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Heterogeneous Transforming Growth Factor (TGF)-β Unresponsiveness and Loss of TGF-β Receptor Type II Expression Caused by Histone Deacetylation in Lung Cancer Cell Lines[1]

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ABSTRACT

Transforming growth factor (TGF)-β strongly inhibits epithelial cell proliferation. Alterations of TGF-β signaling are thought to play a role in tumorigenesis. We show in the present study that most lung cancer cell lines have lost the growth-inhibitory response to TGF-β signal, and that those with TGF-β unresponsiveness can be divided into two major groups, TGF-β type II receptor (TGFβRII)(+)/Smad7(+) and TGFβRII(−)/Smad7(−), suggesting the heterogeneous mechanisms underlying the TGF-β responsiveness. The mechanism of the loss of TGFβRII expression of the latter group was further studied, identifying aberrant DNA methylation of the promoter region in a limited fraction of cell lines. Interestingly, we found that the alteration of chromatin structure because of histone deacetylation may also be involved, showing a good correlation with loss of TGFβRII expression. This notion was supported by the findings of a restriction enzyme accessibility assay, of a chromatin immunoprecipitation assay with anti-acetyl histone antibodies, and of an *in vivo* induction of TGFβRII expression by histone deacetylase inhibitors including trichostatin A (TSA) and sodium butyrate. *In vitro* induction of TGFβRII promoter reporter activity by TSA was also detected and found to require the CCAAT box within the −127/−75 region. A positive regulatory mechanism for TGFβRII expression in a TGF-β-expressing cell line was also investigated, and a TPA-responsive element (TRE)-like motif, TRE2, was detected in addition to the previously reported TRE-like motif Y element in the positive regulatory region. Alterations in two discrete proteins interacting with these two TRE-like motifs were also suspected of being involved in the loss of TGFβRII expression. This is the first study to demonstrate that, in addition to the TSA-responsive region and TRE2 motif in the TGFβRII promoter, the alteration of histone deacetylation may be involved in the loss of TGFβRII expression in lung cancer cell lines.

INTRODUCTION

TGF[3]-β is a multifunctional cytokine that strongly inhibits epithelial cell proliferation (1, 2). It binds to the membrane-bound serine/threonine kinase complex, TGFβRI and TGFβRII. The ligand-binding TGF-β receptor complex phosphorylates TGF-β-restricted Smad, Smad2, and Smad3. Next, the phosphorylated Smad2 and Smad3 translocate into the nucleus interacting with Smad4 and mediate the TGF-β signal to the transcriptional machinery. The loss of the growth-inhibitory response to the TGF-β signal (TGF-β unresponsiveness) is found in many cancers, including lung cancers, and is widely thought to promote tumor development (3, 4). However, several studies have also demonstrated that TGF-β signaling may enhance the migration and invasive behavior of tumor cells without the growth-inhibitory response to TGF-β (2, 4). Therefore, TGF-β unresponsiveness may be heterogeneous in terms of its biological effects and underlying mechanisms and thus needs to be clearly characterized.

Loss of TGFβRII expression has also been reported in many cancers, including lung cancers, which suggests its involvement in TGF-β unresponsiveness. The most frequent type of TGFβRII alteration is a frame-shift mutation of the poly(A) tract caused by the microsatellite instability phenotype (5). However, lung cancers rarely show the microsatellite instability phenotype and such TGFβRII mutations (6, 7). The regulatory mechanism of TGFβRII expression and its alterations in a few cancers have been reported. The TGFβRII promoter consists of two positive elements (PRE1 and PRE2; −219/−172 and +1/+35) and two negative regulatory elements (NRE1 and NRE2; −1240/−504 and −100/−67). Two elements (X, −207/−172; Y, −196/−189) in PRE1 and one element (Z, +11/+25) in PRE2 reportedly interact with nuclear proteins and regulate the TGFβRII expression (8). Several proteins are also reported to interact with the TGFβRII promoter and regulate TGFβRII expression. ATF-1 regulates TGFβRII expression through the Y element during the differentiation of F9 EC cells (9). The ERT (also called ESX/ESE-1) regulates the expression through the Z element (10), whereas another *ets* family gene *FLI1* fused with the *EWSR1* gene down-regulates the TGFβRII expression in Ewing sarcomas (11). The *E1A* oncogene reduces protein interaction with the PRE1 and PRE2 regions and down-regulates TGFβRII expression (12), whereas the Sp1 and Kruppel-like zinc-finger Zf9 up-regulates it (13). Significant negative regulation of TGFβRII expression by NRE2 was also demonstrated in F9 EC cells (9). Apart from the fore-mentioned action of *FL1/EWSR1* fusion gene in Ewing sarcomas, only little is known about how TGFβRII expression is compromised in human cancers.

We previously examined the mutation of *Smad* genes and found mutations of *Smad2* and *Smad4* genes in only about 5∼10% of lung cancers (14, 15). Other mechanisms must therefore be involved in the TGF-β unresponsiveness in lung cancer cell lines. In the study presented here, we studied the expression of the components of TGF-β signaling and found that lung cancer cell lines can be divided into two groups based on the expression of TGFβRII and Smad7. Further investigation of the mechanism of the loss of TGFβRII expression revealed that alteration of the chromatin structure of the TGFβRII promoter region may play a role in the loss of TGFβRII expression.

MATERIALS AND METHODS

Cell Culture, Plasmid Transfection, Northern Blot, and RT-PCR. Lung cancer cell lines were cultured in RPMI 1640 supplemented by 5% FCS. For the construction of the *TGFβRII* gene expression plasmid, the wild-type *TGFβRII* cDNA was inserted into the pcDNA3 vector (Invitrogen, Groningen, the Netherlands). The VMRC-LCD cells were transfected with the *TGFβRII*-

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[3] The abbreviations used are: TGF, transforming growth factor; TGFβRII, TGF-β type II receptor; RT-PCR, reverse transcription-PCR; ChIP, chromatin immunoprecipitation; TSA, trichostatin A; TRE, TPA-responsive element; NF-Y, nuclear factor for Y box; EMSA, electrophoresis mobility shift assay; FN, fibronectin; HDAC, histone deacetylase; EC, embryonal carcinoma; PRE, positive regulatory region; NRE, negative regulatory region; ERT, ets-related transcription factor; NaB, sodium butyrate; RE, restriction enzyme.

pcDNA3 or the empty pcDNA3 plasmid by means of DIMRIE-C (Life Technologies, Inc., Gaithersburg, MD) and selected with the aid of neomycin over a 2-week period to establish the stable clones. Their expression of TGFβRII was confirmed with Northern blotting. For the Smad7 induction study of Calu-6 and SK-LU-1, the culture medium was changed every 12 h for 3 days. For *in vivo* induction of transcription and chromatin structural change of the *TGFβRII* gene, cells were treated with 1 μM TSA(Wako Chemical, Osaka, Japan) and 3 mM NaB (Sigma Chemical Co., St. Louis, MO) for 1 day, before harvesting cells. RNA extraction, Northern blotting, and RT-PCR procedures were performed as described (16).

