Human T-cell Leukemia Virus Type 1 Oncoprotein Tax Represses ZNF268 Expression through the cAMP-responsive Element-binding Protein/Activating Transcription Factor Pathway

Di Wang¹, Ming-Xiong Guo¹, Hai-Ming Hu¹, Zhou-Zhou Zhao¹, Hong-Ling Qiu¹, Huan-Jie Shao¹, Chen-Gang Zhu¹, Lu Xue¹, Yun-Bo Shi³, and Wen-Xin Li¹²

From the ¹State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, China and ²Section on Molecular Morphogenesis, Laboratory of Gene Regulation and Development, NICHD, National Institutes of Health, Bethesda, Maryland 20892

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 the 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 cAMP-responsive element. By using Tax, cAMP-responsive element-binding protein (CREB)-1, CREB-2, and their mutants, we further showed that Tax repressed ZNF268 through the CREB/activating transcription factor pathway. Electrophoretic mobility shift assays and chromatin immunoprecipitation demonstrated the formation of the complex of Tax-CREB-1 directly at the cAMP-responsive element 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 1 (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 and of a neurological progressive inflammatory syndrome called tropical spastic paraparesis or HTLV-1-associated myelopathy (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 the 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), cAMP-responsive element-binding protein/activating transcription factor (CRE/ATF) (8–10), serum response factor (11–13), stimulatory protein 1 (14), and activating protein 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, is 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 have a high similarity with cAMP-responsive element (CRE) and interact with a Tax homodimer. This CREB-Tax-TRE ternary complex can then influence TATA-binding protein to regulate the initiation by RNA polymerase II (22).

The 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 plays a role in the differentia-

mobility shift assay; ChIP, chromatin immunoprecipitation; ATF, activating transcription factor; GFP, green fluorescent protein; DN, dominant negative; IL, interleukin.
tion of blood cells and the development of human fetal liver (25, 26). Analysis of the ZNF268 gene promoter shows that the 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, Krackhardt et al. (28) identified 14 antigens, KW-1 to -14. Among them, KW-4 was found to be one of the several known alternatively spliced transcripts of the ZNF268 gene. These results suggest that the ZNF268 gene plays 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 wild-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 Tax1 and Tax2. The PCR products were cloned into EcoRI and Xhol sites of pCMV-Tag2B to generate plasmid pCMV-Tag2B-Tax. pcEGFP-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 Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO2 incubator. Jurkat and Hut-102 cells (CCTCC) were maintained in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO2 incubator.

*Transfection and Luciferase Assays*—HeLa cells were chosen for stably expressing Tax protein due to the ease with which stably transfected cell lines were established. 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 instructions. Stable transfectants were obtained after 2–3 weeks of selection with 600 μg/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 the indicated recombinant plasmids by mixing 0.2 μg of 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 promoter and 0.4 μg of plasmids with 1.5 μl of Sofast™ transfection reagent (Sunma) according to the manufacturer’s instructions. Jurkat cells were seeded in 24-well plates and transfected a total of 1 × 10⁶ cells/well using DMRIE-C transfection reagent (Invitrogen), following the manufacturer’s instructions. Phytohemagglutinin (final concentration 1 μg/ml; Sigma) and phorbol 12-myristate 13-acetate (final concentration 50 ng/ml; Sigma) were added to each well 4 h after transfection. After incubation for 48 h, the cells were harvested for luciferase assays.

*Semiquantitative RT-PCR*—Total RNA was extracted by using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed with total RNA as the template. Specific mRNA was amplified by RT-PCR using primers PE41 and PECS3 for the ZNF268 gene.
and EgrU and EgrD for the egr-1 gene (Table 1). The PCRs 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 RT-PCR—Quantitative RT-PCR was performed using the Rotor-Gene 2000 real time PCR system (Rotor-Gene, Sydney, Australia) with 20× SYBR Green I PCR mix reagent in a 25-µl volume in triplicate according to the manufacturer’s instructions. 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 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 12,000 × g for 10 min. Supernatants were collected, mixed with an equal volume of SDS-PAGE 2× sample buffer, aliquoted, and stored at −80 °C until used.

