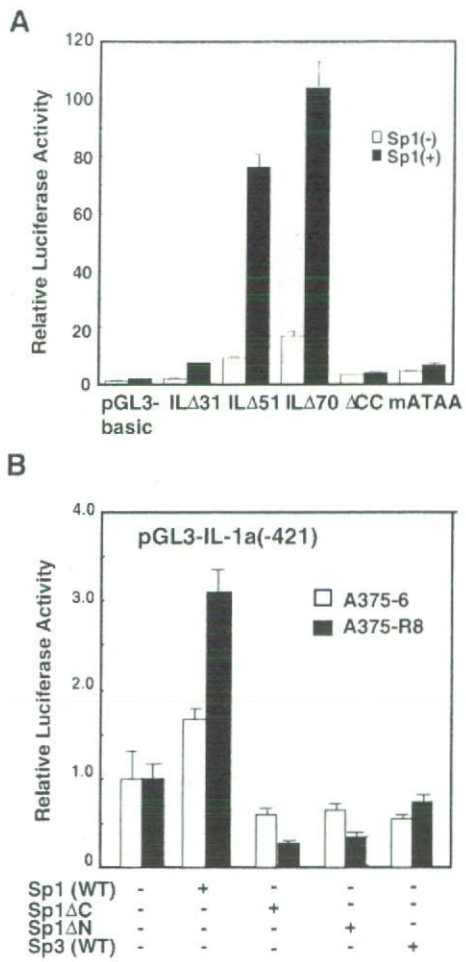


**Fig. 2.** Sp1 activates the IL-1α promoter activity in a manner dependent on the GC box in A375-R8 cells. (A) A375-R8 cells were cotransfected with the indicated reporter plasmids and pCMV-β-gal in the presence or absence of pCIneo-Sp1. (B) A375-6 and A375-R8 cells were cotransfected with pGL3-IL1a(−421) and pCMV-β-gal in the presence of indicated plasmids. Cells were harvested 48 h after transfection, and the luciferase activity was measured. After normalization with β-gal activity, luciferase activity was indicated as the ratio between each activity to that of pGL3-basic-transfected cells without Sp1 expression (A) and pGL3-IL-1a(−421)-transfected cells without Sp1 expression (B). Mean ± SD based on triplicate cultures is shown. WT, Wild-type.

## Comparison of DNA binding activity of Sp1 and Sp3 in A375-6 and A375-R8 cells

Next, we performed EMSA to examine whether Sp1 or Sp3 is bound to the GC box and if there are any differences in the amount of Sp1 or Sp3 bound to the sequence between A375-R8 and A375-6 cells. Nuclear protein extracts from A375-R8 and A375-6 cells were allowed to bind to end-labeled DNA using wild-type oligonucleotide (−56 to −37), with or without excess amount of unlabeled nucleotides (**Fig. 3A**). As demonstrated in Figure 3B, similar specific binding patterns were observed in extracts of A375-R8 and A375-6 cells. Antibodies against Sp1 and Sp3 caused a supershift of the bands, indicating that Sp1 and Sp3 are bound to the sequence (Fig. 3C). Slow migrating complex and fast migrating complex of Sp3 represent a full-length isoform and two N-terminally truncated isoforms, respectively [31]. There were no differences between A375-R8 and A375-6 cells. Competitive binding assay was performed using unlabeled wild-type (−56 to −37) and mutant oligonucleotides. Wild-type oligonucleotide #1 and the nucleotide #2 (−54 to −35) abrogated the binding completely, and mutant m2 inhibited the binding less effectively but significantly. In contrast, none of the nucleotides #3, #4, and m1 inhibited the binding (Fig. 3D). These results indicate that the sequence CGCC (−48 to −45) is critical for Sp1 and Sp3 binding, and the sequence between −52 and −50 is necessary for maximum binding.

## Transactivation activity of Sp1 and Sp3 in A375 cells

To examine whether there are any differences in transactivation activity of Sp1 or Sp3 between A375-6 and A375-R8 cells, a reporter gene assay was performed by transfecting these cells with a plasmid encoding GAL4-dbd (DNA binding domain), -Sp1, or -Sp3 fused with GAL4-dbd together with a reporter plasmid containing five tandem repeats of GAL4 binding sites (**Fig. 4A**). Transactivation activity of Sp1 or Sp3 was markedly augmented in A375-R8 cells as compared with A375-6 cells (Fig. 4B). The activity of GAL4-dbd was also higher in A375-R8 cells, but it was less than those of Sp1 or Sp3. We next performed the reporter gene assay by transfecting the expression plasmid for Sp1 or Sp3 together with reporter genes containing three tandem repeats of the GC-rich sequence in the IL-1α promoter region. As shown in Figure 4D, increasing amount of Sp1 led to activation of the luciferase activity in A375-R8 cells. However, Sp1 augmented the activity only slightly in A375-6 cells. In contrast, Sp3 did not induce the luciferase activity in any cells. These results indicate that Sp1 is activated in A375-R8 cells.

