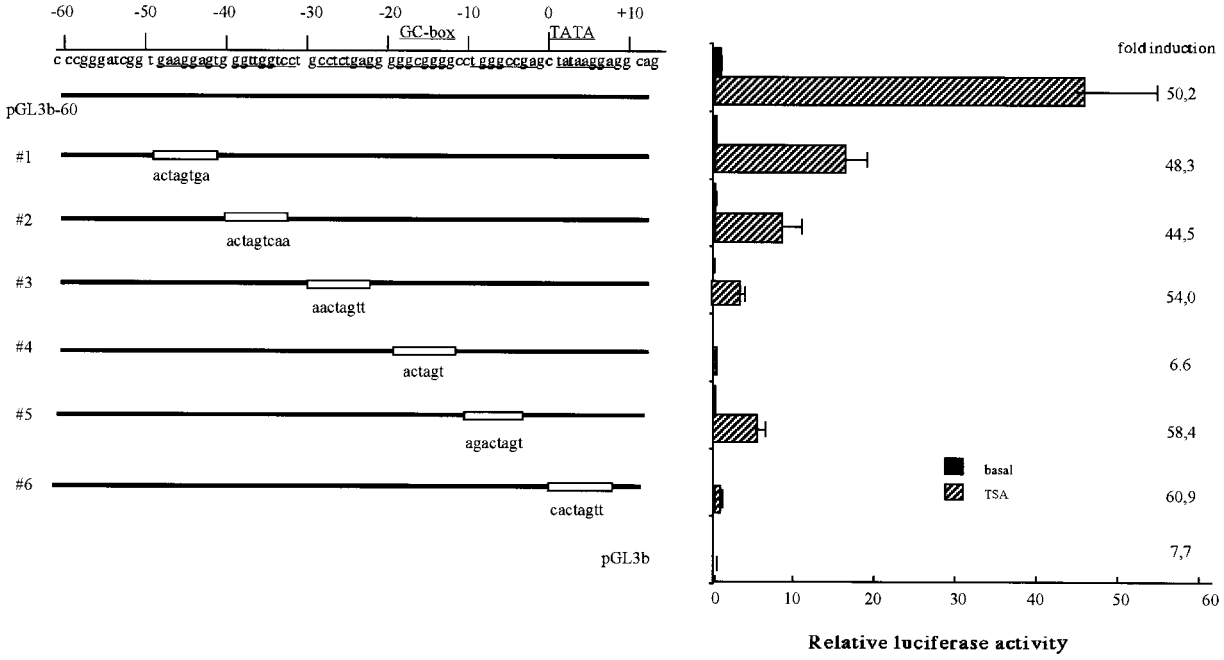


Fig. 5. Mutation analysis of minimal region of p21 promoter. A total of 0.25 μg DNA of wild-type or site-directed mutant p21 minimal promoter constructs were transiently transfected into NIH3T3 cells. Transfected cells were kept in Dulbecco's modified Eagle's medium (DMEM) or treated with 100 ng/ml TSA for 16 h. Luciferase activity was normalized and expressed as described in Figure 4. The results are the mean and standard deviation of six separate transfections from three experiments. The fold induction was calculated by comparing TSA-treated versus untreated cells.

