JBC Papers in Press. Published on March 28, 2008 as Manuscript M706426200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M706426200

HUMAN T-CELL LEUKEMIA VIRUS TYPE 1 ONCOPROTEIN TAX REPRESSES ZNF268 EXPRESSION THROUGH THE CREB/ATF PATHWAY

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Running title: Tax represses ZNF268 expression through CREB/ATF pathway

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Key Words: Tax, CREB, ZNF268, Gene regulation, Leukemia, HTLV

Expression of the human T-cell leukemia virus type 1 (HTLV-1) oncoprotein Tax is correlated with cellular transformation, contributing to the development of adult T-cell leukemia. In this study, we investigated the role of Tax in the regulation of ZNF268 gene, which plays a role in the differentiation of blood cells and the pathogenesis of leukemia. We demonstrated that ZNF268 mRNA was repressed in HTLV-1 infected cells. We also showed that stable and transient expression of HTLV-1 Tax led to repression of ZNF268. In addition, by using reporter constructs that bear the human ZNF268 promoter and its mutants, we showed that Tax repressed ZNF268 promoter in a process dependent on a functional CRE element. By using Tax, CREB-1, CREB-2, and their mutants, we further showed that Tax repressed ZNF268 through the CREB/ATF pathway. Electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) demonstrated the formation of the complex of Tax-CREB1 directly at the CRE both in vitro and in vivo. These findings suggest a role for ZNF268 in aberrant T cell proliferation observed in HTLV-1 associated diseases.

Human T-cell leukemia virus type I (HTLV-1) is the first discovered human retroviral pathogen. It has been firmly implicated with the etiology of an aggressive malignancy known as adult T-cell leukemia (ATL) and of a neurological progressive inflammatory syndrome called tropical spastic paraparesis or HTLV-1 associated myelopathy (TSP/HAM) (1,2). Tax was originally discovered as a trans-activator modulating the synthesis or function of a wide variety of cellular regulatory factors that control gene expression, cell replication and differentiation, cell cycle, apoptosis, and genome stability. Thus, Tax is widely regarded as a key factor in HTLV-1 pathogenic mechanism (3,4). Tax, mediates the transition from latency to virion production by interacting with specific host proteins associated with cellular transcription pathways such as nuclear factor κB (NF-κB) (5-7), cyclic adenosinc monophosphate response element-binding-activating transcription factor (CREB/ATF) (8-10), serum response factor (SRF) (11-13), stimulatory protein 1 (SP1) (14), and activating protein 1 (AP-1) (15,16). Through interactions with cellular transcription factors, Tax potently activates transcription from the viral promoter and enhancer elements of many cellular genes involved in host cell proliferation (17-19). In comparison,

transcriptional repression by Tax on β-polymerase, Lck, c-myb and p53 promoters (20), reported recently from several studies, are less well understood.

Many studies have suggested that regulation through the CREB/ATF pathway by Tax plays an important cellular role (21). A model for Tax-mediated transcription through the CREB/ATF pathway is that a CREB-dimer binds to the Tax-responsive elements (14) which has a high similarity with cyclic adenosinc monophosphate response element (CRE) and interacts with a Tax homodimer. This CREB/Tax/TRE ternary complex can then influence TATA-binding protein (TBP) to regulate the initiation by RNA-polymerase II (RNAPII) (22).

ZNF268 gene is one of the typical KRAB-containing zinc finger genes, cloned and characterized from an early human embryonic cDNA library (23). KRAB-containing zinc finger genes represent a subfamily within a large family of zinc finger genes, and they typically act as transcriptional repressors (24). Several different alternatively spliced transcripts have been isolated for the ZNF268 gene and developmental expression studies have suggested that ZNF268 play a role in the differentiation of blood cells and the development of human fetal liver (25,26). Analysis of ZNF268 gene promoter shows that ZNF268 gene utilizes an intragenic promoter element to control its transcription, similar to some other genes involved in the diseases development and progression such as WT-1. In addition, in HeLa cells, CREB-2 has been shown to play an important role during the regulation of ZNF268 expression (27).

By using a recombinant expression cloning (SEREX) approach to identify tumor-associated antigens in chronic lymphocytic leukemia (CLL), Krackhardt et al. identified 14 antigens, KW-1 - KW-14. Among them, KW-4 was found to be one of the several known alternatively spliced transcripts of ZNF268 gene (28). These results suggest that ZNF268 gene play a role in the differentiation of blood cells and the pathogenesis of leukemia. Thus, considering the ability of Tax to transcriptionally regulate cellular gene expression as a likely mechanism for Tax-mediated transformation and leukemogenesis (22), the aim of this study was to investigate if Tax plays a role in the regulation of ZNF268 expression and to determine the underlying molecular mechanisms. Our results showed that HTLV-1 Tax was able to repress ZNF268 gene expression and that CREB-1 was involved in this repression of ZNF268 by Tax.

EXPERIMENTAL PROCEDURES

Plasmid construction and cell culture - The sequence from -37 to +938 containing the intragenic promoter element and a series of truncation mutants of the human ZNF268 promoter were inserted into the promoterless luciferase expression vector pGL3 (Promega) (27). pGL3(-37/+938)-p53-mut (+596 to +621), pGL3(-37/+938)-Ets-mut (+606 to +631), pGL3(-37/+938)-CREB-mut (+724 to +749), pGL3(-37/+938)-AP1-mut (+722 to +746), and pGL3(-37/+938)-C/EBP-mut (+728 to +752) were constructed by using the overlapping extension PCR method with pGL3(-37/+938) plasmid as described previously (27). pcDNA-Tax expresses the wide-type Tax, M22 expresses a Tax mutant that can activate CREB/ATF but not NF-κB and M47 expresses a Tax mutant that can activate NF-κB but not CREB/ATF (29). The Tax ORF was amplified by PCR from pcDNA-Tax using primers of Tax1 and Tax2. The PCR products were cloned into EcoRI and XhoI sites of pCMV-Tag2B to generate plasmid pCMV-Tag2B-Tax. pEGFP-C1 expresses GFP protein. pcDNA-CREB-1 and pcDNA-CREB-2 express CREB-1 and CREB-2 respectively.

pcDNA-CREB-1-dominant-negative (DN) expresses CREB-1 dominant-negative mutant (S133A) as described (30,31). The corresponding primers used for cloning are listed in Table 1.

HEK293 and HeLa cells (CCTCC, Wuhan, China) were grown in DMEM supplemented with 10% fetal calf serum (Invitrogen), penicillin (100U/ml) and streptomycin (100μg/ml) at 37°C in a 5% CO_2 incubator. Jurkat and Hut-102 cells (CCTCC, Wuhan, China) was maintained in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), penicillin (100U/ml) and streptomycin (100μg/ml) at 37°C in a 5% CO_2 incubator.

Transfection and luciferase assays - HeLa cells were chosen for stably expressing Tax protein due to the easiness with which to establish stably transfected cell lines. The cells were transfected with the Tax expression vector pCMV-Tag2B-Tax or empty vector pCMV-Tag2B using Sofast™ Transfection Reagent (Sunma, China) according to the manufacturer's instruction. Stable transfectants were obtained after 2-3 weeks of selection with 600μg/ml geneticin (G-418) and screened for Flag-tagged Tax protein expression by Western blotting as described below. HEK293 and HeLa cells were seeded in 24-well plates at 75% confluence and co-transfected with luciferase reporter vectors and indicated recombinant plasmids by mixing 0.2μg firefly luciferase reporter vectors and the internal control *Renilla* luciferase reporter construct, pRL-TK (Promega) (firefly luciferase reporter construct and pRL-TK in a ratio of 20:1), which contains the *Renilla* luciferase gene driven by the herpes simplex virus thymidine kinase (TK) promoter and 0.4μg plasmids with 1.5μl Sofast™ Transfection Reagent (Sunma, China) according to the manufacturer's instruction. Jurkat cells were seeded in 24-well plates and transfected at about 1 X 10^5 cells/well using DMRIE-C Transfection Reagent (Invitrogen) following the manufacturer's instruction. PHA (final concentration 1μg/ml, Sigma) and PMA (final concentration 50ng/ml, Sigma) per well were added 4 h after transfection. After incubation for 48h, the cells were harvested for luciferase assays.

Semi-quantitative RT-PCR - Total RNA was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Reverse transcription were performed with total RNA as the template. Specific mRNA was amplified by RT-PCR using primers PE41 and PECS3 for ZNF268 gene, and EgrU and EgrD for Egr-1 gene (Table 1). The PCR reactions were individually optimized so that each reaction fell within the linear range of product amplification. The PCR analysis of β-actin was used as the internal control.

Quantitative real-time PCR (qRT-PCR) - qRT-PCR was performed using Rotor-Gene 2000 real time PCR system (Australia) with 20X SYBR Green I PCR mix reagent in a 25μl volume in triplicate according to the manufacturer's instruction. As a control, the mRNA level of β-actin was determined for each RNA sample and was used to correct for experimental variations. ZNF268 mRNA was amplified by RT-PCR using primers PE41 and PECS3.

Western blot analysis - Lysates of cells were prepared in the lysis buffer containing 100mM NaCl, 10mM Tris-HCl (PH 8.0), 1mM EDTA (pH 8.0), 1% Triton X-100 and 1mM phenyl-methylsulfonyl fluoride, followed by centrifugation at 12,000 X *g* for 10 min. Supernatants were collected, mixed with an equal volume of SDS-PAGE 2X sample buffer, aliquoted, and stored at -80 °C until used.