## Expression of Sp1 and Sp3 in A375-6 and A375-R8 cells

Next, we determined the expression levels of Sp1 and Sp3 in A375-R8 and A375-6 cells by Western blot analysis. As shown in **Figure 5, A** and **B**, the expression levels of Sp1 and Sp3 were comparable between A375-R8 and A375-6 cells. Sp1 activity is regulated by several mechanisms at a post-transcriptional level. As phosphorylation of Ser/Thr is a major mechanism for Sp1 activation [32], we determined the phosphorylation of overexpressed or endogenous Sp1 in these cells; however, there were no differences in the levels of phosphorylated Sp1 between A375-R8 and A375-6 cells (Fig. 5C). These results indicate that phosphorylation does not account for the differential activation of Sp1 in these cells.

TSA augments the IL-1α promoter activity and IL-1α mRNA expression in A375 cells. Recently, it was reported that interaction of Sp1 or Sp3 with HDAC is critical for suppressing induction of genes involved in cell cycle regulation, including $p15^{INK4b}$, $p19^{INK4d}$, and $p21^{WAF1/CIP1}$ [33]. To examine whether HDAC is involved in the differential activation of the IL-1α gene in A375 cells, TSA, an inhibitor of HDAC, was added to the reporter gene assay for the IL-1α promoter activity in A375-6 and A375-R8 cells. The luciferase activity without TSA was higher in A375-R8 cells as compared with A375-6