Cell Proliferation Assay. The cells were plated into 24-well plates at 3×10^3 cells/well and cultured in the absence or presence of TGF-β_1 (0.2, 1, or 5 ng/ml; R&D System, Minneapolis, MN) for 7 days. The cell number was measured with an colorimetric assay reagent, TetraColor One (Seikagaku, Tokyo, Japan), according to the manufacturer's protocol. Cell lines that showed >40% reduction of cell proliferation by TGF-β (5 ng/ml) were determined to be TGF-β responsive. TGF-β responsiveness was confirmed with FN induction and stress fiber formation (17).

FN Induction Assay and Western Blot. The TGF-β responsive HPL1 (17) was cultured for 3 days with TGF-β or the conditioned medium derived from a 3-day culture of SK-LU-1 or Calu6 cells at the indicated concentrations. After harvesting of the cell lysates, Western blot analysis was performed with anti-human FN polyclonal antibody (Collaborative Research Inc.) as described previously (17). To prepare the cytoplasmic and nuclear fractions, cells were resuspended in NP40 hypotonic buffer [0.5% NP40, 10 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM $MgCl_2$] for 3 min at 4°C. The nuclei were pelleted by means of brief centrifugation, and cytoplasmic proteins in the supernatant were precipitated with 10% TCA. The pelleted nuclei and precipitated cytoplasmic proteins were then resuspended in the usual SDS lysis buffer (17). Smad2/3 proteins were detected by the anti-Smad2/3 antibody N-19 (Santa Cruz Biotechnology, Santa Cruz, CA).

DNA Methylation Analysis. Genomic DNA was treated with the bisulfite method as described elsewhere (18). After conversion, the TGFβRII promoter regions were amplified with PCR and sequenced to verify the DNA methylation of CpG sites. The primer sequence was designed on the basis of the following converted sense strand sequence: sense 1, 5′-TGAAGAAAGTTGAGGGGA (−285/−265); antisense 1, 5′-ACTCAACTTCAACTCAAC (+50/+33); sense 2, 5′-AGAGAGGTTTTGTTTAGTTGTTG (−18/+4); antisense 2, 5′-ACCACAAACCCCTAAACAACC (+369/+349).

RE Accessibility Assay. As described (19), the cell nuclei resuspended in ice-cold RSB lysis buffer [0.5% NP40, 10 mM Tris (pH 7.4), 10 mM NaCl, and 5 mM $MgCl_2$] at a concentration of 1.5×10^6 cells/ml were incubated on ice for 10 min. After being subjected to brief spinning, the pelleted nuclei were resuspended in 200 μl of 1× RE buffer with 40 units/10^6 nuclei of *Eco*RI, *Xba*I, or *Pvu*II (New England Biolabs, Beverly, MA) and incubated at 37°C for 30 min. DNA was then extracted with the usual proteinase K/phenol procedure, digested with *Hin*dIII, and analyzed with Southern blotting with the indicated probe. RE accessibility was calculated on the basis of the intensity of undigested and digested bands measured by a phosphorimager BAS-2500 (Fuji Film, Tokyo, Japan).

ChIP. Before harvesting, cells were treated with 1% formaldehyde for 10 min at 37°C to cross-link the histones to DNA. Cells were then harvested and resuspended in 200 μl of SDS lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris-HCl (pH 8.1)] for 10 min on ice. After brief sonication, the anti-acetyl histone H3 or H4 antibodies (Upstate Biotechnology, Lake Placid, NY) were added to the lysate. After overnight incubation, immune complexes were collected with protein A-Sepharose (Amersham Pharmacia, Buckinghamshire, United Kingdom; Ref. 20). The histone-DNA complexes were eluted, extracted with the standard proteinase K/phenol extraction procedures, and used as templates for PCR of *TGFβRII* or *β-actin* gene promoters. The following oligonucleotides were used for PCR: sense primer for *TGFβRII* promoter, GAGAGAGCTAGGGGCTGG; antisense, CTCAACTTCAACTCAGCGCTGC; sense primer for *β-actin* gene promoter, CCAACGCCAAAACTCTCCC; antisense, AGCCATAAAAGGCAACTTTCG.

Reporter Plasmid and Luciferase Assay. TGFβRII promoter fragments (−1887/+50, −1248/+50, −370/+50, −280/+50, and −176/+50) were generated with PCR. The amplified genomic fragments were sequenced and inserted into pGL3-basic luciferase reporter plasmid (Promega Corp., Madison, WI). The internal deletions (−370/+50 del −127/−3) were made with *Pvu*II digestion. Other internal deletion reporters (−370/+50 del −42/−3 or del −127/−75) were created by insertion of genomic fragments (−127/−43 or −74/−3) after *Pvu*II digestion. The reporters with point mutations were generated with the Chameleon Double-stranded Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The reporter activity was measured with the Luciferase Assay System with Reporter Lysis Buffer (Promega). For normalization, the β-galactosidase expression plasmid (pEF-BOS-β-GAL) was cotransfected with luciferase reporters, and β-galactosidase activity was measured as described (21). The normalized luciferase activity was calculated by dividing the raw values for the luciferase activity by those for the β-galactosidase activity to compare the promoter activity between different cell lines (21).

EMSA. Nuclear extract preparation and EMSA analyses were performed as reported elsewhere (22). Oligonucleotide probes were labeled with [α-^{32}P]dCTP (Amersham Pharmacia) and the Klenow polymerase (New England Biolabs) after annealing of the oligonucleotides. The following probe sequences were used: GTGTGCACTTAGTCATTCTTGAGTAAATACTTGGA for the wild type; GTGTGCACTTTACGATTCTTGAGTAAATACTTGGA for the TRE1 mutation; and GTGTGCACTTAGTCATTCTTTCGGAAATACTTGGA for the TRE2 mutation.

RESULTS

Differences in TGF-β Unresponsiveness of Lung Cancer Cell Lines. To verify TGF-β unresponsiveness in lung cancers, we first examined the growth-inhibitory effect of TGF-β on 33 lung cancer cell lines and 2 immortalized normal respiratory tract epithelial cell lines, BEAS2B (23) and HPL1 (17). TGF-β showed strong growth inhibition in BEAS2B and HPL1 cell lines, whereas only 4 cell lines including A549 showed suppression of cell proliferation by the TGF-β stimuli (Table 1 and Fig. 1*A*). Previously, we found that alterations of the *Smad2* or *Smad4* genes occur only in about 5∼10% of lung cancers (14, 15). We therefore looked for other mechanisms of TGF-β unresponsiveness in lung cancer cells by studying the expression of *Smad2*, *Smad4*, *Smad7*, *TGFβRI*, and *TGFβRII* genes. The expression levels of the *Smad2* and *Smad4* genes and the *TGFβRI* gene in the cell lines were similar (data not shown). In contrast, a significant difference was detected in the expression of *TGFβRII* and *Smad7* genes (Fig. 1*B*). SK-LU-1 and Calu6 expressed the *TGFβRII* gene in abundance. *Smad7* was also expressed more abundantly than in BEAS2B, HPL1, and A549. In contrast, no *TGFβRII* signals and very few *Smad7* transcripts were detected in VMRC-LCD and ACC-LC-176. These results showed that the TGF-β-unresponsive cancer cell lines could be divided into two major groups (Table 1 and Fig. 1*B*), one showing abundant expressions of the *TGFβRII* and *Smad7* genes, and the other significantly reduced expressions of the same genes.