Aliquots (50μg) were analyzed on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk for 1 hr at

37°C. Subsequently it was incubated with anti-Tax monoclonal antibody (Tab172) for 2 hr at 37°C. Membranes were then incubated for 1 hr with horseradish peroxidase conjugated goat anti-rabbit antibodies (Zhongshan Biotechnology, China) at 37°C. Protein bands were detected using SuperSignal Chemiluminescent (Pierce, IL).

Electrophoresis mobility shift assay (EMSA) - HEK293 cells incubated in serum-free media for 24h were washed with cold PBS twice and scraped into 1ml of cold PBS. Cells were harvested by centrifugation for 15s and incubated in 2 packed cell volumes of buffer A (10mM, HEPES, pH 8.0, 0.5% Nonidet P-40, 1.5 mM $MgCl_2$, 10 mM KCl, 200 mM sucrose, and 0.5 mM DTT) for 5 min at 4°C with flicking. The crude nuclei were collected by centrifugation for 30s, pellets were rinsed with buffer A, resuspended in 1 packed cell volume of buffer B (20mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, and 1.0 mM DTT), and incubated on a rocking platform for 30min at 4°C. Nuclei were clarified by centrifugation for 5min, and the supernatants were diluted 1:1 with buffer C (20mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol and 1.0 mM DTT). Cocktail protease inhibitor tablets were added to all buffers. Nuclear extracts were frozen in liquid N_2 and stored at -70°C until use. Probes were generated by annealing single-strand oligonucleotides (sequences are listed in Table) containing the cognate promoter regions of the ZNF268 gene and labeled at the ends with [γ-^{32}P] ATP using T4 polynucleotide kinase (TaKaRa).

EMSA were performed with 4μg of nuclear extract in binding buffer (20 mM Hepes, pH 7.9, 0.1mM EDTA, pH 8.0, 75mM KCl, 2.5mM $MgCl_2$, and 1mM DTT) containing 1 μg of poly (dI-dC). To assure the specific binding of transcription factors to the probe, unlabeled wild –type and mutated double-stranded oligonucleotide competitors were preincubated at a 100-fold molar excess for 10 min prior to probe addition. For super-shift experiments, 2μg of anti-Tax monoclonal antibody (Tab172) or anti-CREB1 monoclonal antibody (EPITOMICS) were incubated with nuclear extracts on ice for 30 min before adding to the binding buffer. Samples were then electrophoresed on 5% nondenaturing polyacrylamide, 0.5X Tris Glycin/EDTA gels, and the gels were dried and subjected to autoradiography.

Chromatin Immunoprecipitation (ChIP) - The assay was done as preciously described (27). Monolayer of HEK293 cells (80% confluent) were incubated for 24 h after transfection, and then were serum-starved for 24h. Formaldehyde was added to the culture medium to a final concentration of 1%. The cells were then washed twice in PBS, scraped, and lysed in lyses buffer (1% SDS, 10 mM Tris-HCl, pH 8.0, 1mM PMSF, 50 mg/ml of both aprotinin and leupeptin) for 10 min on ice. The lysates were sonicated on ice and the debris was removed by centrifugation at 12,000 rpm for 15 min at 4°C. One-fourth of the supernatant was used as DNA input control. The remaining supernatant was diluted 10-fold with dilution buffer (0.01% SDS, 1% Triton X-100, 1mM EDTA, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) and incubated with anti-Tax monoclonal antibody or anti-p-CREB-1 (Santa Cruz) overnight at 4°C. Immunoprecipitated complexes were collected using protein A/G agarose beads. The pellets were washed with dialysis buffer (2 mM EDTA, 50 mM Tris-HCl, pH 8.0). Samples were incubated at 67°C for 5 h to reverse formaldehyde cross link. DNA was precipitated with ethanol and extracted three times with phenol/chloroform. Finally, pellets were resuspended in TE buffer and subjected to PCR amplification using primers PECS11/PECA (+594 to +925) (Table 1) for the promoter region containing the CREB/ATF binding site and PU/PDT1A for an upstream region as a negative control (Table 1). The PCR products

were resolved by agarose gel electrophoresis.

RESULTS

Analysis of ZNF268 expression in HTLV-1-infected cell lines and stably transfected Tax-expressing cell line. To determine whether HTLV-1 infection affects the expression of ZNF268, we compared ZNF268 mRNA levels in HTLV-1-infected T cell line Hut-102 with uninfected T cell line Jurkat and HEK293 cell line. The result showed that Hut-102 expressed a much lower level of ZNF268 mRNA compared to Jurkat and HEK293 cell lines. This finding suggested that ZNF268 expression was repressed in HTLV-1-infected cells (Fig. 1A).

To investigate whether Tax from HTLV-1 was responsible for the repression of the expression of ZNF268, we generated stably transfected cells overexpressing the Tax protein in HeLa cells since it is easy to generate stable HeLa cells and ZNF268 is expressed in HeLa cells (32). Transfections were done with pCMV-Tag2B-Tax for stably expressing Tax protein and the empty vector pCMV-Tag2B as a control. The transfected cells were cultured in the medium containing 600μg/ml geneticin (G-418), and the G418-resistant clones were obtained after 2-3 weeks. Tax expression was confirmed by Western blotting of the Flag-tagged Tax protein expression (Fig. 1C). Analysis of ZNF268 expression in the transfected cells showed that the sc-Tax cell line has a reduced level of ZNF268 mRNA compared to sc-vector cell line (Fig. 1B), suggesting that Tax was responsible for the repression of ZNF268 in HTLV-1 infected cells.

Tax represses ZNF268 promoter. To investigate the regulation of ZNF268 by Tax, we transfected the Tax-expression plasmid pcDNA-Tax with a reporter plasmid carrying the luciferase gene under the control of ZNF268 promoter into HEK293 cells, Jurkat cells and HeLa cells, respectively. Cells were cultured for 24 hrs, then serum-starved for an additional 24 hrs and harvested. Cell lysates were analyzed for luciferase activity. Luciferase assay demonstrated that Tax dramatically repressed the ZNF268 promoter in all three different cell lines when compared to transfection with the empty vector pcDNA3.1 (Figs. 2A, 2B and 2C). Because of the higher levels of endogenous ZNF268 gene expression in HEK293 cells (Fig. 1) and the easiness with which to tranfect HEK293 cells, most of our transfection studies below were done in HEK293 cells.

To determine whether this repression of ZNF268 promoter by Tax was dependent on the amount of Tax, different concentrations of pcDNA-Tax plasmid along with plasmid carrying the reporter gene were co-transfected into HEK293 cells. Luciferase activity assays showed that ZNF268-promoter activity decreased as the concentration of plasmid DNA increased (Fig. 2D), indicating the repression of ZNF268 promoter by Tax was dose-dependent. To confirm the expression of Tax in transfected cells, transfected cells were harvested as described above. Western blot analysis was carried out using monoclonal antibody against Tax (Tab172). The amount of Tax expressed increased with increasing amounts of the plasmid DNA (Fig. 2D).

Tax represses endogenous ZNF268 mRNA level. To determine whether transient expression of Tax also affected the endogenous ZNF268 gene in HEK293 cells as observed above with the stably transfected HeLa cells, plasmid (pcDNA-Tax) expressing Tax or control plasmid (pcDNA3.1) was transfected into HEK293 cells. Total RNA of the transfected cells was isolated and used for semi-quantitative RT-PCR and quantitative real-time PCR (Figs. 3A and 3B) as described above. The results showed that Tax repressed the amount of endogenous ZNF268 mRNA in the HEK293 cells (Figs. 3A and 3B). To

determine the specificity of the repression of ZNF268 by Tax, we carried out similar analysis in Jurkat cells. It has been shown that in Jurkat cells, Tax activates Egr-1 (33). Consistently, we found that transient transfection of Tax expression plasmid led to increased expression of Egr-1 (Fig. 3C). In these same cells, Tax also repressed ZNF268, just like in HEK293 cells (Fig 3C). These results demonstrate gene specific repression of ZNF268 by Tax.

Again, to confirm the expression of Tax in the transfected cells, transfected cells were treated and harvested as described above. Western blot analysis was carried out using monoclonal antibody against Tax (Tab172). Tax was detected in cells transfected with the plasmid pcDNA-Tax, but not present in cells transfected with control plasmid pcDNA-3.1 (Fig. 3B, insert).

CRE element is required for the repression of ZNF268 expression by Tax. To define the *cis*-regulatory element that was responsive to Tax, a series of mutants with truncation or site-specific mutations in the ZNF268 promoter were generated (Figs. 4 and 5). HEK293 cells were co-transfected with a plasmid carrying Tax gene (pcDNA-Tax) and plasmids containing the luciferase reporter gene driven by mutated ZNF268 promoters. Results of luciferase assays indicated that +540 to +760 of ZNF268 promoter was necessary and sufficient for the repression by Tax (Fig. 4). This repression by Tax was specific as transfection with a GFP expression plasmid did not affect the promoter activity (Fig. 4, bottom row). Furthermore, site specific mutational analysis revealed that mutations in the CRE element significantly diminished the repression of ZNF268 promoter activity by Tax (Fig. 5).