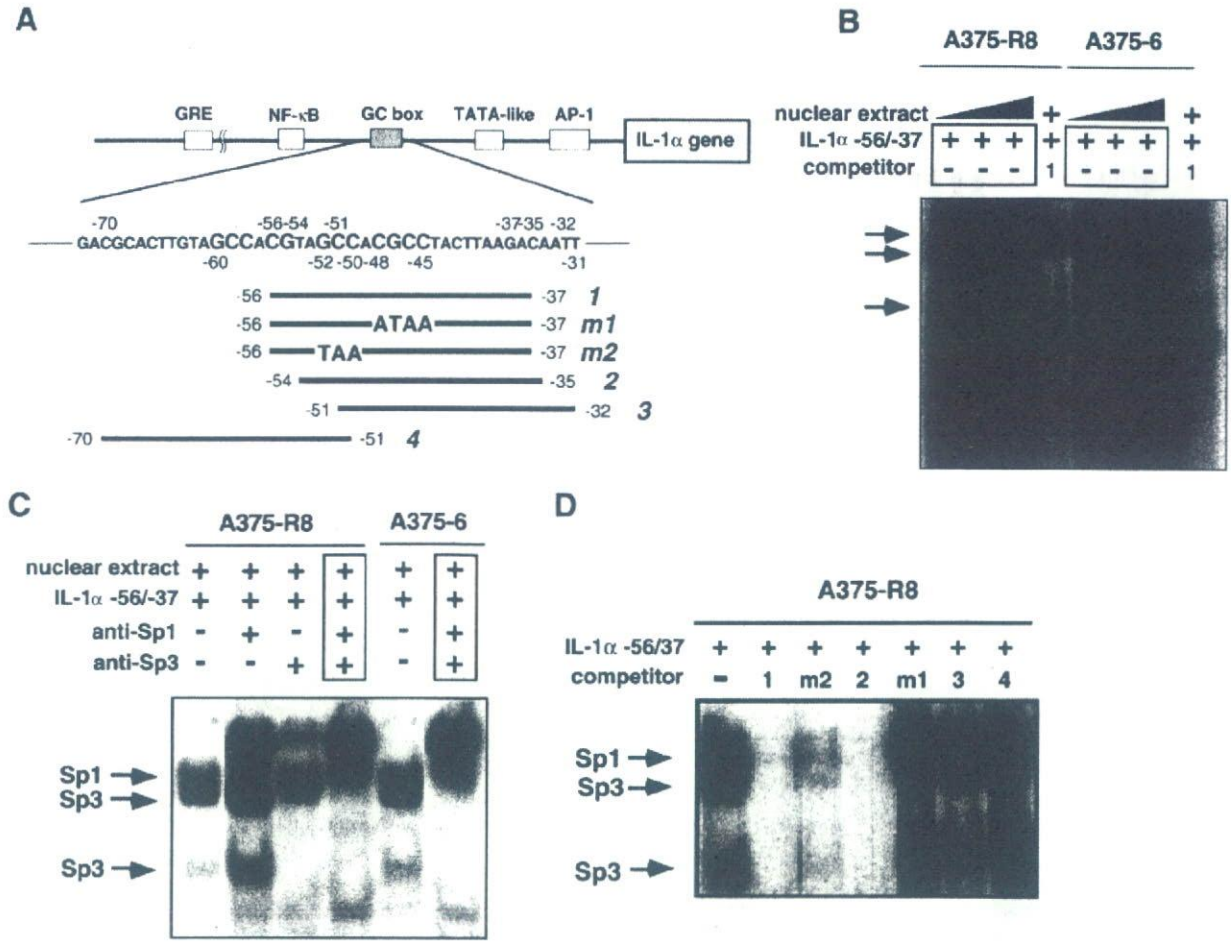


**Fig. 3.** Binding of nuclear proteins from A375 cells to the GC-rich region of the IL-1α promoter gene. (A) Sequences of probes are presented. (B) DNA binding activity of nuclear proteins from A375 cells to the GC-rich region of the IL-1α promoter gene. EMSA was performed using $^{32}$P-labeled probe 1 and the nuclear extracts of A375 cells. Competition analysis was performed to confirm the specific binding in the presence of 250-fold excess unlabeled probe 1. (C) Binding of Sp1 and Sp3 to the GC-rich region of the IL-1α promoter gene. EMSA was performed using $^{32}$P-probe 1 and the nuclear extracts of A375 cells. Supershift analysis was performed using anti-Sp1 or anti-Sp3 antibodies. Supershifted bands are indicated with arrows on the left. (D) Sequences required for the Sp1 and Sp3 binding in the GC-rich region of the IL-1α promoter gene. EMSA was performed using $^{32}$P-probe 1 and the nuclear extracts of A375-R8 cells in the presence of 250-fold excess of each unlabeled probe designated as probes 1, 2, 3, 4, m1, or m2 to determine the Sp1 and Sp3 binding sequence. The bands for specific binding are indicated with arrows on the left. Representative data of more than three experiments are shown.

cells (**Fig. 6A**). Increasing doses of TSA dramatically augmented the promoter activity in A375-6. TSA also augmented the promoter activity in A375-R8 cells, and the maximum level of the promoter activity in TSA-treated A375-6 cells was comparable with that of TSA-treated A375-R8 cells. TSA, however, did not augment the luciferase activity of the GC box mutants pGL3-IL-1a(421)ΔCC or pGL3-IL-1a(421)mATAA (Fig. 6B) and pGL3-basic but augmented that of pGL3-(IL1a-GC)$_3$, which contained three tandem repeats of the GC-rich sequence in the IL-1α promoter region (Fig. 6C). These results indicate that the augmenting effect of TSA was specific to the GC box in the IL-1α promoter region. We then determined the effect of TSA on IL-1α mRNA expression in A375 cells. As shown in Figure 6D, TSA induced IL-1α mRNA in A375-6 cells but not A375-R8 cells. These results suggest that HDAC is critical for the differential IL-1α mRNA expression at the transcriptional level in A375-R8 and A375-6 cells.

We examined whether HDAC is involved in the differential transactivation activity of Sp1 and Sp3. A375 cells were transfected with an expression plasmid encoding Sp1 or Sp3 fused with GAL4-dbd, together with 5× GAL4-Luc, and then luciferase activity in the cell lysates was determined. As shown in **Figure 7, A** and **B**, transactivation activity of Sp1 and Sp3 without TSA was higher in A375-R8 cells as compared with A375-6 cells. TSA also augmented the transactivation activity of Sp1 and Sp3 in A375-6 and A375-R8 cells, especially in A375-6 cells. The transactivation activity of Sp1 was approximately tenfold higher than Sp3 in A375-6 or A375-R8 cells. The response of GAL4-dbd to TSA was not changed at the highest dose of TSA in A375-R8 cells (1.06-fold), and a small increase of the activity (2.03-fold) was observed in A375-6 cells. These results suggest that HDAC is also critical for the differential transactivation activity of Sp1 and Sp3 in A375-6 and A375-R8 cells.