Aliquots (50 µg) were analyzed on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk for 1 h at 37 °C. Subsequently, it was incubated with anti-Tax monoclonal antibody and EgrU and EgrD for the egr-1 gene (Table 1). The PCRs 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 RT-PCR—Quantitative RT-PCR was performed using the Rotor-Gene 2000 real time PCR system (Rotor-Gene, Sydney, Australia) with 20× SYBR Green I PCR mix reagent in a 25-µl volume in triplicate according to the manufacturer’s instructions. 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 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 12,000 × g for 10 min. Supernatants were collected, mixed with an equal volume of SDS-PAGE 2× sample buffer, aliquoted, and stored at −80 °C until used.

Aliquots (50 µg) were analyzed on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk for 1 h at 37 °C. Subsequently, it was incubated with anti-Tax monoclonal antibody and EgrU and EgrD for the egr-1 gene (Table 1). The PCRs 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 RT-PCR—Quantitative RT-PCR was performed using the Rotor-Gene 2000 real time PCR system (Rotor-Gene, Sydney, Australia) with 20× SYBR Green I PCR mix reagent in a 25-µl volume in triplicate according to the manufacturer’s instructions. 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 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 12,000 × g for 10 min. Supernatants were collected, mixed with an equal volume of SDS-PAGE 2× sample buffer, aliquoted, and stored at −80 °C until used.

Aliquots (50 µg) were analyzed on a 10% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk for 1 h at 37 °C. Subsequently, it was incubated with anti-Tax monoclonal antibody and EgrU and EgrD for the egr-1 gene (Table 1). The PCRs 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.
Tax Represses ZNF268 Expression through the CREB/ATF Pathway

FIGURE 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 promoterless 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.

TABLE 1

| Deletion Mutant | Activity (Relative to Control) |
|-----------------|--------------------------------|
| +398            | 0.5                              |
| +540            | 0.2                              |
| +760            | 0.05                            |

Fold repression (GFP/YFP) = 3.0

RESULTS

Analysis of ZNF268 Expression in HTLV-I-infected Cell Lines and Stably Transfected Tax-expressing Cell Line—To determine whether HTLV-I infection affects the expression of ZNF268, we compared ZNF268 mRNA levels in HTLV-I-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 with Jurkat and HEK293 cell lines. This finding suggested that ZNF268 expression was repressed in HTLV-I-infected cells (Fig. 1A).

To investigate whether Tax from HTLV-I 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
Tax Represses ZNF268 Expression through the CREB/ATF Pathway

To determine whether this repression of the 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. 2), indicating that the repression of ZNF268 promoter by Tax was concentration-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 (pcDNA-3.1) was transfected into HEK293 cells. Total RNA of the transfected cells was isolated and used for semiquantitative RT-PCR and quantitative real time PCR (Fig. 3, A and B) as described above. The results showed that Tax repressed the amount of endogenous ZNF268 mRNA in the HEK293 cells (Fig. 3, A and B). To determine the specificity of the repression of ZNF268 by Tax, we carried out a 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, inset). The CRE 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 the Tax gene (pcDNA-Tax) 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 promoterless negative control. Promoter activities were determined as before. Note that only mutations in the CRE significantly reduced the repression by Tax.

**FIGURE 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 promoterless negative control. Promoter activities were determined as before. Note that only mutations in the CRE significantly reduced the repression by Tax.
CREB-2 eliminated the repression of ZNF268, which suggests that CREB-1 plays an important role in the repression of this promoter in HeLa cells (27). This result indicates that the lack of effect of CREB-1 on this promoter in HeLa cells (27) is not due to a lower level of repression. In the absence of Tax, dominant negative CREB-1 and Tax inhibited the repression of the ZNF268 gene by Tax, whereas the overexpression of CREB-1 at low concentrations enhanced repression by low levels of Tax (29). Transient transfection studies demonstrated that Tax M22 strongly repressed the 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 is involved in the repression of the 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 the ZNF268 gene by Tax, we constructed an expression plasmid for a dominant negative mutant of CREB-1 (S133A), which can bind to the CRE, whereas it 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 with 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, nonsignificant 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 suggests that CREB-1 plays an important role in the repression of ZNF268 gene by Tax.