Table 1 *The expression of the TGFβRII and Smad7 genes in lung cancer cell lines*

	NSCLC[a]	SCLC	Total
TGF-β responsive[b]	4 (17%)[c]	0	4 (12%)[d]
TGFβRII(+)	4 (17%)	0	4 (12%)
Smad7(+)	1 (4%)	0	1 (3%)
Smad7(−)	3 (13%)	0	3 (9%)
TGFβRII(−)	0	0	0
TGF-β unresponsive[b]	20 (83%)	9	29 (88%)
TGFβRII(+)	8 (33%)	0	8 (24%)
Smad7(+)	5 (21%)	0	5 (15%)
Smad7(−)	3 (13%)	0	3 (9%)
TGFβRII(−)	12 (50%)	9	21 (64%)
Smad7(+)	1 (4%)	2	3 (9%)
Smad7(−)	11 (46%)	7	18 (55%)

[a] NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.
[b] TGF-β responsiveness was examined with cell proliferation assay. Cell lines showed >40% reduction of cell proliferation by TGF-β (5 ng/ml) were determined as TGF-β responsive. TGF-β responsiveness was also confirmed with stress fiber formation and FN induction (17).
[c] The percentages in NSCLC are shown.
[d] The percentages in all examined cell lines are shown.

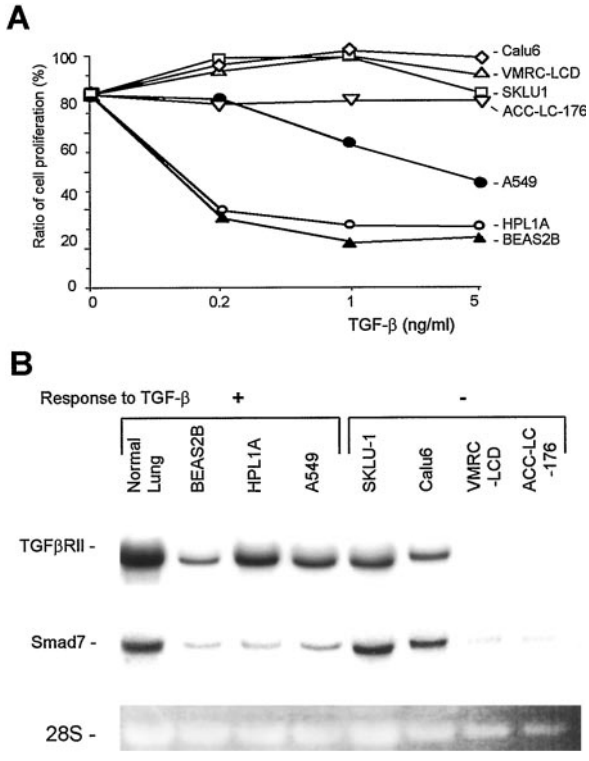


Fig. 1. *A*, the abrogation of growth-inhibitory effect of TGF-β signal in lung cancer cell lines. Cell growth was measured with the colorimetric assay, TetraColor One, and expressed as the ratio of TGF-β treated cells to nontreated cells. Thirty-five cell lines were examined, and representative results of 7 cell lines are shown. *B*, Northern blot analyses of *TGFβRII* and *Smad7* genes in lung cancer cell lines. Correlation of TGFβRII and Smad7 expression was observed in the TGF-β unresponsive cell lines, which can be divided into two groups, TGFβRII(+)/Smad7(+) (SK-LU-1 and Calu-6) and TGFβRII(−)/Smad7(−) (VMRC-LCD and ACC-LC-176). The results of normal lung tissue and normal lung cell lines are also shown.

We studied the mechanism of unresponsiveness of the former group (Calu-6 and SK-LU-1). Because Smad7 can inhibit TGF-β signaling, we examined whether the expression of Smad7 was constitutively abnormal or normally inducible by TGF-β stimuli. We found that with frequent medium changes (every 12 h), *Smad7* gene expression became inducible by TGF-β stimuli in SK-LU-1 and Calu-6, as was observed in the TGF-β-responsive cell lines, BEAS2B and A549 (Fig. 2*A*). We checked the localization of Smad molecules with Western blotting of the fractionated cell lysate (cytoplasmic and nuclear fractions) and found that Smad2 and Smad3 were localized in the nuclei in Calu-6 and SK-LU-1, regardless of the exogenous TGF-β signal (Fig. 2*B*). TGF-β activity in the conditioned medium of SK-LU-1 and Calu-6 was also measured with a FN induction assay using HPL1 cell lines (17). The conditioned medium of these cell lines showed strong TGF-β activity (Fig. 2*C*). These results suggest that, although these TGF-β-unresponsive cells retain their ability to mediate the TGF-β signal, they do not respond to the exogenous TGF-β because of the abundantly expressed TGF-β.

Alteration of Chromatin Structure in Lung Cancer Cell Lines with Loss of TGFβRII Expression. The lung cancer cell lines of the latter group (ACC-LC-176 and VMRC-LCD) showed loss of the *TGFβRII* gene expression, which presumably results in disruption of the TGF-β signaling. In fact, neither ACC-LC-176 nor VMRC-LCD showed Smad7 induction or nuclear localization of Smad2 and Smad3 proteins after TGF-β stimulation (Fig. 2, *A* and *B*). To verify the biological significance of the loss of TGFβRII expression, the *TGFβRII* gene was introduced into the VMRC-LCD cell line. TGFβRII expression in all clones examined was confirmed with Northern blot analysis. The growth-inhibitory effect of TGF-β was clearly demonstrated in TGFβRII-expressing stable clones, whereas no effect was observed in clones transfected with an empty vector. This result suggests that loss of TGFβRII expression may be responsible for TGF-β unresponsiveness (Fig. 3*A*). Therefore, the mechanism of loss of TGFβRII expression was further studied.

The genetic alteration of the TGFβRII promoter and coding regions was reported previously to be very rare in lung cancers (7). We therefore studied epigenetic mechanism as a cause of loss of TGFβRII expression. DNA methylation and chromatin structure are now thought to be significantly involved in transcriptional regulation (24). DNA methylation was studied with the bisulfite conversion technique in a panel of lung cancer cell lines with or without TGFβRII expression. After conversion, the TGFβRII promoter and exon 1 noncoding region containing CpG clusters were amplified and sequenced. Three of the 13 cell lines without TGFβRII expression demonstrated heavy DNA methylation at almost all CpG sites, whereas other cell lines without TGFβRII expression and all cell lines with TGFβRII expression showed DNA methylation in a very limited number of CpG sites. These results suggest the potential involvement of DNA methylation in loss of TGFβRII expression in a small fraction of lung cancer cell lines (Fig. 3*B*).