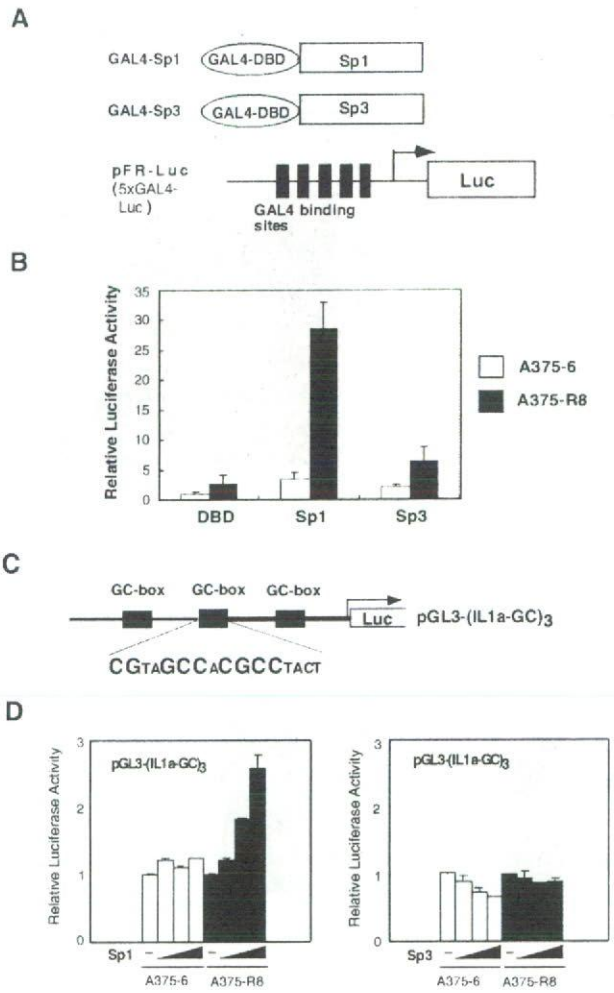


**Fig. 4.** Contribution of constitutive Sp1 activity to the activation of gene expression in A375-R8 cells. (A and B) A375 cells were cotransfected with pFR-Luc and pCMV-β-gal in the presence of GAL4-dbd, GAL4-Sp1, or GAL4-Sp3 plasmids. (C and D) A375 cells were cotransfected with pGL3-$(IL1a\text{-}GC)_3$ and pCMV-β-gal in the presence of varying doses of pCIneo-Sp1 or pCIneo-Sp3. The ratio of the amount of DNA of pCIneo-Sp1 or pCIneo-Sp3 to pGL3-$(IL1a\text{-}GC)_3$ was 0, 1, 2, and 4 from left to right. Cells were harvested 48 h after transfection, and the luciferase activity was measured. After normalization with β-gal activity, luciferase activity was indicated as the ratio between each activity to that of GAL4-dbd-transfected A375-6 cells (B) and as the ratio between each activity to that of A375-6 or A375-R8 cells without Sp1 or Sp3 expression (D). Mean ± SD based on triplicate cultures is shown.

## Interaction of Sp1 or Sp3 with HDAC1 in A375-6 and A375-R8 cells

Eight types of HDAC are identified to date, and HDAC1 and HDAC2 bind to Sp1 or Sp3 [34]. To determine whether the differential transactivation activity of Sp1 and Sp3 in A375-R8 and A375-6 cells was a result of the difference in expression levels of HDAC, cell lysates were subjected to Western blot analysis by using antibody against HDAC1. As shown in **Figure 8A**, there were no differences in the expression levels of HDAC1 between A375-R8 and A375-6 cells. The expression level of HDAC2 was low and comparable between A375-6 and A375-R8 cells as well (data not shown). Next, we determined whether Sp1 or Sp3 interacts physically with HDAC1. Cell lysates were subjected to immunoprecipitation with antibodies against Sp1 or Sp3, and then Western blotting was performed with antibodies against HDAC1, Sp1, or Sp3. As shown in Figure 8B and C, Sp1 and Sp3 appeared to interact physically with HDAC1. There were no significant differences in their interactions between A375-R8 and A375-6 cells. The interaction of HDAC2 with Sp1 or Sp3 was not observed (data not shown).

## Sp1 and HDAC1 bind to the promoter region of the IL-1α gene in A375 cells

To determine whether Sp1 and HDAC1 are actually involved in the regulation of IL-1α gene activation, ChIP assay was performed. As shown in **Figure 9A**, Sp1 and HDAC1 appeared to bind to the promoter region of the IL-1α gene in A375-R8 and A375-6 cells. However, the binding level of Sp1 and HDAC1 was not significantly different between A375-R8 and A375-6 cells. To confirm whether HDAC is critical for regulating the transactivation activity of Sp1, HDAC activity was measured. The activity of HDAC associated with Sp3 was higher as compared with Sp1 (Fig. 9B). HDAC activity bound to Sp1 or Sp3 was lower in A375-R8 cells as compared with A375-6 cells. In addition, the total activity of HDAC1 was lower in A375-R8 cells as compared with A375-6 cells. These results suggest that the reduced activity of HDAC1 is critical for the constitutive gene activation of IL-1α.