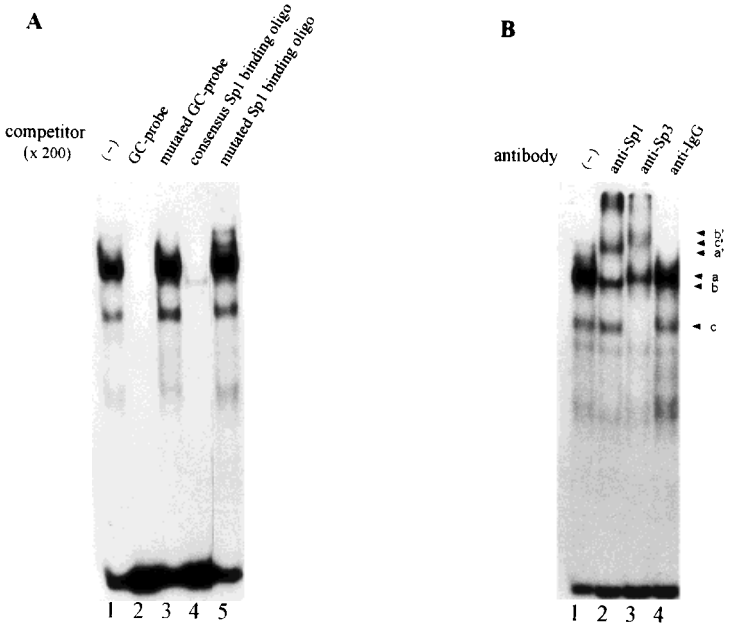


Fig. 6. Electrophoretic mobility shift assay (EMSA) of p21 promoter GC-box binding proteins. Nuclear extracts from NIH3T3 cells were incubated with ^{32}P-labeled GC-probe corresponding to −2 to −40 of the wild-type p21 promoter sequence. **A:** Competition analysis: protein binding reactions were conducted with or without different oligonucleotide competitors. No competitor (**lane 1**), GC-probe (**lane 2**), mutated GC-probe (**lane 3**), consensus Sp1-binding oligo (**lane 4**), and mutated consensus Sp1-binding oligo (**lane 5**). **B:** Supershift assay: protein binding reactions were conducted with or without different antibodies. No antibody (**lane 1**), anti-Sp1 (**lane 2**), anti-Sp3 (**lane 3**), and anti-IgG (**lane 4**).

(data not shown), cotransfection of Sp1 and Sp3 with the parental vector, pGL3-basic, also markedly enhanced the luciferase activity. This means that this kind of vector is not suitable for cotransfection of the Sp1 or Sp3. After searching for a vector with a relatively low level of response to Sp1 or Sp3 cotransfection, we found that the pRL-null vector is a good selection. Hence, cotransfection of Sp1 and/or Sp3 expression constructs (pCGN-Sp1, or pCGN-Sp3) with p21 minimal promoter-driven *Renilla* luciferase expression plasmid (pRL-60) were carried

out. The results demonstrated that the basal activity of p21 was enhanced by Sp1 or Sp3 in a dose-dependent manner (Fig. 7A) and both Sp1 and Sp3 enhanced TSA-induced promoter activity as well (Fig. 7B). More importantly, since the effects of Sp1 and Sp3 on GC box-mutated minimal promoter (pRL-#4) were much weaker than those on wild-type minimal promoter (pRL-60) but the same as vector alone (pRL-null) (Fig. 7C), we obtained the functional evidence that the Sp1-and Sp3-induced transactivation of the p21 promoter is dependent on the GC-box located near TATA box. These results are consistent with the in vitro evidence from EMSA experiments (Fig. 6). To clarify further that both Sp1 and Sp3 are functional transactivators for the p21 promoter, we inserted antisense Sp1 or Sp3 in a CMV promoter-driven vector and examined whether the constructs can influence p21 promoter activity. Cotransfection of antisense Sp1 or Sp3 plasmids with pRL-60 resulted in decreased p21 promoter activity dose dependently (Fig. 8A). Despite the effect on basal promoter activity, antisense Sp1 or Sp3 RNA dramatically decreased TSA-induced transactivation of the p21 promoter (Fig. 8B). Furthermore, the effects of these antisense RNA revealed a GC-box dependency because these antisense RNA did not significantly influence the transcription of the GC-box mutated minimal promoter-driven construct (RL-#4) and the parental vector (RL-null). All results from these cellular experiments strongly demonstrate that both Sp1 and Sp3 are responsible for the basal and TSA-induced p21 transcription in NIH3T3 cells.

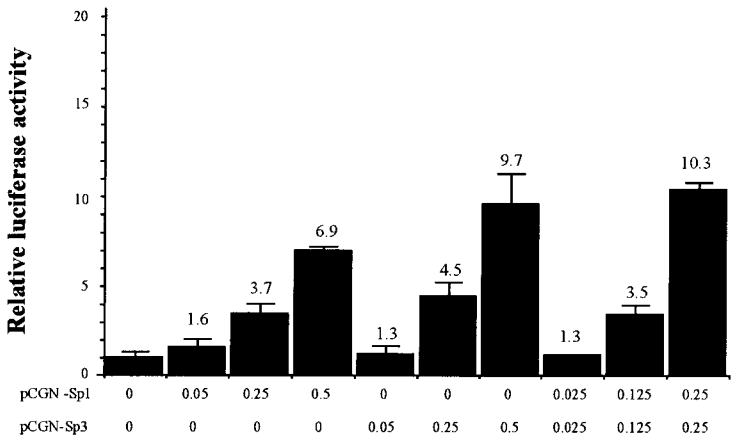
DISCUSSION

In this paper, we report that one of the histone deacetylase inhibitors, TSA, can induce histone hyperacetylation and p21 expression effectively in NIH3T3 cells; the minimal region of the mouse p21 promoter, containing from −60 bp to +40 bp relative to the TATA box, is essential and sufficient for induction of p21 in NIH3T3 cells by TSA. We report also that a GC-box in this region is critical for both basal and TSA-induced promoter activity, and that Sp1 and Sp3 are the functional activators of this GC-box. We consider the significance of these findings as follows: (1) they confirm the efficacy of TSA as an inhibitor of histone deacetylase on NIH3T3 cells and the transcriptional responsiveness of p21 to TSA in this system; (2) they provide the first evidence that besides the human p21 promoter, the mouse p21 promoter is also transcriptionally regulated through a GC-box near the TATA box, reflecting the similar structural and functional features of human and mouse p21 promoters; and (3) they define the TSA-responsive element in this promoter, as well as the transcription activators responsible for TSA response.