The chromatin structure was studied with the RE accessibility assay (Ref. 19; Fig. 4). Three REs, *Eco*RI, *Xba*I, and *Pvu*II, were used. The *Pvu*II sites are located (−127 and −2) in the vicinity of the transcription initiation site, whereas the *Eco*RI (−1101) and *Xba*I (−506) sites are far away (Fig. 4*A*). *Pvu*II enzyme digestion demonstrated readily detectable digestion of the nuclear DNA of the A549 expressing *TGFβRII* gene (Fig. 4*A*, A549 *Lane N* in the *Pvu*II panel), whereas VMRC-LCD and ACC-LC-176 without TGFβRII expression were completely resistant. The other two REs did not show any digestion in any of these cell lines (Fig. 4*A*). These results suggest that the chromatin structure is open near the transcriptional initiation site only in the TGFβRII-expressing cells and closed at remote locations, regardless of the expression level of the *TGFβRII* gene. To verify this observation, other cell lines were examined. The strong correlation between *Pvu*II accessibility and TGFβRII expression was confirmed, suggesting that the alteration of the chromatin structure may affect the expression of the *TGFβRII* gene (Fig. 4*B*).

The ChIP assay was performed by using anti-acetyl histone H3 or H4 antibodies in ACC-LC-176, VMRC-LCD, and A549 (Ref. 20; Fig. 5*A*). For the positive control, the quantity of β-actin promoter in the precipitates was examined using the primers for the β-actin promoter. The β-actin promoter region was similarly amplified in the three cell lines, regardless of TGFβRII expression. In contrast, significant reduction of amplification of the TGFβRII promoter was observed in ACC-LC-176 and VMRC-LCD but not in A549, suggesting that the acetylation of histones H3 and H4, which were combined with the TGFβRII promoter region, was significantly reduced in the cell lines without TGFβRII expression. To further confirm the alteration in histone acetylation and chromatin structure at the TGFβRII promoter region, the effects of the HDAC inhibitors TSA and NaB were examined. ACC-LC-176 and VMRC-LCD were treated with TSA (1 μM) or NaB (3 mM) for 1 day, after which, the transcription level and chromatin structure of the endogenous *TGFβRII* gene was examined with RT-PCR (Fig. 5*B*) and *Pvu*II RE accessibility assay (Fig. 5*C*). TGFβRII expression was not detected even with RT-PCR in ACC-LC-176, whereas the expression was strongly induced by TSA or NaB treatment. Similar results were obtained also for VMRC-LCD (Fig. 5*B*). The accessibility of *Pvu*II RE to the promoter region was increased in response to the TSA and NaB treatments (Fig. 5*C*), although full accessibility was not achieved in comparison with those of cell lines with abundant *TGFβRII* transcripts (Fig. 4). These results

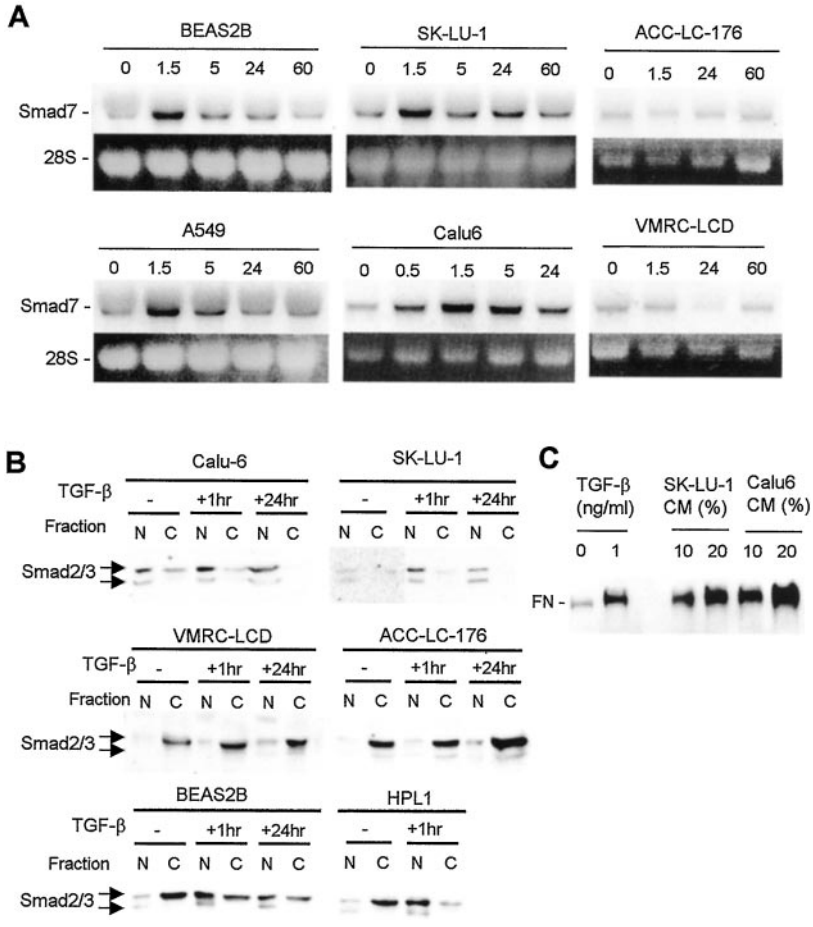


Fig. 2. *A*, Northern blot of Smad7 induction by TGF-β signal. RNA samples from the indicated cell lines were harvested after TGF-β treatment for the indicated incubation periods (h). TGF-β unresponsive cell lines, SK-LU-1 and Calu-6, showed prompt induction of Smad7 expression 1.5 h after the TGF-β stimuli as the TGF-β responsive cell lines BEAS2B and A549, whereas no transcriptional response of *Smad7* gene was observed in ACC-LC-176 or VMRC-LCD. *B*, Western blot of Smad2 and Smad3 proteins in Calu-6 and SK-LU-1. In Calu-6 and SK-LU-1, Smad2/3 were constitutively located in nuclei regardless of the exogenous TGF-β stimuli. TGF-β unresponsive cell lines, BEAS2B and HPL1, showed prompt nuclear localization of Smad2/3 proteins 1.5 h after the TGF-β stimuli, whereas no change of Smad2/3 protein localization was observed in ACC-LC-176 or VMRC-LCD. *N*, nuclear fraction; *C*, cytoplasmic fraction. *Panel C*, TGF-β activity in the conditioned mediums (*CM*) of SK-LU-1 and Calu-6. The cellular FN induction in HPL1 was measured with Western blot analysis using in the anti-human FN antibody. Significant induction of FN in response to the conditioned medium of SK-LU-1 and Calu-6 was clearly demonstrated, indicating that SK-LU-1 and Calu-6 secreted active TGF-β abundantly.