To further elucidate the mechanism of this repression we employed two Tax mutants which have the missense mutations in Tax that functionally segregated two pathways: Tax M22, which is capable of activating CREB/ATF but not the NF-κB transcription factors, and Tax M47, which can activate NF-κB but not CREB/ATF (29). Transient transfection studies demonstrated that Tax M22 strongly repressed ZNF268 promoter, whereas Tax M47 was ineffective in this respect (Fig. 6). These results, together with the findings from the promoter mutants above, indicate that Tax exerts its effect on the ZNF268 promoter through the CREB/ATF pathway.

CREB-1 plays a role in the repression of ZNF268 expression by Tax. To identify whether CREB-1 or CREB-2 involved in the repression of ZNF268 gene by Tax, we co-transfected Tax expression plasmids for Tax, CREB-1, and CREB-2 into HEK293 cells with different concentrations and combinations together with the reporter DNA. The results indicated that the overexpression of CREB-1 stimulated the repression of ZNF268 by low levels of Tax, whereas the overexpression of CREB-2 eliminated the repression of ZNF268 by Tax (Fig. 7A).

To further investigate whether CREB-1 is involved in the repression of ZNF268 gene by Tax, we constructed an expression plasmid for a dominant-negative mutant of CREB-1 (S133A), which can bind to CRE element while has no transcriptional activity (34). Co-transfection of cells with this dominant-negative CREB-1 and Tax inhibited the repression of the ZNF268 promoter by Tax (Fig. 7B) (The lower level of Tax expression plasmid used here compared to that in Fig. 2 led to a lower level of repression). In the absence of Tax, dominant-negative CREB-1 had little effect (a small, non significant increase in Fig. 7B) on the ZNF268 promoter, consistent with the lack of effect of CREB-1 on this promoter in HeLa cells (27) (see Discussion for a possible explanation). This result supports that CREB-1 plays an important role in the repression of ZNF268 gene by Tax.

Corecruitment of Tax and CREB1 to the CRE site of the ZNF268 promoter represses

ZNF268 gene expression. The CRE sequence +732 5' ATGACGCAAT 3' +741 of ZNF268 promoter has a high level of similarity with Tax response element (TxRE) (35). Existing evidence suggests that Tax need not to directly bind to DNA to accomplish its function but rather that it can act through binding to CREB-1 bound to the TxRE element (22). To define the association between the complex of Tax-CREB1 and ZNF268 promoter, we conducted electrophoresis mobility shift assay (EMSA). HEK293 cells were transfected with a control plasmid (Fig. 8A, lanes 5 and 6) or a plasmid containing the Tax gene (Fig. 8A, lanes 1, 2 and 3; Fig. 8B, lanes 2, 3, 4 and 5). Nuclear extracts were prepared from the transfected cells and EMSA was performed with 4μg of nuclear extract and labeled CRE probe. A strong CRE complex(es) was observed (Fig. 8A, lane 1; Fig. 8B, lane 2). To assure specific binding of transcription factors to the probe, unlabeled wide-type double-strand oligonucleotide competitors (Fig. 8A, lane 2; Fig. 8B, lane 3) and unlabeled mutated double-strand oligonucleotide competitors (Fig. 8B, lane 4) were added prior to the addition of labeled probe. To determine whether Tax protein or CREB1 protein was bound to the promoter, anti-Tax monoclonal antibody (Tab172) or anti-CREB1 monoclonal antibody was incubated with nuclear extracts before adding the binding buffer (Fig. 8A, lane 3; Fig. 8B, lane 5). The CRE complex(es) was super-shifted by both antibodies, indicating the presence of Tax and CREB1 in the CRE complex(es). When similar experiments were carried out with nuclear extracts from cells without Tax transfection, no super-shifted bands by the anti-Tax antibody were observed (Fig. 8A, lane 6). It is interesting to note that the anti-Tax and anti-CREB1 antibodies super-shifted nearly all of the CRE complexes, suggesting that in the presence of Tax, Tax-CREB1 was the predominant complex bound to the CRE, at least under our in vitro binding conditions. These results indicate that both Tax and CREB1 bind the CRE in vitro.

To further confirm Tax-ZNF268 promoter binding, chromatin immunoprecipitation assay (ChIP) was performed. Chromatin fragments were prepared from HEK293 cells transfected with plasmid expressing Tax and immunoprecipitated with specific anti-Tax monoclonal antibody (Tab172) or anti-p-CREB-1. The immunoprecipitated DNA was amplified by PCR with primers PECS11/PECA (+594 to +925) (Table 1) for the promoter region containing the CRE element. A fragment of the expected size of 332 bp was detected when anti-Tax or anti-p-CREB-1 antibody was used (Fig. 9A, lanes 2, 4 and 5), but no signal was detected when anti-GFP antibody was used for the ChIP assay (Fig. 9A, lane 8). In addition, no signal was detected for the anti-Tax antibody ChIP assay with the immunoprecipitated DNA from the cells transfected without any plasmid or with the empty vector pcDNA3.1 (Fig. 9A, lane 3 and 7). Furthermore, when no antibody was included in the ChIP assay as a negative control, no signal was detected (Fig. 9A, lane 6). Finally, no signal was detected when PCR amplification of the precipitated DNA was done for a negative control region with primers PU/PCT1A (-1790 to -1381) (Table 1) (Fig. 9B). These results indicated that both Tax and CREB-1 were bound to the CRE element in the ZNF268 promoter, suggesting the formation of the Tax-CREB1-CRE complex in vivo.

DISCUSSION

The HTLV-1 Tax is crucial for viral replication and for initiating malignant cell transformation leading to leukemogenesis (22) . There are data demonstrating that Tax uses the CREB/ATF factors to repress the expression of genes such as cyclin A, cyclin D3 and DNA

polymerase alpha. This CRE-dependent effect of Tax on such cellular genes may contribute to the initiation of an oncogenic process by impairing the cell and growth control (22). Many cellular genes contain in their promoters CRE elements and are regulated by signals that elevate the cellular cAMP level (22). However, the ability of Tax to regulate transcription via CRE site is context specific since at many other CREB-binding sites, where Tax-CREBs complex formation may occur, transcriptional regulation by Tax is not seen (35). Our previous studies suggested that ZNF268 played a role in the differentiation of blood cells during early human embryonic development and the pathogenesis of leukemia (25,26,32). Here, we first showed that ZNF268 mRNA was repressed in HTLV-1-infected cell line and cells stably expression Tax, suggesting that Tax is responsible for the repression of ZNF268 genes in HTLV-1-infected cells. We also found that Tax repressed the ZNF268 promoter in different cell lines. Finally, we showed that ZNF268 was transcriptionally regulated by the HTLV-1 encoded Tax through the CREB/ATF pathway and that both Tax and CREB-1 are bound to the CRE in the ZNF268 promoter in vitro and in vivo.

Our luciferase assays showed that Tax repressed ZNF268 promoter in a dose-dependent manner. Semi-quantitative RT-PCR and quantitative real-time PCR confirmed this repression of ZNF268 at the mRNA level by Tax. There are at least three potential protein products encoded by ZNF268 gene: ZNF268a, ZNF268b1 and ZNF268b2, due to alternative splicing (32). Since Tax represses the ZNF268 promoter, all three ZNF268 products are likely reduced in the presence of Tax, although further studies are needed to confirm this. Studies with mutants of the ZNF268 promoter and Tax revealed that Tax specifically recognized the CRE regulatory element in the ZNF268 promoter. Based on our results and previously findings, it is reasonable to suggest that Tax represses ZNF268 gene expression through CREB/ATF pathway. Indeed, EMSA and ChIP assays confirmed the binding of both Tax and CREB1 to the ZNF268 CRE site in vitro and in vivo, respectively. These results suggest the formation of CREB-1/Tax complex at the CRE site of the promoter, which would be consistent with the luciferase assay showing that dominant negative CREB-1 inhibited the repression by Tax.

Historically, Tax was first characterized as a potent activator of gene expression. Among the genes include transcription factors (such as c-fos, c-jun, c-myc, egr-1, egr-2), cytokines (such as IL-1α, IL-2, IL-4, IL-6, IL-8) and cell cycle regulators (such as Cyclin D1, Cyclin D2) (33). Recently, however, several prototypic transcription activators such as p53, E2F, E1a have been shown to also function as potent transcriptional repressors (20). Thus, it may not be surprising to find that HTLV-1 Tax can also function as repressors as shown recently by several studies describing its repressive activity on genes such as cyclin A, Lck, Bax and c-Myb (20). Here, we have extended this repressive effect of Tax to a new target gene, ZNF268, and more importantly, revealed that Tax can repress through the CREB/ATF pathway.