## DISCUSSION

Our study is the first to show that the binding of Sp1 or Sp3 to the GC box in the promoter of the IL-1α gene, interaction of HDAC1 with Sp1 or Sp3, and reduction of HDAC1 activity are critical for the constitutive activation of the IL-1α gene. Consistent with our previous report [26], the sequence between −103 and −70 of the IL-1α gene contained a positive regulatory element(s), and the sequence between −421 and −103 contained a negative regulatory element(s). The NF-κB site between −103 and −70 has been shown to be important for an autocrine effect of IL-1α. The positive regulatory element(s) were also found in the sequence between −70 and −31, which contained the GC box. In our previous study, we found that this region, −70 to −31, is not involved in the autocrine effect of IL-1α. Similar but less-effective responses were observed in A375-6 cells. There are no differences in the sequence of the promoter of the IL-1α genomic gene, −70 to −1, between A375-6 and A375-R8 cells (data not shown). Recently, it is reported that methylation of the adjacent Sp1 binding site inhibits Sp1 binding to DNA [35]. However, treatment of A375-6 cells with 5-aza-CdR, an inhibitor of methylation, for 6 days did not induce IL-1α mRNA, suggesting that methylation cannot account for the differential IL-1α mRNA expression either (data not shown).

We analyzed the region critical for the constitutive activation of the IL-1α promoter. By deletion and substitution mutation analyses, the sequence CGCC at −48 to −45 appeared to

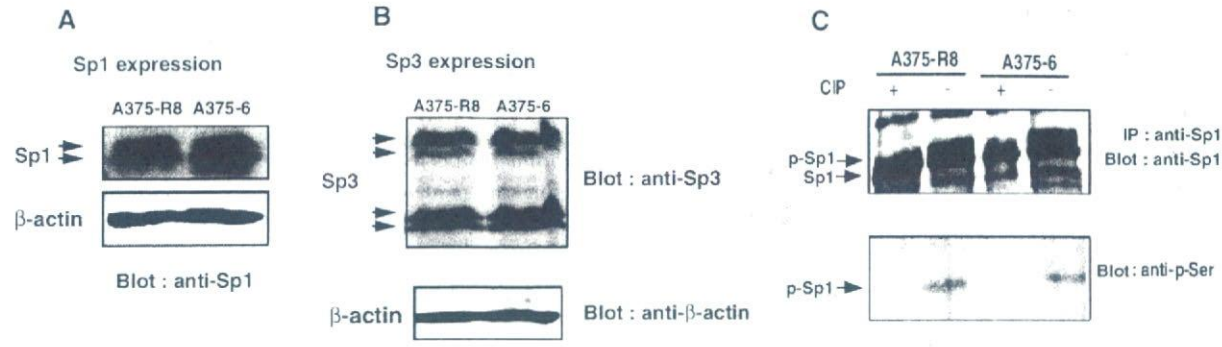


**Fig. 5.** Expression levels of Sp1 and Sp3 and phosphorylation level of Sp1. A375 cells were lysed, and an equal amount of protein was subjected to SDS-PAGE. Immunoblotting (Blot) was performed with (A) anti-Sp1 or (B) anti-Sp3 antibodies. The expression level of each protein was assessed by immunoblotting of cell lysates with anti-β-actin (lower). (C) Cell lysates were immunoprecipitated (IP) with anti-Sp1 antibody and then treated with or without calf intestine phosphatase (CIP; 0.5 units/μg) and immunoblotted with anti-Sp1 or anti-phosphorylated (p)-Ser antibodies.

be important for the promoter activity. EMSA analysis revealed that Sp1 and Sp3 are bound to the nucleotide, −56 to −37, containing the GC box. In addition to the sequence CGCC, the sequence GCC at −52 to −50 contributed to the maximum binding of Sp1 and Sp3 to the sequence. In agreement with the findings, the transactivation activity of plasmid IL$\Delta$51, which lacks G at −52, was lower as compared with the wild-type plasmid IL$\Delta$71.

We next determined the effect of Sp1 or Sp3 on the transactivation activity of the IL-1α promoter. Overexpression of wild-type Sp1, but not mutant Sp1, up-regulated the promoter activity in A375-R8 cells and less effectively in A375-6 cells. In contrast, overexpression of wild-type Sp3 did not up-regulate the promoter activity. Therefore, Sp1 but not Sp3 appeared to contribute to the constitutive transactivation of the IL-1α promoter. As the augmenting effect of Sp1 is higher in A375-R8 cells as compared with A375-6 cells, it was suggested that there are differences in the mode of activation of Sp1 and Sp3 between A375-6 and A375-R8 cells.