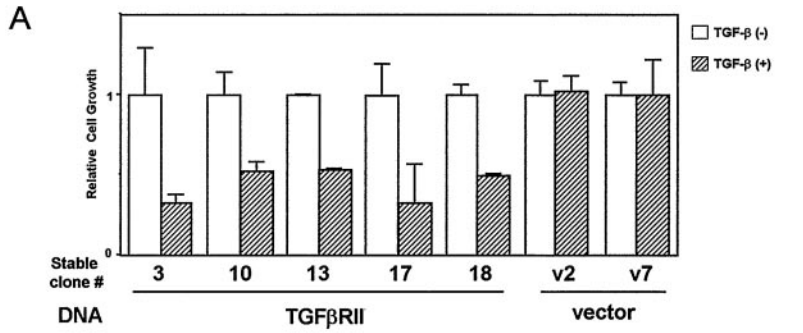
imply that the alteration in histone acetylation resulting in a closed chromatin structure may cause the loss of TGFβRII expression.

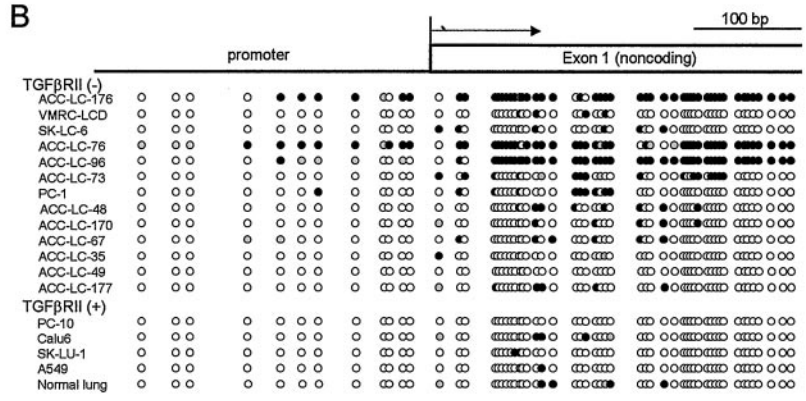
TSA Responsiveness of TGFβRII Promoter Reporters. We studied the transcriptional activity of TGFβRII promoter reporters to further clarify the regulation of histone acetylation within the TGFβRII promoter and the effect of HDAC inhibitors on its transcriptional activity. We first constructed reporters containing TGFβRII promoter regions of various lengths (−1887/+50, −1248/+50, −370/+50, −280/+50, and −176/+50) and measured their transcriptional activity in A549 and ACC-LC-176 (Fig. 6*A*). The luciferase activity of all TGFβRII reporters was very strong in A549, whereas ACC-LC-176 showed very weak transcriptional activity, suggesting changes in the epigenetic regulation and/or transcriptional factors, but not genetic alterations, as the cause of the loss of TGFβRII expression in ACC-LC-176 (Fig. 6*A*). Similar results were obtained in VMRC-LCD (data not shown).

Because the endogenous *TGFβRII* gene expression was induced by TSA and NaB, the effect of TSA on the −370/+50 reporter activity was studied. In A549, TSA showed a weak effect on transcriptional activity, whereas in ACC-LC-176, TSA strongly enhanced transcription in a manner similar to its *in vivo* effect (Fig. 6*B*), suggesting that the TGFβRII promoter might be negatively regulated by HDAC in these cell lines without TGFβRII expression. To further study TSA responsiveness of the TGFβRII reporter, the effect of TSA was also examined by using reporters with various internal deletions. The −370/+50 reporter with internal deletions of the −127/−3 or −127/−75 regions showed complete elimination of the TSA response in ACC-LC-176, whereas the reporter with the −43/−3 internal deletion still exhibited a TSA response similar to that of the wild-type −370/+50 reporter. According to these results, the transcriptional induction of the TGFβRII reporter by TSA was proved to depend strongly upon the −127/−75 region (Fig. 6*B*). The DNA sequence showed that the CCAAT box was located (−81/−77) within this TSA responsive region.

To further investigate the mechanism of TSA induction, we tried to determine the molecules involved in this transcriptional induction by using the reporter with the CCAAT box point mutation. The TSA response in ACC-LC-176 (33.7-fold increase in the wild-type −370/+50 reporter) was significantly reduced by the CCAAT box mutation (7.7-fold) or −127/−75 internal deletion (2.5-fold; Fig. 6*C*), suggesting that the CCAAT box and CCAAT box-interacting proteins are involved in this TSA induction. Because the NF-Y was thought to be a major CCAAT box binding factor (25), we studied the effect of wild-type and dominant-negative NF-YA, a subunit of NF-Y. However, the cotransfected wild-type or dominant-negative *NF-YA* gene did not show any effect of the TSA response, suggesting that other proteins may be involved (data not shown).

Transcriptional Activity of TRE-like Motifs. The findings presented here suggest that alterations of the chromatin structure may be responsible for loss of TGFβRII expression in lung cancer cell lines, but that alterations in the expression of transcription factors may also be involved in loss of the expression. Because the reporter assay (Fig. 6*A*) indicated that the transcriptionally active region in A549 was located in the −280/−176 region, which almost corresponds to PRE1 (8), we also investigated the potential importance of this region in the regulation of TGFβRII expression in lung cancers. A previous study has demonstrated the existence of the X (−207/−197) and Y (−196/−189; TTAGTCAT) elements in PRE1 (8). The Y element, called





	DNA methylation		total
	(+)	(-)	
TGFβRII (-)	3	10	13
TGFβRII (+)	0	12	12

Fig. 3. *A*, restoration of growth inhibition by TGF-β signal in TGFβRII stable transfectants of VMRC-LCD. All stable transfectants expressing the *TGFβRII* gene demonstrated growth inhibition by TGF-β, whereas no transfectants of the empty vector did. The average cell number of each transfectant without TGF-β treatment is shown as *1*. *B*, DNA methylation of TGFβRII promoter. Results for 25 lung cancer cell lines are shown in the table under the figure. Results for all 13 lung cancer cell lines without TGFβRII expression [*TGFβRII (−)*], representative four lung cancer cell lines with TGFβRII expression [*TGFβRII (+)*], and 1 normal lung tissue are demonstrated. *Circles*, CpG sites. ●, complete preservation of cytidine residue after the bisulfate conversion; ◍, partial preservation. Three cell lines, ACC-LC-176, ACC-LC-76, and ACC-LC-96, showed heavy DNA methylation of almost all CpG sites within the promoter and noncoding region of exon 1. ACC-LC-73 and PC-1 showed weak DNA methylation, whereas that of other cell lines including that of normal lung was very limited.