Our previous in vitro and in vivo binding studies showed that in HeLa cells, CREB-2 but not CREB-1 binds to the CRE element within the minimal promoter and over-expression of CREB-2 but not CREB-1 dramatically enhances ZNF268 promoter activity (27). Interestingly, here we were able to detect CREB-1 binding to the CRE in the absence of Tax. It is possible that the binding of CREB-1 to the CRE is stronger in HEK293 cells than in HeLa cells. Alternatively, weak binding of CREB-1 in HeLa cells was not detected under the previous experimental conditions. While the mechanism underlying the differences between CREB-1 and CREB-2 remains to be determined, the promoter context

within the ZNF268 gene may also affect either the function or the binding of CREB-1 and -2 to the CRE in vivo. In addition, it remains possible that the levels of CREB-1 and -2 in HeLa and HEK293 cells with or without transfection may be different to account for the observed results. Finally, there may be a delicate balance of the CRE element binding activity between CREB-1 and CREB-2. In normal cells in the absence of Tax, CREB-2 may be the main CREB bound to the CRE at ZNF268 promoter in the context of this gene and activates it by recruiting co-activators, which would be consistent with the lack of significant effects of overexpression of CREB-1 (27) or CREB-1 DN on ZNF268 promoter in the absence of Tax (Fig. 7B). In the presence of Tax, CREB1/Tax complex may now be able to compete effectively against CREB2 for binding to the promoter. For unknown reason, e.g, due to the particular conformation of the complex at the promoter, both CREB1 and Tax cannot activate the promoter, thus leading to repression. It is also possible that CREB1/Tax may recruit other co-repressors in a complex to actively repress the promoter. Thus, the repression of ZNF268 by Tax may be due to a combination of two effects: the loss of the activation by CREB-2 and the additional repressive activity of Tax. Clearly, further studies, included a detailed quantitative analysis of CREB-1 and CREB-2 binding to the CRE in the presence and absence of Tax, are needed to determine the exact mechanism.

As a factor very likely important for blood cell and leukemia development, abnormal expression of ZNF268 is potentially an important event in cell transformation induced by Tax. The unexpected observation that Tax represses ZNF268 provides a further illustration of the intimate relationship between viruses and host factors. It additionally highlights the delicate balance between positive and negative events in maintaining cellular homeostasis.

REFERENCES

1. Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K. I., Shirakawa, S., and Miyoshi, I. (1981) *Proc Natl Acad Sci U S A* **78**(10), 6476-6480
2. Poiesz, B. J., Ruscetti, F. W., Mier, J. W., Woods, A. M., and Gallo, R. C. (1980) *Proc Natl Acad Sci U S A* **77**(11), 6815-6819
3. Robek, M. D., and Ratner, L. (1999) *J Virol* **73**(6), 4856-4865
4. Grassmann, R., Berchtold, S., Radant, I., Alt, M., Fleckenstein, B., Sodroski, J. G., Haseltine, W. A., and Ramstedt, U. (1992) *J Virol* **66**(7), 4570-4575
5. Napolitano, M., Modi, W. S., Cevario, S. J., Gnarra, J. R., Seuanez, H. N., and Leonard, W. J. (1991) *J Biol Chem* **266**(26), 17531-17536
6. Tsukahara, T., Kannagi, M., Ohashi, T., Kato, H., Arai, M., Nunez, G., Iwanaga, Y., Yamamoto, N., Ohtani, K., Nakamura, M., and Fujii, M. (1999) *J Virol* **73**(10), 7981-7987
7. Brauweiler, A., Garrus, J. E., Reed, J. C., and Nyborg, J. K. (1997) *Virology* **231**(1), 135-140
8. Zhao, L. J., and Giam, C. Z. (1992) *Proc Natl Acad Sci U S A* **89**(15), 7070-7074
9. Zhao, L. J., and Giam, C. Z. (1991) *Proc Natl Acad Sci U S A* **88**(24), 11445-11449
10. Suzuki, T., Fujisawa, J. I., Toita, M., and Yoshida, M. (1993) *Proc Natl Acad Sci U S A* **90**(2), 610-614
11. Alexandre, C., and Verrier, B. (1991) *Oncogene* **6**(4), 543-551
12. Fujii, M., Niki, T., Mori, T., Matsuda, T., Matsui, M., Nomura, N., and Seiki, M. (1991) *Oncogene* **6**(6), 1023-1029
13. Fujii, M., Tsuchiya, H., Chuhjo, T., Akizawa, T., and Seiki, M. (1992) *Genes Dev* **6**(11), 2066-2076
14. Trejo, S. R., Fahl, W. E., and Ratner, L. (1996) *J Biol Chem* **271**(24), 14584-14590
15. Fu, W., Shah, S. R., Jiang, H., Hilt, D. C., Dave, H. P., and Joshi, J. B. (1997) *J Neurovirol* **3**(1), 16-27
16. Joshi, J. B., and Dave, H. P. (1992) *Proc Natl Acad Sci U S A* **89**(3), 1006-1010
17. Tanaka, A., Takahashi, C., Yamaoka, S., Nosaka, T., Maki, M., and Hatanaka, M. (1990) *Proc Natl Acad Sci U S A* **87**(3), 1071-1075
18. Ishiguro, N., Abe, M., Seto, K., Sakurai, H., Ikeda, H., Wakisaka, A., Togashi, T., Tateno, M., and Yoshiki, T. (1992) *J Exp Med* **176**(4), 981-989
19. Hoshino, H., Tanaka, H., Shimotohno, K., Miwa, M., Akatsuka, T., and Odaka, T. (1985) *Virology* **147**(1), 223-226
20. Kibler, K. V., and Jeang, K. T. (2001) *J Virol* **75**(5), 2161-2173
21. Matsuoka, M. (2003) *Oncogene* **22**(33), 5131-5140
22. Azran, I., Schavinsky-Khrapunsky, Y., and Aboud, M. (2004) *Retrovirology* **1**(1), 20
23. Gou, D. M., Sun, Y., Gao, L., Chow, L. M., Huang, J., Feng, Y. D., Jiang, D. H., and Li, W. X. (2001) *Biochim Biophys Acta* **1518**(3), 306-310
24. Gebelein, B., and Urrutia, R. (2001) *Mol Cell Biol* **21**(3), 928-939
25. Sun, Y., Gou, D. M., Liu, H., Peng, X., and Li, W. X. (2003) *IUBMB Life* **55**(3), 127-131
26. Sun, Y., Shao, H., Li, Z., Liu, J., Gao, L., Peng, X., Meng, Y., and Li, W. (2004) *Int J Mol Med* **14**(6), 971-975
27. Guo, M. X., Wang, D., Shao, H. J., Qiu, H. L., Xue, L., Zhao, Z. Z., Zhu, C. G., Shi, Y. B., and

Li, W. X. (2006) *J Biol Chem* **281**(34), 24623-24636
28. Krackhardt, A. M., Witzens, M., Harig, S., Hodi, F. S., Zauls, A. J., Chessia, M., Barrett, P., and Gribben, J. G. (2002) *Blood* **100**(6), 2123-2131
29. Smith, M. R., and Greene, W. C. (1990) *Genes Dev* **4**(11), 1875-1885
30. Nguyen, L. Q., Kopp, P., Martinson, F., Stanfield, K., Roth, S. I., and Jameson, J. L. (2000) *Mol Endocrinol* **14**(9), 1448-1461
31. Pathak, S. K., Bhattacharyya, A., Pathak, S., Basak, C., Mandal, D., Kundu, M., and Basu, J. (2004) *J Biol Chem* **279**(53), 55127-55136
32. Shao, H., Zhu, C., Zhao, Z., Guo, M., Qiu, H., Liu, H., Wang, D., Xue, L., Gao, L., Sun, C., and Li, W. (2006) *Int J Mol Med* **18**(3), 457-463
33. Wycuff, D. R., and Marriott, S. J. (2005) *Front Biosci* **10**, 620-642
34. Amorino, G. P., Mikkelsen, R. B., Valerie, K., and Schmidt-Ullrich, R. K. (2003) *J Biol Chem* **278**(32), 29394-29399
35. Cox, J. M., Sloan, L. S., and Schepartz, A. (1995) *Chem Biol* **2**(12), 819-826

FOOTNOTES

The Tax, M22, M47 expression plasmids were kind gifts from Warner C. Greene (Gladstone Institute of Virology and Immunology, UCSF). The anti-Tax monoclonal antibody (Tab172) was a kind gift from John N. Brady (National Cancer Institute, NIH). We thank Dr. Xue Zhang for experimental technology. This research was supported by the National High Technology Research and Development Program of China (863 Program) (2006AA02A306), the Programme of Introducing Talents of Discipline to Universities (B06018), the Key Project of the Ministry of Science and Technology of China (973 Program) (2005CB522903) and the National Natural Science Foundation of China (30500266). This research was also supported in part by the Intramural Research Program of the National Institute of Child Health and Human Development, NIH.

The abbreviations used are: HTLV-1, human T-cell leukemia virus type 1; ZNF, zinc finger; CREB, cyclic AMP-responsive element-binding protein; CRE, cyclic AMP-responsive element; TxRE, Tax-responsive element; RT, reverse transcription; EMSA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation.

FIGURE LEGENDS

Fig.1. ZNF268 mRNA was repressed in HTLV-1 infected cells and in cells stably expressing Tax. (A) Specific mRNA was amplified by RT-PCR using primers PE41 and PECS3 for ZNF268 gene. As an internal control, the mRNA level of β-actin was also analyzed. ZNF268 mRNA level in Hut-102 T cell line infected by HTLV-1 was compared to non-infected Jurkat T cell line and HEK293 cell line. (B) Stable cell lines were generated from transfection of HeLa cells with pCMV-Tag2B-Tax for expressing Tax protein (sc-Tax) and the empty vector pCMV-Tag2B (sc-vector) as a control. Total RNA was isolated from the transfected cells and was amplified by RT-PCR using primers PE41 and PECS3 for ZNF268 gene. Again, the mRNA level of β-actin was used as an internal control. (C) Western blot analysis showing the expression of Flag-tagged Tax in sc-Tax but not in sc-vector transfected cell line.