Sp1 and Sp3 are expressed in a variety of tissues and play an important role in regulation of the activation of genes involved in the development, proliferation, and differentiation of cells and inflammation [36]. Therefore, the expression of Sp1 and Sp3 is strictly regulated at multiple levels, including transcription, translation, and the proteasome-dependent degradation pathway. As it is reported that Sp1 and Sp3 are degraded through a ubiquitin-proteasome pathway [37], the protein levels of Sp1 and Sp3 were compared between A375-R8 and A375-6 cells. However, there were no differences between these cells, indicating that the differential transactivation activity for IL-1α promoter activity was not a result of differential expression levels of Sp1 and Sp3.

Constitutive activation as well as a high level expression of Sp1 is reported to be essential for constitutive production of an

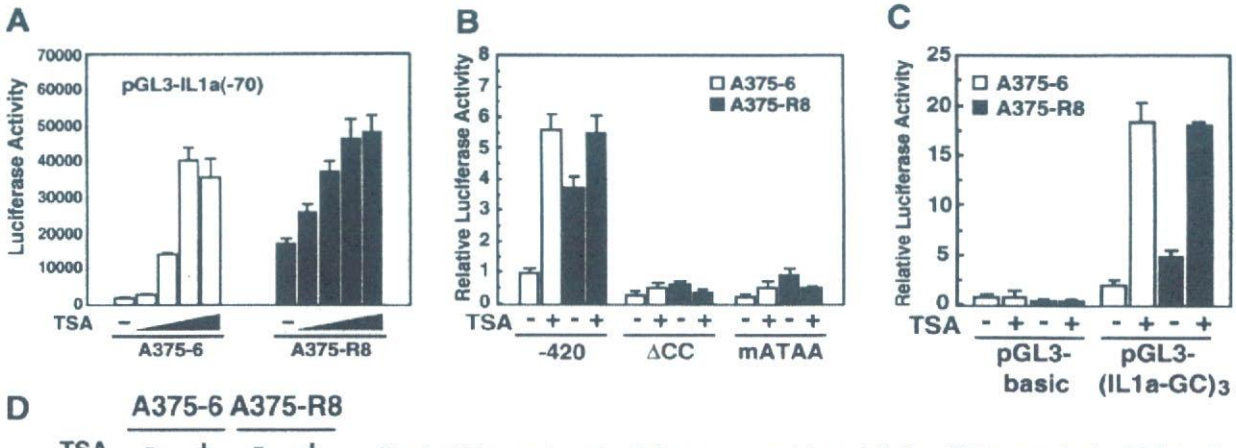


**Fig. 6.** TSA potentiates the IL-1α promoter activity and IL-1α mRNA expression in A375-6 cells. (A–C) A375 cells were transiently transfected with (A) pGL3-IL1a(−70), (B) pGL3-IL1a(−421), pGL3-IL1a(−421)ΔCC, or pGL3-IL1a(−421)mATAA, and (C) pGL3-basic or pGL3-(IL1a-GC)$_3$ and pCMV-β-gal. After 48 h culture, cells were treated without or with 50, 100, 200, or 500 nM TSA, from left to right (A) or 500 nM TSA (B and C) for 16 h, and luciferase activity was measured. Luciferase activity was indicated after normalization with β-gal (A) or as the ratio between each activity to that of A375-6 cells transfected with pGL3-IL1a(−70) (B) or pGL3-basic (C) without TSA. Mean + SD based on triplicate culture is shown. (D) A375 cells treated with or without 500 nM TSA for 16 h and total RNA were isolated. The expression levels of IL-1α and GAPDH mRNA were determined by RT-PCR. Representative data of three experiments are shown.

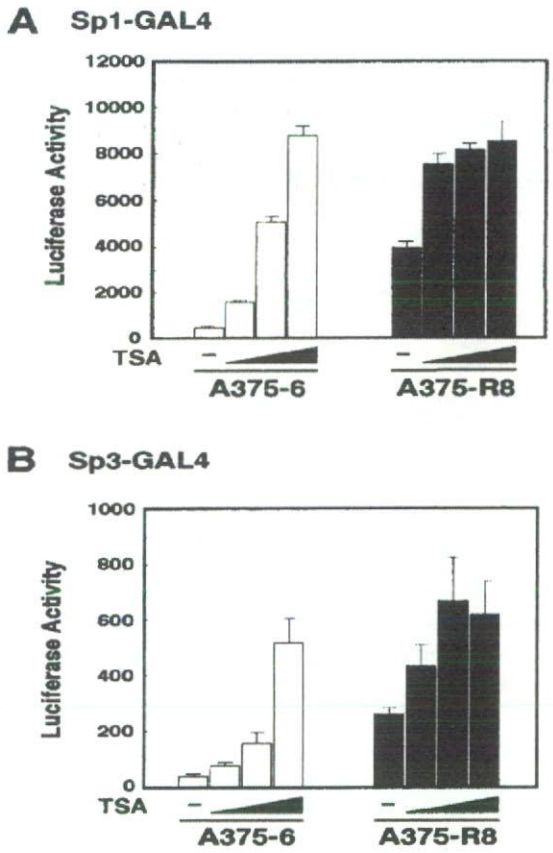


**Fig. 7.** TSA up-regulates Sp1 and Sp3 transactivating activity in A375 cells. (A and B) A375 cells were transiently cotransfected with pFR-Luc and pCMV-β-gal in the presence of GAL4-Sp1 (A) or GAL4-Sp3 plasmids (B). After 48 h culture, cells were treated without or with 100, 200, or 500 nM TSA, from left to right, for 16 h, and luciferase activity was measured. After normalization with β-gal activity, the luciferase activity was indicated. Mean ± SD based on triplicate culture is shown.