Corecruitment of Tax and CREB-1 to the CRE Site of the ZNF268 Promoter Represses ZNF268 Gene Expression—The CRE sequence +732 to +741 (5′-ATGACGCAAT-3′) of the ZNF268 promoter has a high level of similarity with the Tax-responsive element (35). Existing evidence suggests that Tax need not directly bind to DNA to accomplish its function but rather that it can act through binding to CREB-1 bound to the Tax-responsive element (22). To define the association between the complex of Tax-CREB-1 and ZNF268 promoter, we conducted an EMSA. HEK293 cells were transfected with a control plasmid (Fig. 8A, lanes 1–3) or a plasmid containing the tax gene (Fig. 8A, lanes 4–6) or a plasmid containing the tax gene (Fig. 8A, lanes 1–3, and B, lanes 2–5). Nuclear
Tax Represses ZNF268 Expression through the CREB/ATF Pathway

extracts were prepared from the transfected cells, and EMSA was performed with 4 μg of nuclear extract and labeled CRE probe. A strong CRE complex(es) was observed (Fig. 8, A, lane 1, and B, lane 2). To ensure specific binding of transcription factors to the probe, unlabeled wild-type double-stranded oligonucleotide competitors (Fig. 8, A, lane 2, and B, lane 3) and unlabeled mutated double-stranded oligonucleotide competitors (Fig. 8B, lane 4) were added prior to the addition of labeled probe. To determine whether Tax protein or CREB-1 protein was bound to the promoter, anti-Tax monoclonal antibody (Tab172) or anti-CREB-1 monoclonal antibody was incubated with nuclear extracts before adding the binding buffer (Fig. 8, A, lane 3, and B, lane 5). The CRE complex(es) was supershifted by both antibodies, indicating the presence of Tax and CREB-1 in the CRE complex(es). When similar experiments were carried out with nuclear extracts from cells without Tax transfection, no bands supershifted by the anti-Tax antibody were observed (Fig. 8A, lane 6). It is interesting to note that the anti-Tax and anti-CREB-1 antibodies supershifted nearly all of the CRE complexes, suggesting that in the presence of Tax, Tax-CREB-1 was the predominant complex bound to the CRE, at least under our in vitro binding conditions. These results indicate that both Tax and CREB-1 bind the CRE in vitro.

To further confirm Tax-ZNF268 promoter binding, a ChIP assay 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-phospho-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. A fragment of the expected size of 332 bp was detected when anti-Tax or anti-phospho-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 pcDNA-3.1 (Fig. 9A, lanes 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 in the ZNF268 promoter, suggesting the formation of the Tax-CREB-1-CRE complex in vivo.

Samples were electrophoresed on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography. Note that the anti-Tax and anti-CREB-1 antibodies supershifted nearly all of the CRE complexes. It is possible that in the presence of Tax, Tax-CREB-1 is the predominant complex binding to the CRE, whereas little CREB-2 is bound to the CRE, at least under our in vitro binding conditions. CRE, complexes containing the CRE probe. Supershift, complexes supershifted by the anti-Tax or anti-CREB-1 antibody. (Note that the CRE complex(es) formed with nuclear extract with or without Tax transfection had similar mobility (A, 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 CRE-CRE complexes under our gel conditions.)

FIGURE 8. Both Tax and CREB-1 bind to the CRE in the ZNF268 promoter in vitro. A, EMSA was performed with nuclear extracts (NE) from HEK293 cells transfected with pcDNA-Tax (lanes 1–3) or with the empty vector pcDNA-3.1 (lanes 4 and 6) as a control. Labeled CRE probe was added to all reactions (lanes 1–6). Unlabeled wild-type double-stranded oligonucleotide competitors were added during preincubation prior to probe addition (lane 2). For supershift 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 1) was used as negative control.