TRE1 in this report for reasons of convenience, is similar to the TRE motif [TGA(C/G)TCA]. Examining the DNA sequence showed that an additional TRE-like motif, termed TRE2 (TGAGTAA), is also present at −185/−179 in the PRE1 (Fig. 6*A*). To study the functional significance of these TRE-like motifs, the −370/+50 reporter with mutations of TRE1 or TRE2 was constructed. The reporter assay demonstrated that the reporter activity was significantly reduced to 23.5% for the TRE1 mutant and 28.7% for the TRE2 mutant in A549 (Fig. 7*A*), suggesting that both TRE-like motifs play significant roles in the positive transcriptional regulation of the *TGFβRII* gene. For further examination, we performed an EMSA with probes containing wild-type TRE1/2, mutant TRE1, or mutant TRE2 (Fig. 7*B*). The nuclear extract from A549 showed two intense shifted bands, whereas the mutant TRE1 probe demonstrated only the lower shifted band and the mutant TRE2 probe only the upper shifted band, suggesting that the upper and lower bands correspond to, respectively, the TRE1 and TRE2 interacting proteins. To further verify the interaction, cold competitors were added to the wild-type TRE1/2 probe. The wild-type competitor significantly diminished both bands, whereas mutant TRE1 and mutant TRE2 competitors completely eliminated the lower and upper bands, respectively, suggesting again that the upper and lower shifted bands correspond to discrete proteins interacting specifically with TRE1 and TRE2 motifs. The nuclear extracts from ACC-LC-176 and VMRC-LCD were analyzed with the same probes (Fig. 7*B*). Both ACC-LC-176 and VMRC-LCD showed patterns different from that of A549. In ACC-LC-176, the upper band was predominant, whereas the intensity of the lower band was very weak. In contrast, the lower band was predominant in the VMRC-LCD extract. These results suggest that the lack of either of the TRE1/2 interacting proteins may contribute to the loss of TGFβRII expression in these two cell lines.

To further clarify the characteristics of molecules involved in the TRE1/2 motifs, we studied the involvement of c-JUN, which binds to a TRE motif, by using the dominant-negative c-JUN-expressing construct. However, this mutant c-JUN did not show any reduction in reporter activity in A549 (data not shown). The ATF/cyclic AMP-responsive element binding protein family gene, *ATF-2*, which may interact with these TRE-like motifs (26), did not demonstrate any positive effect on the reporter activity in ACC-LC-176 or VMRC-LCD (data not shown).

DISCUSSION

Our study demonstrated loss of growth-inhibitory response to the TGF-β signal (TGF-β unresponsiveness) in the majority of lung cancer cell lines. Two major groups could be distinguished among these cell lines, *i.e.*, TGFβRII(+)/Smad7(+) and TGFβRII(−)/Smad7(−). The former appeared to retain the ability to respond to TGF-β, whereas abundant expression of TGF-β may have saturated the TGF-β signaling pathway, thus diminishing responsiveness. It is worth noting that TGF-β paradoxically enhances the invasive and metastatic potential of tumor cells and induces angiogenesis in certain tumors (2, 27). Therefore, autocrine stimulation of TGF-β may play a role in the enhancement of the growth and/or progression of the tumors of the TGFβRII(+)/Smad7(+) group *in vivo*, although the precise role of this group in lung cancer development remains to be determined, because the difference in the mechanism of TGF-β unresponsiveness may well become an important marker for decision-making regarding cancer therapy.

In contrast, cell lines of the TGFβRII(−)/Smad7(−) group exhibited lack of transcriptional response to TGF-β stimuli (Fig. 2, *A* and *B*), suggesting that the underlying mechanism of TGF-β unrespon-

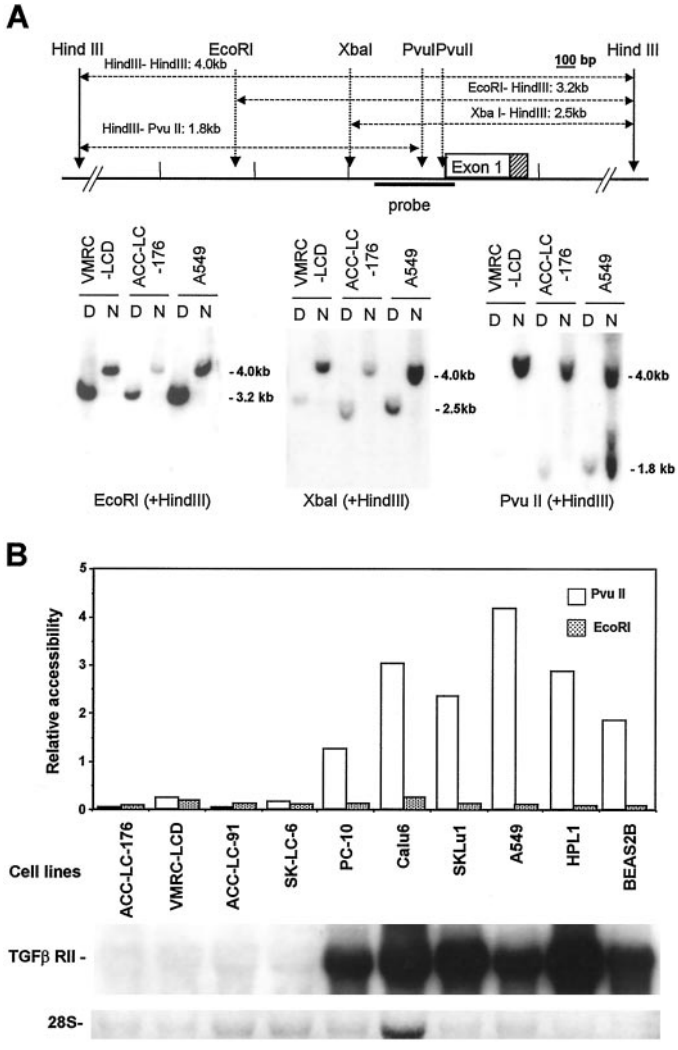


Fig. 4. *A*, accessibility of REs in the nucleus. The enzyme sites for *Eco*RI, *Xba*I, and *Pvu*II within the TGFβRII promoter are indicated, and Southern blotting results of ACC-LC-176, VMRC-LCD, and A549 with *Eco*RI, *Xba*I, and *Pvu*II are shown. The intense 1.8-kb band in *Pvu*II-treated A549 nuclei (*Lane N*) indicates high accessibility resulting from the open chromatin structure around the *Pvu*II site. *Lane N* shows results for RE-treated nuclei of each cell line, whereas *Lane D* shows results for RE-digested purified DNA from each cell line. *B*, correlation between *Pvu*II accessibility and TGFβRII expression. *Eco*RI accessibility was not detected in any cell lines regardless of TGFβRII expression, and *Pvu*II accessibility was observed only in TGFβRII-expressing cell lines, indicating the correlation between chromatin structure and TGFβRII expression. The results of Northern blotting with the TGFβRII probe are also shown.

siveness was completely different from that of the TGFβRII(+)/Smad7(+) group. Although loss of TGFβRII expression in lung cancer cells was reported previously (28, 29), its mechanism has not been fully identified. Ours is thus the first demonstration of a significant correlation between alteration in the chromatin structure of TGFβRII promoter, presumably caused by histone deacetylation and the loss of TGFβRII expression. The alteration in the chromatin structure may be caused by direct or indirect recruitment of HDAC to the TGFβRII promoter in cancer cells without TGFβRII expression. Treatment with HDAC inhibitors induced transcription of the *TGFβRII* gene (Fig. 5*B*) and increased the accessibility of *Pvu*II RE to the TGFβRII promoter (Fig. 5*C*). These data suggest that the chromatin structure of the TGFβRII promoter plays a significant role and that the chromatin structure might directly cause the loss of TGFβRII expression, although it remains possible that some other event blocked receptor expression and was then reinforced by subsequent changes in the chromatin structure. In general, histone deacetylation and subsequent chromatin structural alterations are thought to follow changes in the expression or function of DNA binding factors (transcription factors or repressors), because the HDAC molecules do not have direct DNA binding activity. Therefore, further studies on the HDAC-recruiting mechanism will be interesting. Heavy DNA methylation at the promoter and exon 1 of TGFβRII was detected only in cell lines without TGFβRII expression, suggesting that DNA methylation may be partly involved in the loss of TGFβRII expression in a fraction of such cell lines.