endothelial growth factor in human pancreatic adenocarcinoma [38]. Therefore, we sought to determine whether Sp1 or Sp3 is constitutively activated in A375-R8 cells. As expected, Sp1 was markedly activated in A375-R8 cells. Although Sp3 was also activated in A375-R8 cells more than A375-6 cells, the activation level was lower as compared with Sp1 in any cells. The functions of Sp1 and Sp3 are regulated by post-translational modifications and an interaction with other regulatory molecules. The former type regulation includes phosphorylation [32, 39], acetylation [40], and glycosylation [41]. The DNA binding ability of Sp1 is regulated by phosphorylation of Ser at the N-terminal Glu-rich region, and Sp1 is phosphorylated by ERK1/2 or JNK upon stimulation with fibroblast growth factor, hepatocyte growth factor, peroxide, or a variety of extracellular stimuli [32, 42]. As hyperphosphorylation is found in melanoma, leukemia cells, and lung carcinoma, the DNA binding ability of Sp1 or Sp3 was determined in A375 cells. However, there were no differences in the amount of DNA-bound Sp1 and Sp3 between A375-6 and A375-R8 cells. In addition, Sp1 was phosphorylated constitutively and comparably in these two cell clones. Therefore, the differential transactivation activity of Sp1 was not a result of phosphorylation.

HDAC negatively regulates transcription of genes by inducing the conformational changes through removing the acetyl group from the histones comprising the nucleosome. It also regulates the activity of transcription factors, including p53, GATA-1, and TFIIE, through deacetylation and does so by recruitment of corepressors, such as m-Sin3A, nuclear receptor corepressor, and silencing mediator of retinoid and thyroid receptors, to Sp1 or NF-κB [34, 43, 44]. Aberrant regulation of HDAC recruitment is implicated in the pathogenesis of tumors, as inhibitors of HDAC cause growth arrest, cell differentiation, and/or apoptosis of tumor cells [45]. For instance, HDAC1 interacts with Sp1 bound to the promoter region of $p21^{WAF1/CIP1}$; consequently, it inhibits the transactivation activity of Sp1, which leads to down-regulation of p21 expression [33]. In this study, TSA up-regulated the promoter activity of IL-1α in A375-6 and A375-R8 cells, which appeared to be specific to the GC box of the promoter. It is interesting that the activation rate was dramatically high (40-fold) in A375-6 cells, and the maximum level of the promoter activity in TSA-treated A375-6 cells was comparable with that of TSA-treated A375-R8 cells. Furthermore, treatment with TSA induced IL-1α mRNA expression in A375-6 cells. Therefore, HDAC appeared to be critical for the regulation of IL-1α expression in A375 cells. As TSA also activated the transactivation activity of Sp1 and Sp3, it was suggested that recruitment of HDAC to Sp1 or Sp3 or activity of HDAC recruited to Sp1 or Sp3 was inhibited by TSA.