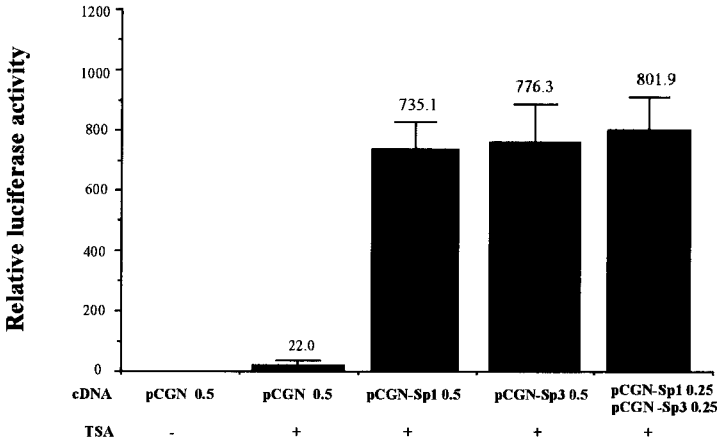
The inhibitors of histone deacetylase induce transactivation though histone hyperacetylation [Schlake et al., 1994; Yamamoto et al., 1996]. Earlier studies have suggested that histone hyperacetylation directs an allosteric change in nucleosome conformation, destabilizes higher-order structure and renders nucleosomal DNA more accessible to transcription factors [Lee et al., 1993; Garcia-Ramirez et al., 1995]. However, the nucleosome or gene specificity of hyperacetylation in vivo long remained unclear. Recent studies demonstrated that there are various kinds of histone acetylases and deacetylases in mammalian cells. These enzymes work as cofactors though association with different sequence-specific DNA binding proteins and recruited by them to local promoter regions. Thus, determining the genes responsive to agents that affect the state of histone acetylation will be very meaningful in revealing the molecular events involved in histone acetylation-related transcriptional regulation. In parallel with the study of human p21 [Nakano et al., 1997], the fact that both TSA (this paper) and butyrate [Xiao et al., 1997] upregulate p21 expression in mice system shows that p21 is a target gene of histone deacetylase inhibitors and that the p21 promoter is a useful

Fig. 7. Cotransfection with Sp1 and Sp3 cDNA expression constructs. **A:** Cells were incubated with a mixture of DNA and the superfect reagents (Qiagen) for 2 h. The DNA mixture contained 0.2 μg of minimal p21 promoter-Renilla luciferase reporter construct (pRL-60), sufficient pCGN-Sp1 and/or pCGN-Sp3, and pCGN vector to adjust the total amount of DNA to 0.7 μg. Cells were then washed and incubated for an additional 46 h. **B:** Cells were transfected with 0.2 μg pRL-60 and 0.5 μg pCGN vector, pCGN-Sp1, or pCGN-Sp3, followed by treatment with or without 100 ng/ml TSA for 16 h. **C:** Cells were transfected with 0.2 μg pRL-60, pRL-#4, or pRL vector and with 0.5 μg pCGN vector, pCGN-Sp1, or pCGN-Sp3. Transfection and incubation was carried out as in A. The luciferase activities shown above were normalized by protein concentration and expressed as relative luciferase activity. The results are the mean and standard deviation of three experiments (n = 6). The fold induction of luciferase activity was calculated by dividing each value by the basal activity without Sp1 or Sp3 transfection.