Our study demonstrates that HDAC inhibitors can increase the transcriptional activity of TGFβRII *in vivo* and *in vitro* in human lung cancer cell lines. Moreover, TSA responsiveness was shown for the first time to be dependent upon the CCAAT box within the −127/−75 region. TSA induction of TGFβRII expression may be applicable for lung cancer treatment or prevention, although additional studies are necessary to fully understand the precise mechanism. In this regard, NF-Y is thought to be the most specific CCAAT box binding factor and to be involved in the chromatin remodeling by recruiting p300/CBP associated factor (PCAF) (25). However, preliminary experiments with the dominant-negative NF-YA did not indicate the involvement of NF-Y in TSA responsiveness of the TGFβRII promoter (data not shown). Interestingly, TSA responsiveness was not specific for cell lines without TGFβRII. The truncated reporter −175/+50 showed TSA responsiveness in A549 (9.0-fold increase), whereas reporters with longer inserts (−1887/+50 to −280/+50) did not demonstrate comparably strong TSA responses (1.8–1.4-fold). These

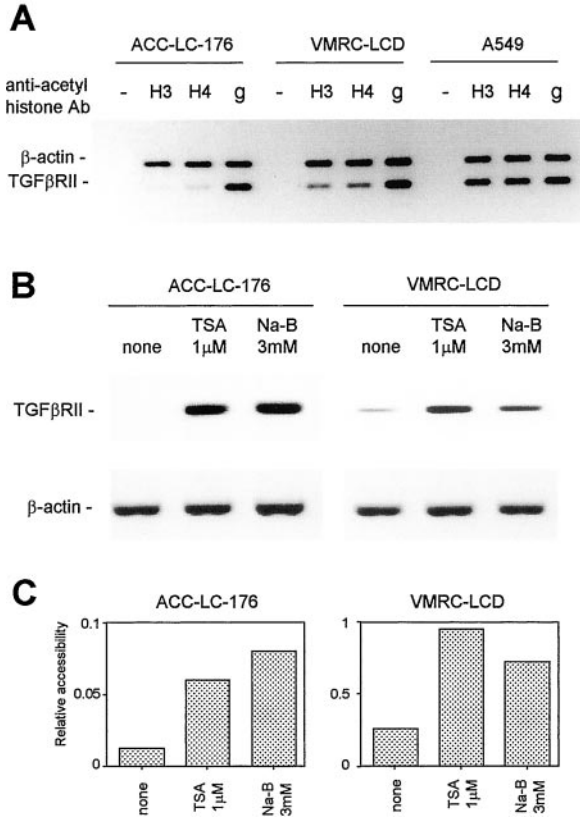


Fig. 5. *A*, ChIP assay with anti-acetyl histone antibodies. The TGFβRII and β-actin promoter regions were amplified using immunoprecipitates of ACC-LC-176, VMRC-LCD, and A549 as PCR templates. The TGFβRII promoter was significantly reduced in ACC-LC-176 and VMRC-LCD, whereas the β-actin promoter was similarly amplified in all three cell lines. *B* and *C*, the *in vivo* induction of transcription and open chromatin structure of endogenous *TGFβRII* gene by TSA and NaB. The expression of the endogenous *TGFβRII* gene was induced *in vivo* by the histone deacetylase inhibitors, TSA and NaB, in ACC-LC-176 and VMRC-LCD (*B*). Changes of chromatin structure by TSA and NaB were also observed with *Pvu*II RE accessibility assay in these cell lines (*C*).