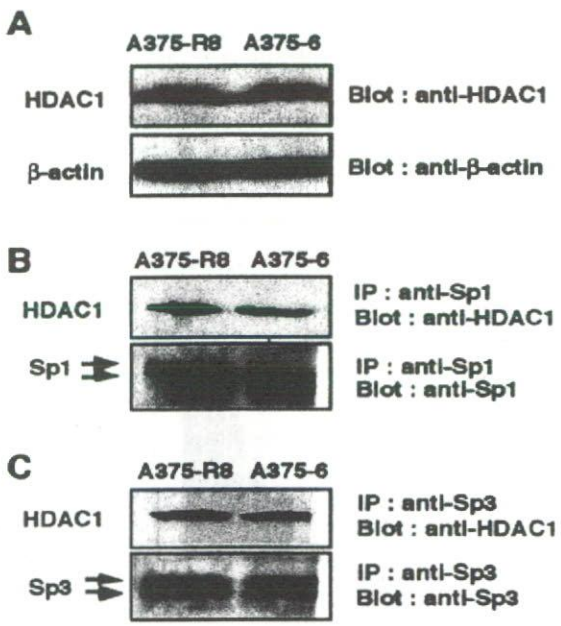


**Fig. 8.** Expression of HDAC1 and its interaction with Sp1 or Sp3 in A375 cells (A), which were lysed, and an equal amount of protein was subjected to SDS-PAGE. Immunoblotting was performed with anti-HDAC1 antibody. The expression level of each protein was assessed by immunoblotting cell lysates with anti-β-actin (lower). (B and C) A375 cells were lysed and immunoprecipitated with (B) anti-Sp1 or (C) anti-Sp3 antibodies and immunoblotted with anti-HDAC1 antibody. The same Western blot filters were probed with (B) anti-Sp1 or (C) anti-Sp3 antibodies to estimate the immunoprecipitation efficiency (lower). The same experiment as representative data of more than three experiments are shown.

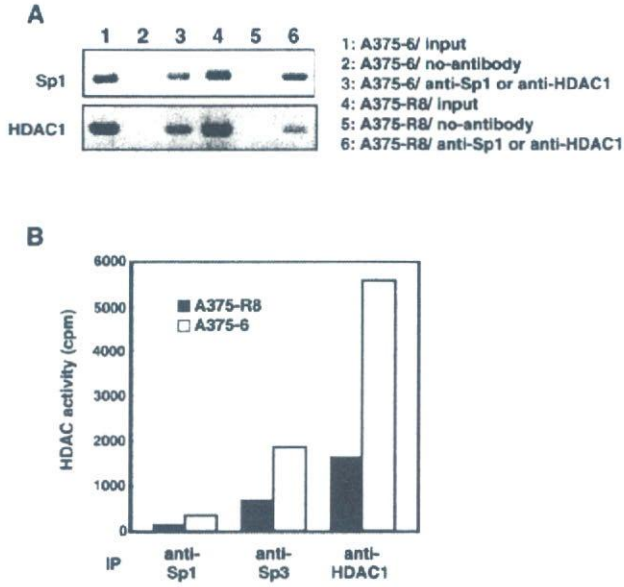


**Fig. 9.** Sp1 and HDAC1 bind to the promoter region of the IL-1α gene in A375 cells. (A) ChIP of the α gene with anti-Sp1 or anti-HDAC1 antibodies from A375 cells detected by PCR targeting the promoter region. Input corresponds to PCR mixtures containing 4% of the total amount of proteins used in immunoprecipitation reactions. (B) A375 cells were lysed, and an equal amount of protein was immunoprecipitated with anti-Sp1, anti-Sp3, or anti-HDAC1 antibodies. HDAC activity was measured as described in Materials and Methods. Representative data of three experiments are shown.

Among HDAC family members, HDAC1 and HDAC2 are shown to interact with Sp1 or Sp3 [34]. The expression levels of Sp1 and Sp3 were comparable between A375-R8 and A375-6 cells, and there were no differences in the levels of HDAC1 interaction with Sp1 or Sp3 in these cells. HDAC1 indirectly binds to DNA through the complex or transcription factors including Sp1 and Sp3 [34]. ChIP assay confirmed the binding of Sp1 and HDAC1 to the promoter region of the IL-1α gene in A375-R8 and A375-6 cells. It is interesting that our study revealed that the activity of HDAC, associated with Sp1 or Sp3, and that of HDAC1 are lower in A375-R8 cells as compared with A375-6 cells. In addition, the activity of HDAC associated with Sp3 was higher than that with Sp1. Therefore, not only the interaction between HDAC1 and Sp1 or Sp3 but also the activity of HDAC1 are critical for the differential activation of the IL-1α gene. The mechanism of HDAC activity reduction in A375-R8 cells is not known. It is hypothesized that HDAC activity is reduced by oxidative stress, including NO and its derivatives [16]. Therefore, it is tempting to speculate that oxidative stress is induced in A375-R8 cells, which may down-regulate HDAC activity.

The GC box in the promoter of the IL-1α gene has been reported to be important for the constitutive gene expression of IL-1α in many cells, including monkey kidney cell line COS-7, human osteosarcoma cell line MG-63, and human esophageal carcinoma cell line EC-GI [1]. Alveolar macrophages and blood monocytes from patients with asthma exhibit enhanced cytokine production and reduced activity of HDAC [17]. In patients of chronic obstructive pulmonary disease, alveolar macrophages are critical in orchestrating the chronic inflammation through the release of proteases and inflammatory cytokines [16]. Alveolar macrophages of cigarette smokers show a reduction in HDAC activity and expression of HDAC2 compared with cells from healthy individuals [18]. Therefore, it is possible that the GC box, Sp1, Sp3, and HDAC are similarly important in the aberrant inflammatory cytokine production in these cells as well.

## ACKNOWLEDGMENTS

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