A


Relative luciferase activity
20
15
10
5
0
1.6
3.7
6.9
1.3
4.5
9.7
1.3
3.5
10.3
pCGN -Sp1 0 0.05 0.25 0.5 0 0 0 0.025 0.125 0.25
pCGN-Sp3 0 0 0 0 0.05 0.25 0.5 0.025 0.125 0.25

B


Relative luciferase activity
1200
1000
800
600
400
200
0
22.0
735.1
776.3
801.9
cDNA pCGN 0.5 pCGN 0.5 pCGN-Sp1 0.5 pCGN-Sp3 0.5 pCGN-Sp1 0.25 pCGN -Sp3 0.25
TSA - + + + +

C

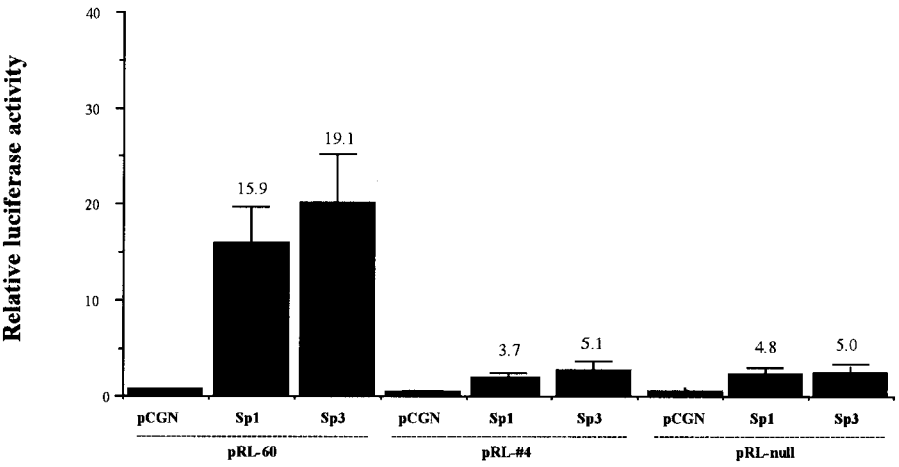

Relative luciferase activity
40
30
20
10
0
15.9
19.1
3.7
5.1
4.8
5.0
pCGN Sp1 Sp3 pCGN Sp1 Sp3 pCGN Sp1 Sp3
pRL-60 pRL-#4 pRL-null

Figure 7.

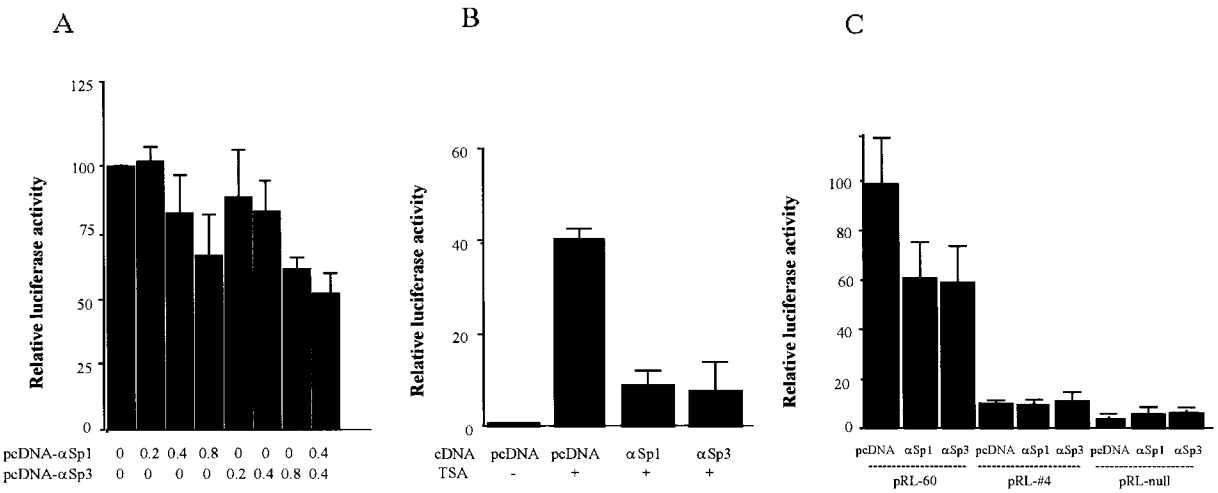


Fig. 8. Cotransfection of antisense Sp1 or Sp3. **A:** Cells were transfected with 0.2 μg pRL-60, sufficient pcDNA3.1-αSp1 and/or pcDNA3.1-αSp3, and pcDNA3.1 to adjust the total amount of DNA to 1.0 μg. Cells were transfected and incubated as described in Fig. 7A. **B:** Cells were transfected with 0.2 μg pRL-60 and 0.8 μg pcDNA3.1 vector, pcDNA3.1-αSp1 or pcDNA3.1 −Sp3, then treated with 100 ng/ml TSA as described in Figure 7B. **C:** Cells were transfected with 0.2 μg pRL-60, pRL-#4, or pRL vector and with 0.8 μg pcDNA3.1 vector, pcDNA3.1-αSp1, or pcDNA3.1-αSp3. Transfections and incubation were carried as described in Figure 7A. Luciferase activities were normalized by protein concentration and expressed as relative luciferase activity. The results are the mean and standard deviation of three experiments (n = 6).

model for studying histone acetylation-regulated transcription.