Fig.2. HTLV-1 Tax represses ZNF268 promoter. HEK293 (A), Jurkat (B), HeLa (C) cells were co-transfected with plasmid expressing Tax (0.4 μg in 24-well plates) and the reporter plasmid in which the luciferase gene is under the control of the ZNF268 promoter. The empty vector pcDNA-3.1 was used as a control. Relative luciferase activity was determined with standard procedures. Values correspond to an average of at least three independent experiments done in duplicate. (D) Dose-dependent repression by Tax. HEK293 cells were co-transfected with different amounts of the plasmid expressing Tax along with the reporter plasmid and relative luciferase activity was determined. Values correspond to an average of at least three independent experiments done in duplicate. At the bottom, the 40kD Tax was detected by Western blot from the same transfected cells.

Fig.3. HTLV-1 Tax represses endogenous ZNF268 gene in HEK293 and Jurkat cells. (A) Semi-quantitative RT-PCR analysis of the expression of ZNF268 mRNA. Cells were transfected with Tax-expression plasmid or the empty vector pcDNA-3.1 as a control. Specific mRNA was amplified by RT-PCR using primers PE41 and PECS3 for ZNF268. As an internal control, the mRNA level of β-actin was also analyzed. Note the reduced level of ZNF268 mRNA in the Tax-transfected cells. (B) Quantitative real-time PCR analysis of the expression of ZNF268 mRNA in cells transfected with Tax expression plasmid or control pcDNA-3.1. The mRNA level of β-actin was determined by real-time PCR for each RNA sample and was used to normalize the ZNF268 expression. ZNF268 mRNA was amplified by using primers PE41 and PECS3. Insert: the 40kD Tax was detected in the cells transfected with pcDNA-Tax but not pcDNA-3.1 (control). (C) Semi-quantitative RT-PCR analysis shows that endogenous ZNF268 is also repressed by Tax in Jurkat cells while a known Tax-inducible gene, Egr-1, is upregulated as expected. Cells were transfected with the empty vector pcDNA-3.1 or Tax expression plasmid. Specific mRNA was amplified by RT-PCR using primers PE41 and PECS3 for ZNF268 and EgrU and EgrD for Egr-1. As a control, the mRNA level of β-actin was also analyzed.

Fig.4. Deletion analysis of cis-regulatory elements of the ZNF268 promoter important for repression by Tax. Diagrams of deletion mutants of ZNF268 promoter are shown on the left and the transfection results are shown on the right. Tax expression plasmid or the empty vector pcDNA-3.1 (control) and plasmids containing the luciferase reporter gene driven by individual ZNF268 promoter mutants were

co-transfected into HEK293 cells. GFP expression plasmid was used as an unrelated protein control to confirm the specificity of the repression of ZNF268 by Tax. The pGL3-Basic plasmid was transfected as the promoter-less negative control. Promoter activities were determined by measuring the relative luciferase activity in transfected-cell lysates from three independent experiments. To determine the relative repression, the promoter activity from cells transfected with the empty vector pcDNA-3.1 was divided by that from Tax transfected cells. Note that repression was observed for all promoter constructs containing the sequences from +540 to +760.

Fig.5. Site-specific mutation analysis of cis-regulatory elements in the ZNF268 promoter important for repression by Tax. (A) Schematic diagrams of the promoter constructs with various transcription factor binding sites mutated as indicated. (B) Tax expression plasmid and plasmids containing the luciferase reporter gene driven by individual ZNF268 promoter mutants were co-transfected into HEK293 cells. The pGL3-Basic plasmid was transfected as the promoter-less negative control. Promoter activities were determined as before. Note that only mutations in the CRE element significantly reduced the repression by Tax.

Fig.6. Effects of Tax mutants on ZNF268 promoter. The pcDNA-3.1 (empty vector control) and plasmids expressing Tax (wild type, W.T. Tax), its mutants M22, which can affect transcriptional activation by CREB/ATF but not NF-κB, or M47, which can affect transcriptional activation by NF-κB but not CREB/ATF, were co-transfected with the luciferase reporter plasmid driven by the ZNF268 promoter into HEK293 cells. Promoter activities were determined by measuring the relative luciferase activity in transfected-cells. Luciferase activities correspond to an average of at least three independent experiments and the data are shown as mean values with standard errors. *, $p<0.05$; **, $p<0.02$, standard T-test.

Fig.7. A role of CREB-1 in the repression of ZNF268 promoter by Tax. (A) The indicated amounts of expression plasmids for Tax, CREB-1, and CREB-2 were transfected into HEK293 cells together with the plasmid containing luciferase reporter gene driven by ZNF268 promoter. Promoter activities were determined as before. Note that overexpresion of CREB-1 at low concentrations enhanced repression by low levels of Tax. (B) The plasmids expressing Tax (0.3 μg in 24-well plates) and CREB-1 DN (dominant negative) (0.2 μg in 24-well plates) were transfected together with the plasmid containing luciferase reporter gene driven by ZNF268 promoter into HEK293 cells respectively. Promoter activities were determined by as before. *, $p<0.05$; **, $p<0.02$, standard T-test. Note that the dominant negative CREB-1 inhibited the repression by Tax.

Fig.8. Both Tax and CREB-1 bind to the CRE element in the ZNF268 promoter in vitro. (A) Eletrophoretic mobility shift assay (EMSA) was performed with nuclear extracts (NE) from HEK293 cells transfected with pcDNA-Tax (lanes 1, 2 and 3) or with the empty vector pcDNA-3.1 (lanes 5 and 6) as a control. Labeled CRE probe was added to all reactions (lanes 1 to 6). Unlabeled wild-type double-stranded oligonucleotide competitors were added during pre-incubation prior to probe addition (lane 2). For super-shift experiments, anti-Tax (lanes 3 and 6) was incubated with nuclear extracts before adding to the reaction. Free probe without any nuclear extracts or antibody (lane 4) was used as a negative control. Samples were electrophoresed on 5% non-denaturing polyacrylamide gel and visualized by autoradiography. (B) EMSA was performed with nuclear extracts (NE) of HEK293 cells

transfected with pcDNA-Tax (lanes 2, 3, 4 and 5). Labeled CRE probe was added to all reactions (lanes 1 to 5). Unlabeled wild-type double-stranded oligonucleotide competitors and mutated double-strand oligonucleotide competitors were added during pre-incubation prior to probe addition (lanes 3 and 4). For super-shift experiments, anti-CREB1 antibody (lane 5) was incubated with nuclear extracts before adding to the reaction. Free probe without any nuclear extracts or antibody (lane 1) was used as negative control. Samples were electrophoresed on 5% non-denaturing polyacrylamide gel and visualized by autoradiography. Note that the anti-Tax and anti-CREB1 antibodies super-shifted nearly all of the CRE complexes. It is possible that in the presence of Tax, Tax-CREB1 is the predominant complex binding to the CRE while little CREB2 is bound to the CRE, at least under our in vitro binding conditions. CRE: complexes containing the CRE probe. Super shift: complexes super-shifted by the anti-Tax or anti-CREB1 antibody. (Note that the CRE complex(es) formed with nuclear extract with or without Tax transfection had similar mobility (Fig. 8A, lanes 1 and 5). It is unclear why. However, the mobility of complexes in native gels is affected by many factors and is not simply related to the mass of the complexes. It is possible that Tax-CREB-CRE complexes had similar mobility as CREB-CRE complexes under our gel conditions).

Fig.9. ChIP assay shows that both Tax and CREB-1 are bound to the CRE region of ZNF268 promoter in vivo. HEK293 cells transfected without any plasmid (lanes 3 and 5) or with empty vector pcDNA-3.1 (lane 7) or pcDNA-Tax (lanes 1, 2, 4, 6 and 8) were lysed and subjected to ChIP assay. PCR amplification of DNA precipitated with anti-Tax (lane 2, 3 and 7), anti-p-CREB-1 (lane 4 and 5) and anti-GFP (lane 8) by using primers for ZNF268 promoter, PECS11 and PECA, flanking the CRE element (+594 to +925). PCR amplification of DNA precipitated without any antibody (lane 6) was used as the negative control. Input lane (lane 1) shows product after PCR amplification of chromatin DNA prior to immunoprecipitation. PCR with primers PU and PDT1A for an upstream region (-1790 to -1381) of the ZNF268 promoter was done as a negative control.