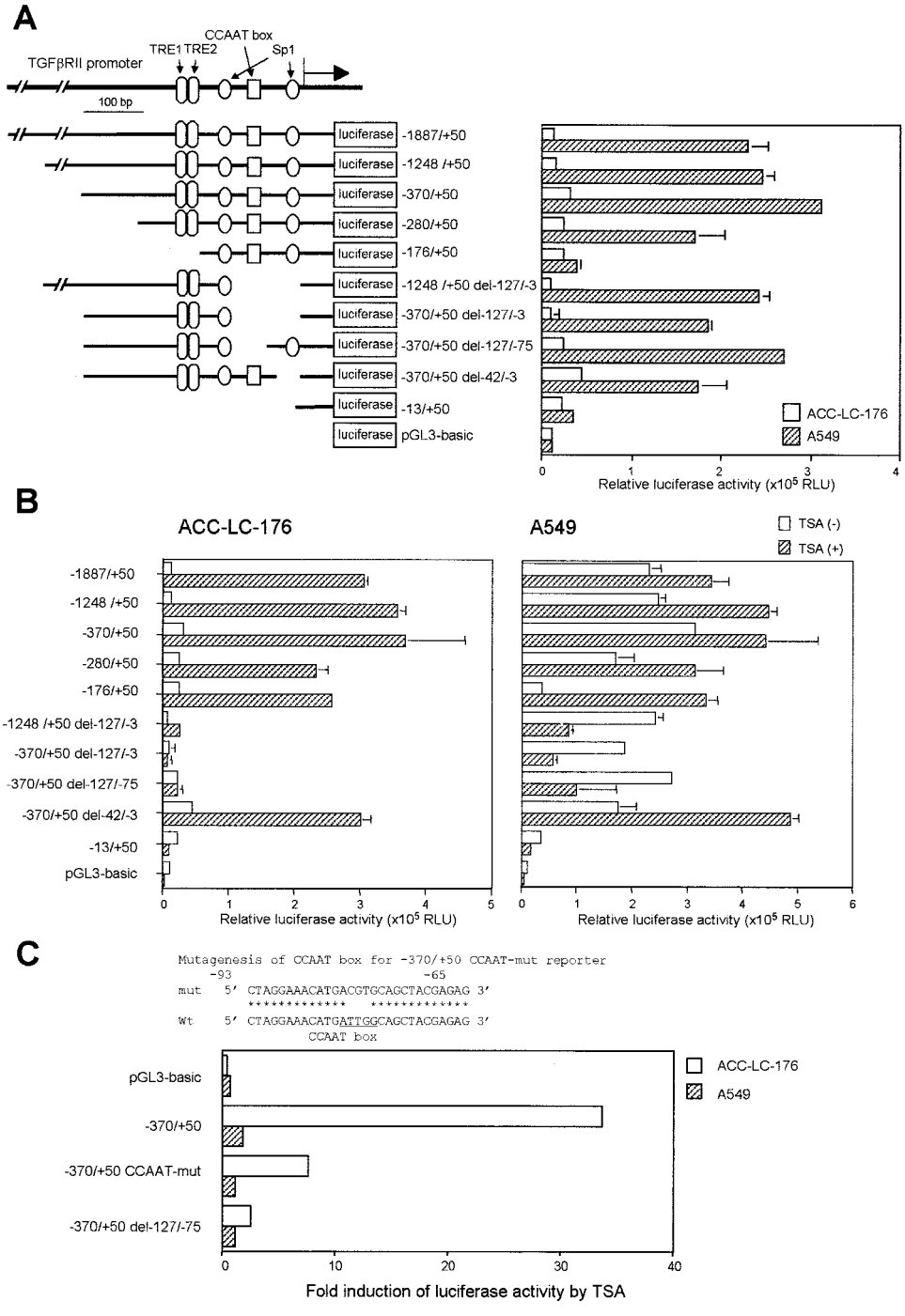


Fig. 6. *A*, luciferase assay with TGFβRII promoter reporters containing truncations or internal deletions. ACC-LC-176 showed limited transcriptional activity, whereas strong reporter activity was observed in A549. The major transcriptional activity in A549 was localized within −280/−176. Luciferase reporter activity was normalized with co-transfected β-galactosidase activity. *Bars*, SD. *B*, induction of transcriptional activity by TSA. TSA significantly enhanced the reporter activity dependent upon the −127/−75 region in ACC-LC-176, whereas TSA induction was much weaker in A549. *Bars*, SD. *C*, CCAAT box influence on TSA induction. In comparison with the wild-type −370/+50 reporter (33.7-fold increase in response), the −370/+50 reporter with the CCAAT box point mutation (7.7-fold increase) did not respond to the TSA treatment in ACC-LC-176, nor did the −370/+50 with internal deletion −127/−75 (2.5-fold increase).

results suggest that the upstream positive regulatory region may inhibit the binding of HDAC recruiting molecules to the TSA-responsive region and induce chromatin remodeling and transcriptional activity. During the preparation of this manuscript, another HDAC inhibitor, MS-275, was reported to induce TGFβRII expression in breast cancer cell lines (30), with the suggestion that the altered regulation of the chromatin structure of TGFβRII might be common in human cancers.

The study presented here demonstrates that TRE1 (Y element) and TRE2 motifs were important for transcriptional activity of the *TGFβRII* gene in the TGFβRII-expressing lung cancer cell line. The TRE1 motif reportedly plays a significant role in TGFβRII expression (8) and interacts with the ATF-1 transcriptional factor in differentiated F9 EC cells (9), whereas the importance of TRE2 has not yet been demonstrated. Our findings indicate that TRE2 also regulates TGFβRII expression. Although TRE1 and TRE2 are similar to the TRE motif, the EMSA analysis demonstrated that different proteins (or protein complexes) interacted with the motifs and that the expression pattern of these proteins was altered in TGFβRII-negative cell lines. Preliminary studies showed that none of the antibodies against c-JUN, c-FOS, or JUNB affected the bandshift patterns, whereas ATF-2, which is theoretically capable of interacting with the TRE-like motifs (26), did not demonstrate any positive effect on the reporter activity (data not shown). The further identification of the TRE1/2-interacting proteins may further clarify the mechanism of the loss of TGFβRII expression. Overexpression of the *ERT* gene has been

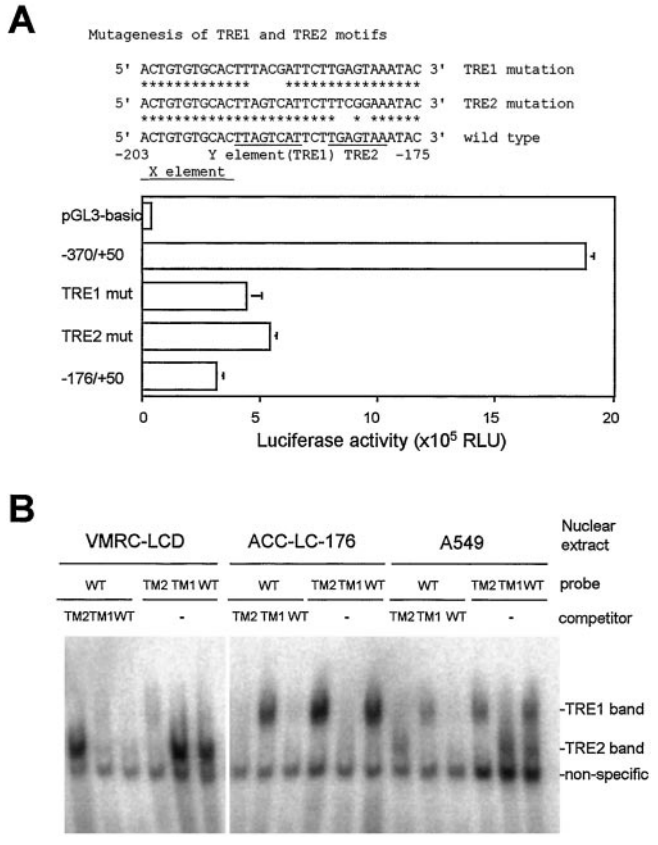


Fig. 7. *A,* transcriptional activity of the reporters with TRE mutants in A549. A significant reduction in luciferase activity was observed in the TRE1 (23.5%) and TRE2 (28.7%) mutant reporters as well as in the −176/+50 reporter (16.7%). *Bars,* SD. *B,* EMSA assay with TRE1/TRE2 probes. *WT, TM1,* and *TM2* indicate oligonucleotides of wild-type, TRE1 mutant, and TRE2 mutant, respectively. A549 showed two discrete bands. The results with probes and cold competitors of the TRE1 or TRE2 mutant showed that the *upper* and *lower bands* corresponded to the TRE1 and TRE2 motifs, respectively. The upper band was predominant in ACC-LC-176, whereas the lower band was predominant in VMRC-LCD.

shown to induce TGFβRII expression in a breast cancer cell line (31). However, the −3/+50 reporter containing the ERT-interacting Z element did not show clear transcriptional activity in lung cancer cell lines. This finding could be explained by the fact that ERT is hardly expressed in the lung (32).

In summary, our study has demonstrated for the first time that the alteration in the chromatin structure may be involved in the loss of TGFβRII expression, implying that the induction of chromatin remodeling by HDAC inhibitors may be a potentially interesting alternative for treatment of lung cancers without TGFβRII expression. Our findings provide important clues to a better understanding of the precise mechanism of positive and negative regulation of TGFβRII expression in lung tissue and lung cancers and may also provide a tool for restoring TGFβRII expression for the treatment and prevention of lung cancer.

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