As shown in Figure 4, the basal activity of the p21 minimal promoter is lower than that of the three longest promoters. This may in part result from deletion of p53 responsive sites located near −2800 bp and −1900 bp from the TATA box. The fact that the TSA-responsiveness of pGL3–60 was not reduced even when these two p53 binding sites were lost confirmed that histone deacetylase inhibitor-induced activation of p21 is p53 independent, as we and others have reported [Xiao et al., 1997; Nakano et al., 1997].

The human p21 promoter has six Sp1 binding sites near the TATA box, which function cooperatively or individually under different conditions. Butyrate sensitivity requires Sp1–3 site in conjunction with the Sp1–5 site and Sp1–6 site, with Sp1–3 site being critical [Nakano et al., 1997]. There are also several GC clusters in the minimal region of the mouse p21 promoter but, on the basis of the results reported in this paper, only one typical GC-box is functional because mutations in this GC-box abolished almost all of the Sp1/Sp3 binding capacity (Fig. 6, mutated GC-probe) and the Sp1/3-mediated transcription (Figs. 7 and 8, RL-#4).

Although the GC-rich region of the human p21 promoter was demonstrated to be the responsive element for many agents, such as phorbol esters, Ca^{2+}, TGF-β, and butyrate [Biggs et al., 1996; Datto et al., 1995; Prowse et al., 1997; Nakano et al., 1997], the proteins responsible for transcriptional activation through this region are not always identical. Although both Sp1 and Sp3 transcription factors from different cells bind to this region in vitro, the data from limited functional assays show discrepancies. For example, exogenous Sp1 was found to induce the p21 promoter in *Drosophila* Schnieder SL2 cells [Biggs et al., 1996]; but only Sp3 acted on the p21 promoter in the keratinocyte differentiation system induced by calcium [Prowse et al., 1997]. Given these discrepancies and another statement that Sp3 may repress Sp1-mediated transcriptional activation under some conditions [Hagen et al., 1994; Dennig et al., 1996], we believe that investigating the functional transcription factors for TSA-induced p21 promoter activity is important for further study.

From DNA cotransfection experiments, we found that transfection of Sp1 or Sp3 strongly enhanced the firefly luciferase activity of the pGL3-basic vector itself (data not shown), implying that the pGL3-basic vector itself is sensitive to Sp1 and Sp3 overexpressionn through an unknown mechanism. We speculate that there may be a GC-rich region in this vector where

Sp1 or Sp3 can bind, or overexpressed Sp1 and Sp3 may influence other steps in transcription, such as elongation and mRNA stability. After searching for a vector that was not markedly enhanced by transfection of Sp1 or Sp3, we found that the pRL-null vector is preferred one. Our results from cotransfection of Sp1 and/or Sp3 with p21 promoter constructs in the pRL vector clearly show that either Sp1 or Sp3 works as a transcription factor influencing p21 minimal promoter activity at both the basal and TSA-induced levels. We also noted that transfection of antisense Sp1 or Sp3 was more effective in reducing TSA-induced p21 minimal promoter activity than basal activity. The different efficiencies of antisense Sp1/Sp3 to basal and TSA-induced activities raised the possibility that other proteins in addition to Sp1 and Sp3 may also bind to the GC-box of the p21 promoter under basal transcription condition, but Sp1 and Sp3 may be the main factors responsible for TSA sensitivity.

To understand the precise mechanism underlying TSA-induced p21 transcription, one question must be answered: how does TSA alter Sp1/Sp3-mediated transcription? As the protein expression and DNA binding capacity of Sp1 and Sp3 did not change after TSA treatment (data not shown), we hypothesize that the interaction of Sp1/Sp3 with other proteins may be critical for transcriptional regulation of the p21 promoter. Given the recent findings that histone acetylases and deacetylases may exert their effects on transcriptional regulation by associating with DNA binding proteins, such as mad, p53, MyoD, and YY1 [Gu et al., 1997; Avantaggiati et al., 1997; Puri et al., 1997; Yang et al., 1997], and these associations may vary in different cellular contexts, one of our speculations is that Sp1 and Sp3 may form complexes with histone acetylase or deacetylase directly or indirectly. The question of whether Sp1/Sp3-mediated transcription is involved in this manner of regulation is a subject of ongoing investigation.

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