Table 1. Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide sequence (5′-3′)	Location [a]
PDT8	CGAGGTACCCTCTGTGAATGTCACCTC	-37 to -20
PD2T1	GTTAAGCTTCTCCTCCAAACCCTGAAG	+938 to +921
PD2T2	GTTAAGCTTCTACGTATGTCGCACAGG	+760 to +743
PD2T3	GTTAAGCTTGACACCAATGGCTCAACG	+540 to +523
p53-wt-F	CTGGCCAGGAAG**GCCT**GAGCTTCCGG	+596 to +621
p53-mut-F	CTGGCCAGGAAG**TAAG**GAGCTTCCGG	+596 to +621
Ets-wt-F	AGGCCTGAGCT**TCC**GGGTCATCTTAG	+606 to +631
Ets-mut-F	AGGCCTGAGCT**GAA**GGGTCATCTTAG	+606 to +631
CRE-wt-F	GCCTCTCCATG**ACG**CAATTCCTGTGC	+724 to +749
CRE-mut-F	GCCTCTCCATG**CAT**CAATTCCTGTGC	+724 to +749
AP1-wt-F	TTGCCTCTCCA**TGA**CGCAATTCCTG	+722 to +746
AP1-mut-F	TTGCCTCTCCA**GTC**CGCAATTCCTG	+722 to +746
C/EBP-wt-F	CTCCATGACGC**AA**TTCCTGTGCGAC	+728 to +752
C/EBP-mut-F	CTCCATGACGC**CC**TTCCTGTGCGAC	+728 to +752
PECS3	GCAGATATGAGAATCCAGCT	+287 to +306
PE41	GCTACGTATGTCGCACAGGAATTG	+761 to +738
PECS11	ACCTGGCCAGGAAGGCCTGAG	+594 to +614
PECA	TGAAGGGGCAGCAGAATAGA	+925 to +906
PU	CGAGGTACCAGAAGACATACAAATGGCCAAC	-1790 to -1769
PDT1A	CTACTGTGACTGATGTAAGA	-1381 to -1400
Tax1	CTAATTGAATTCGGAATTCGATCCACCATGGC	
Tax2	GCCGCGGTCTCGAGTTTTCAGACTTCTGTTTCG	
CREBU	GGAGAAGCTTGTACCACCGGTAACTAAATGAC	
CREBD	GAGAGCGGCCGCTTATTAATCTGATTTGTGGCAGTAAA	
CREB-DN-F	CTTTCAAGGAGGCCTGCCTACAGGAAAATTT	
CREB-DN-R	AAATTTTCCTGTAGGCAGGCCTCCTTGAAAG	
EgrU	ACCGAATTCATGGCCGCGGCCAAGGCCGAGATGC	
EgrD	ATCGTCGACCTTAGCAAATTTCAATTGTCCTGGG	

[a] Shown are the oligonucleotide positions, where +1 is the transcription start site of the *ZNF268* gene.

Figure 1

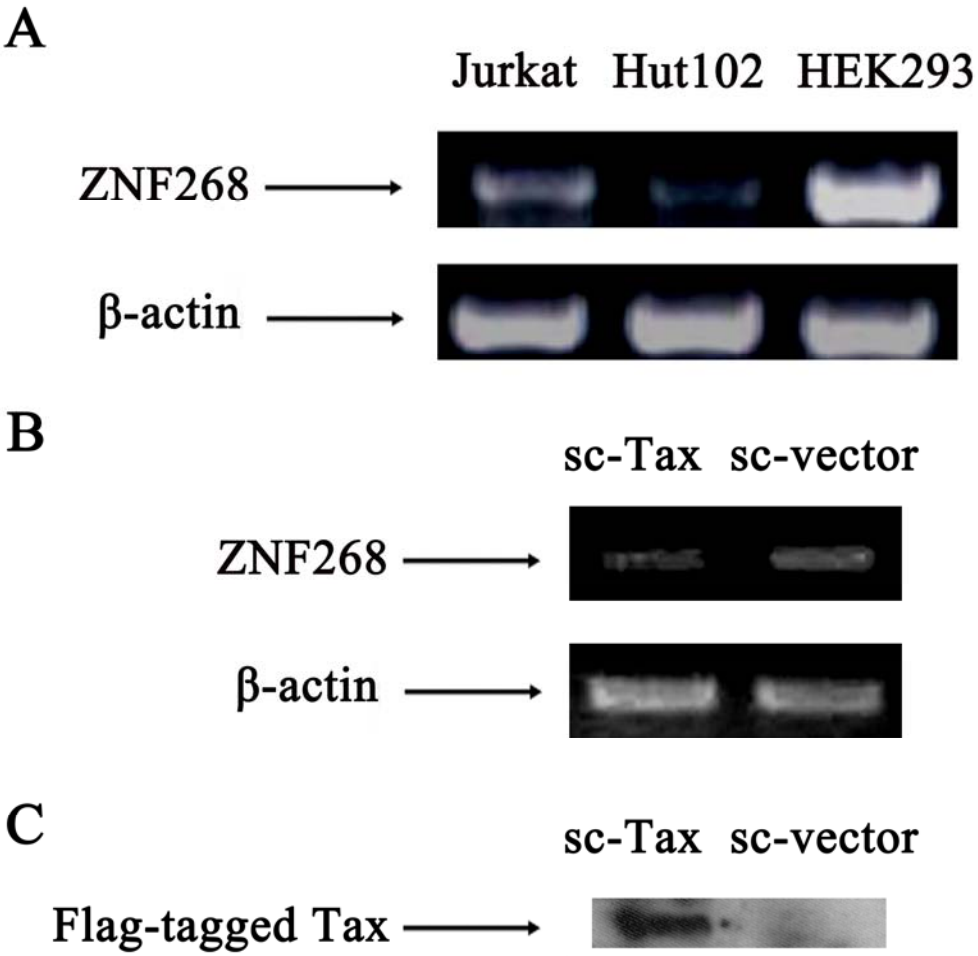

Figure 2

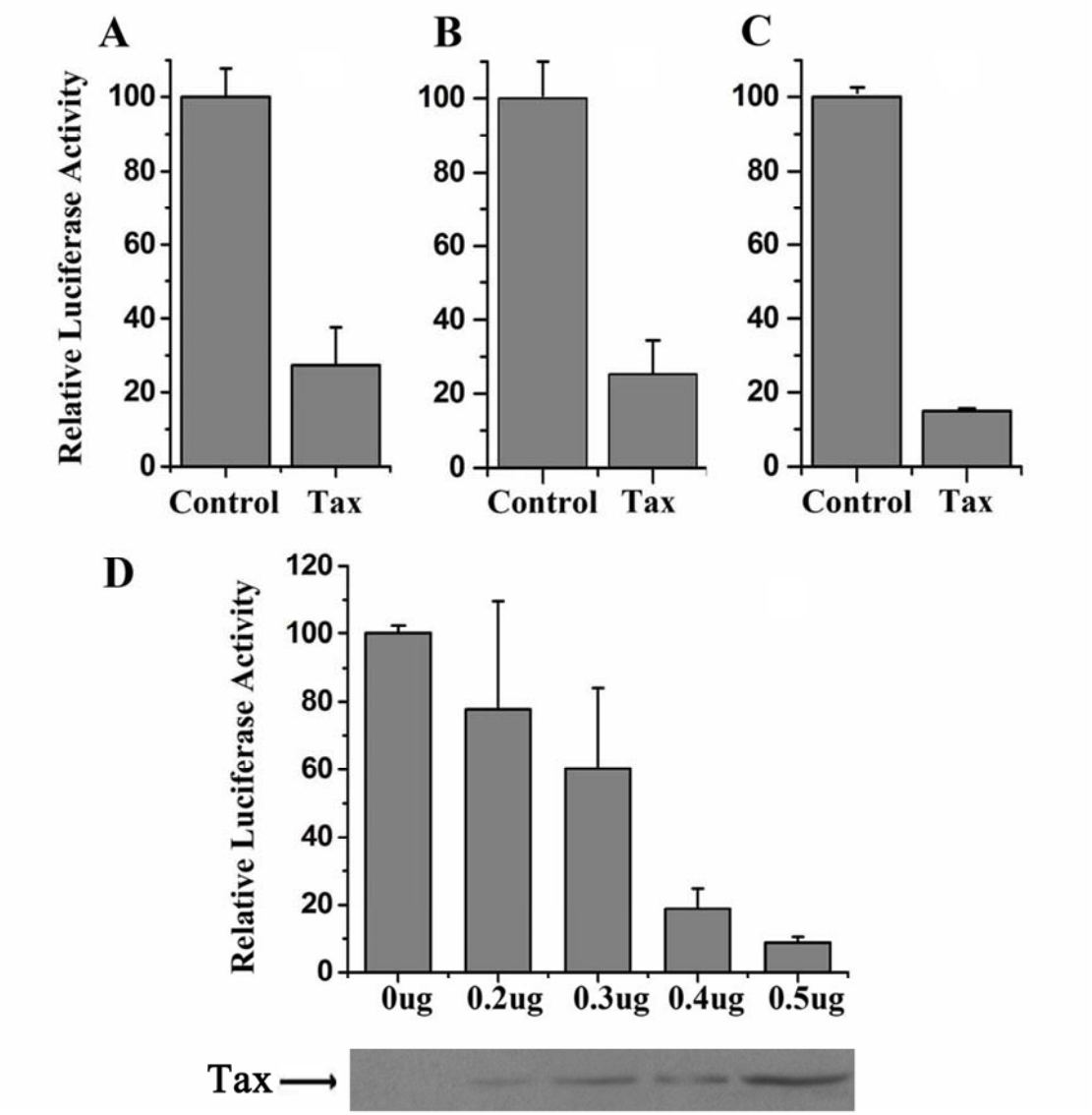

Figure 3

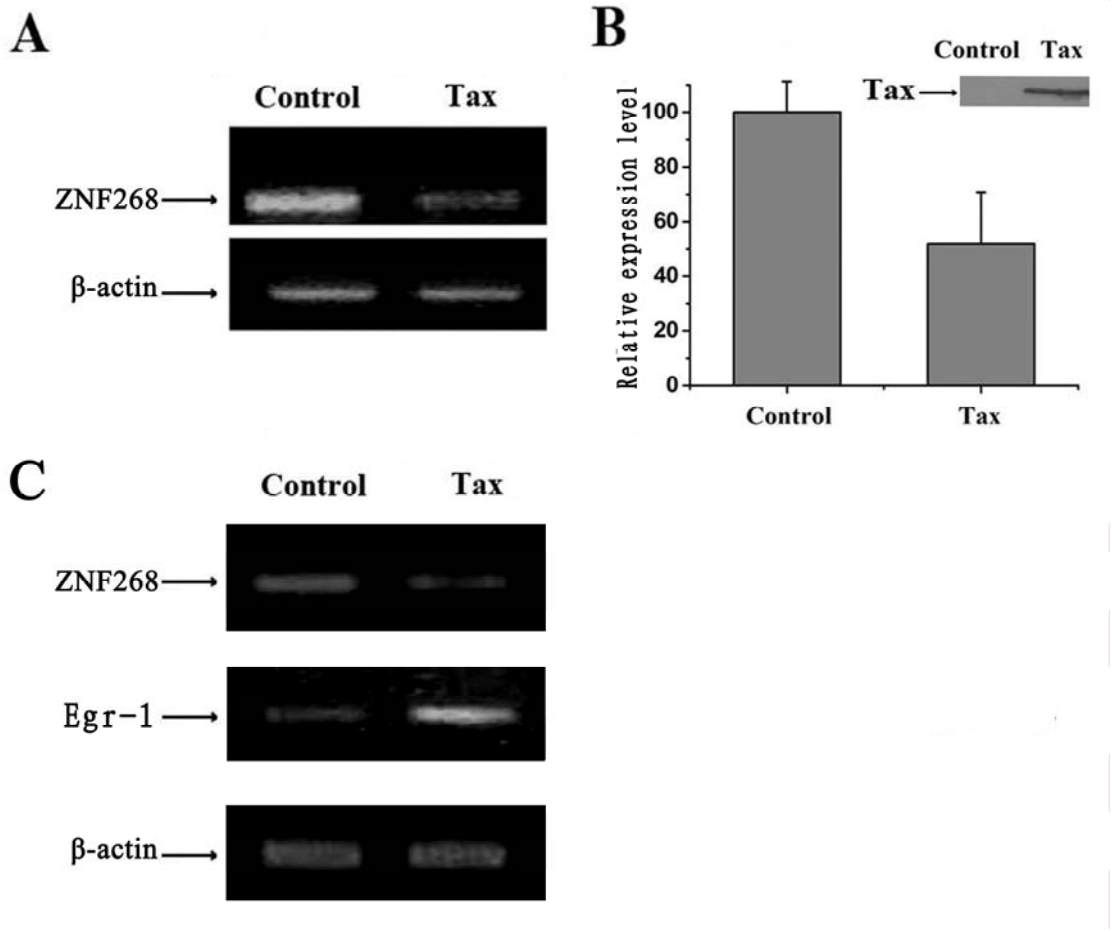

Figure 4

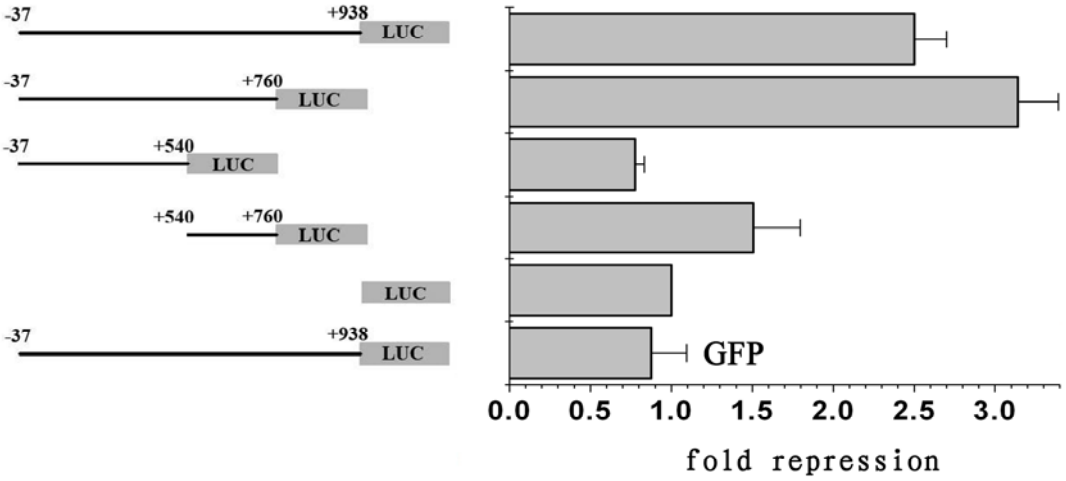

Figure 5

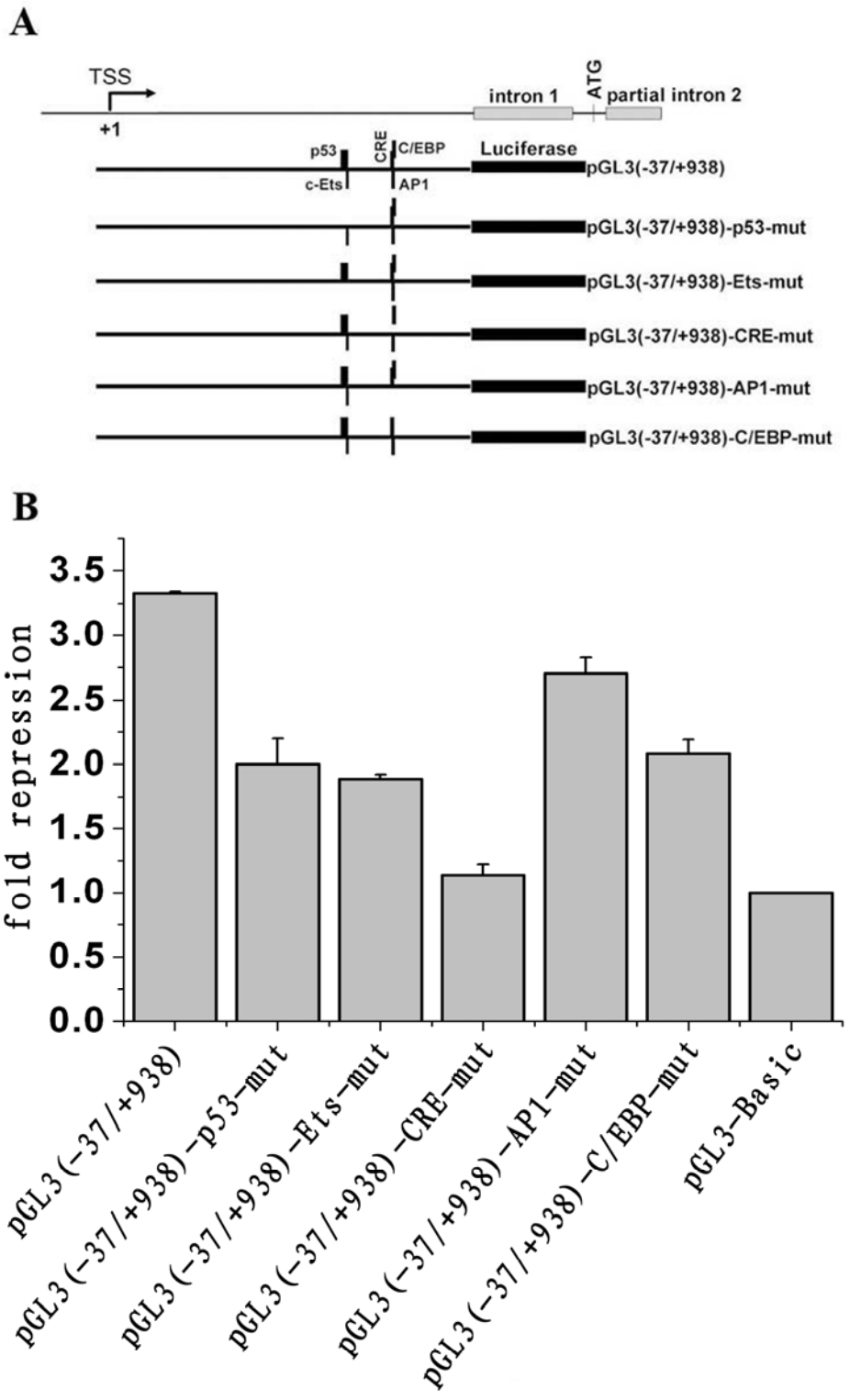

Figure 6

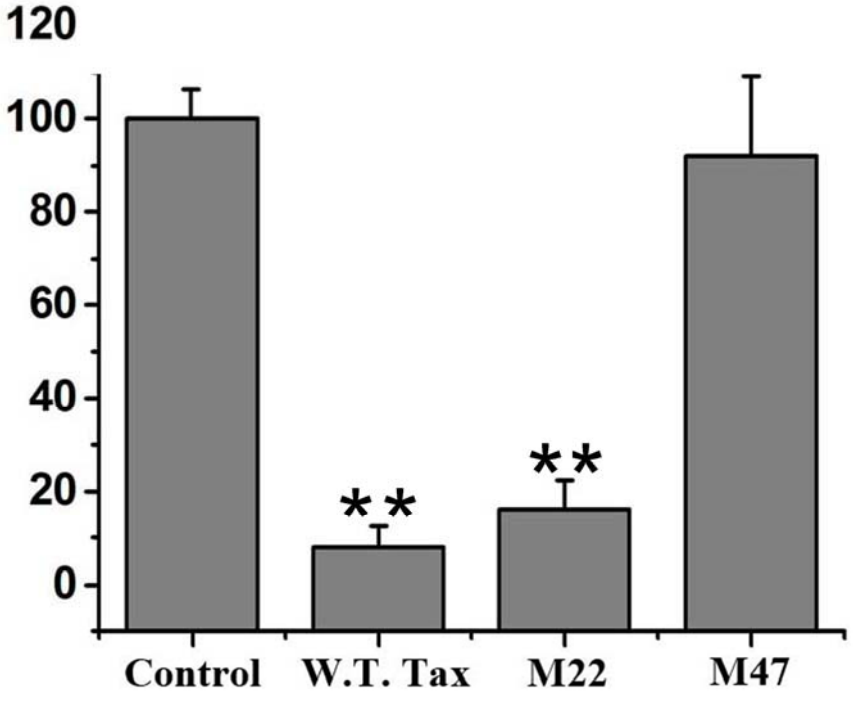

Figure 7

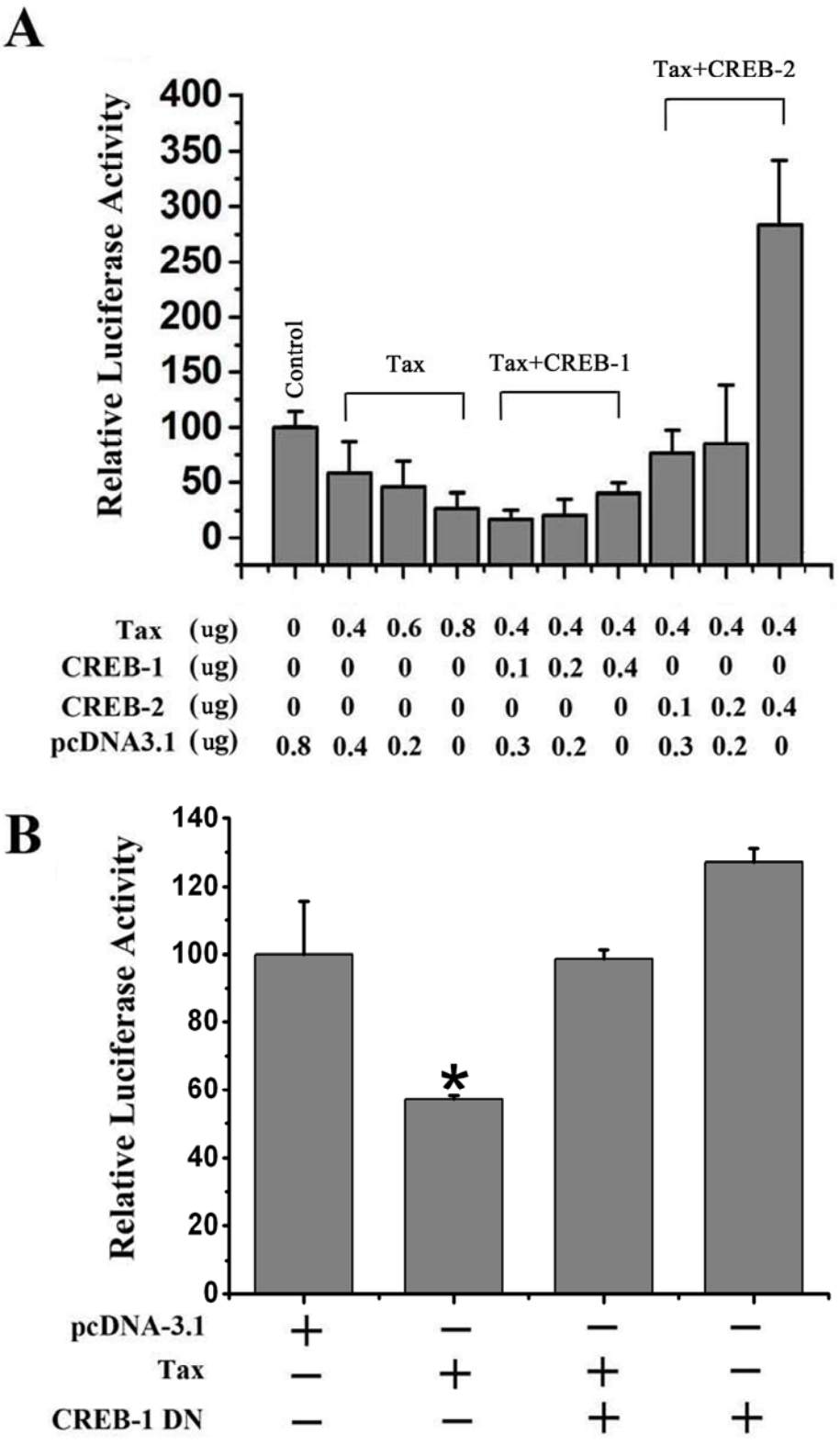

Figure 8

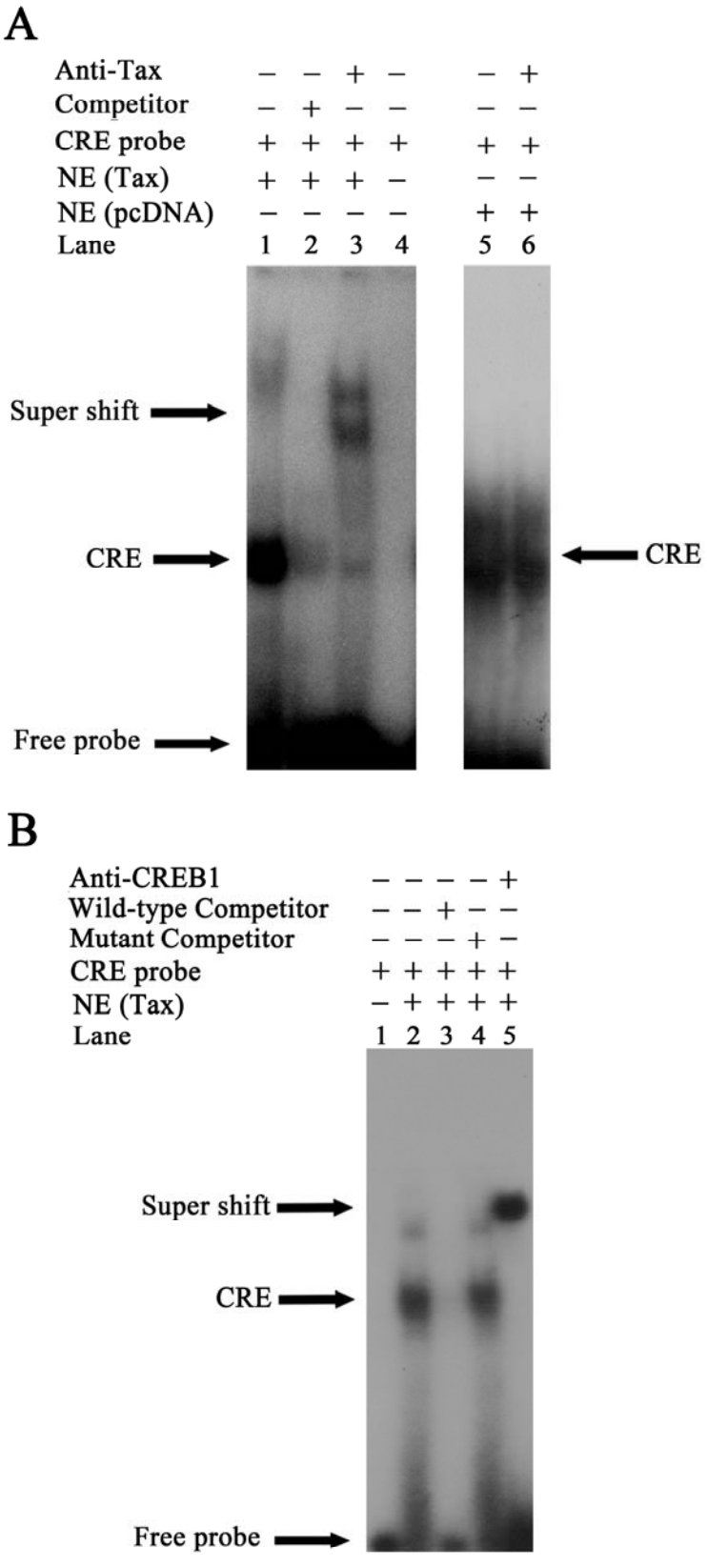

Figure 9

Anti-Tax	-	+	+	-	-	-	+	-
Anti-CREB-1	-	-	-	+	+	-	-	-
Anti-GFP	-	-	-	-	-	-	-	+
Vector	-	-	-	-	-	-	+	-
Agrose beads	-	+	+	+	+	+	+	+
Tax	+	+	-	+	-	+	-	+
Lane	1	2	3	4	5	6	7	8