Free probe — — — — — +
CRE + + + + + +
NE (Tax) + + + + + +
NE (pcDNA) + + + + + +
Lane 1 2 3 4 5 6

Super shift
CRE

Free probe — — — — +
CRE + + + + + +
NE (Tax) + + + + + +
NE (pcDNA) + + + + + +
Lane 1 2 3 4 5 6

Super shift
CRE

B
Anti-CREB1
Wild-type Competitor
Mutant Competitor
CRE probe
NE (Tax)
Lane
1 2 3 4 5
— — — — +
— — — — +
— — — — +
+ + + + +
— — — — +
1 2 3 4 5

Supershift
CRE

Free probe — — — — +
CRE + + + + + +
NE (Tax) + + + + + +
NE (pcDNA) + + + + + +
Lane 1 2 3 4 5 6

Supershift
CRE

A
Anti-Tax
Competitor
CRE probe
NE (Tax)
NE (pcDNA)
Lane
1 2 3 4 5 6
— — — — +
— — — — +
+ + + + +
+ + + + +
+ + + + +
1 2 3 4 5 6

Supershift
CRE

Free probe — — — — +
CRE + + + + + +
NE (Tax) + + + + + +
NE (pcDNA) + + + + + +
Lane 1 2 3 4 5 6

Supershift
CRE

JUNE 13, 2008 • VOLUME 283 • NUMBER 24
Tax Represses ZNF268 Expression through the CREB/ATF Pathway

### TABLE 1
Oligonucleotides used in this study

| Oligonucleotide | Oligonucleotide sequence (5' to 3') | Location<sup>a</sup> |
|-----------------|-----------------------------------|---------------------|
| PDT8            | CGAAGTATCCCTTGTAAGATCTGGTGACTGGTG | −37 to −20          |
| PDT7            | GTTAACTTTTTGCTTCACCTGCACACCTG    | +938 to +921         |
| PDT6            | GTTAACTTTTTGCTTCACCTGCACACCTG    | +760 to +743         |
| p53-mut-F       | CTGGCAAGGAGGGCTGAAGTCTGCGAATGG   | +540 to +523         |
| p53-WT-F        | CTGGCAAGGAGGGCTGAAGTCTGCGAATGG   | +596 to +621         |
| Ets-mut-F       | AGGCTGAGGCTGCTGCTCACTG         | +606 to +631         |
| Ets-WT-F        | AGGCTGAGGCTGCTGCTCACTG         | +606 to +631         |
| CRE-WT-F        | GCCCTCCACGGCCCTTGGAGGGATTTG    | +724 to +749         |
| CRE-mut-F       | GCCCTCCACGGCCCTTGGAGGGATTTG    | +724 to +749         |
| AP1-WT-F        | TTCCCTCTCTCAAGCTGTTG           | +722 to +746         |
| AP1-mut-F       | TTCCCTCTCTCAAGCTGTTG           | +722 to +746         |
| C/EBP-WT-F      | CTCCAGAGGCTTCATCTGCGAC         | +728 to +752         |
| C/EBP-mut-F     | CTCCAGAGGCTTCATCTGCGAC         | +728 to +752         |
| PECS3           | GCAGATGAGGAGGGTATCTG            | +287 to +306         |
| PECA            | GCGAGATGAGGAGGGCTG             | +761 to +738         |
| PECS11          | ACCGCGCGGAGGAGGGCTG            | +594 to +614         |
| ZNF268          | GAGCTGGGAGGGTGCTGAATGGAGGAATTTG| +925 to +906         |
| Tax1            | CTGACTTGGGAGGGTATCTG            | −1790 to −1799       |
| CREU            | GAGGCTGAGGCTGCTGCTCACTG         | −1790 to −1799       |
| CREBD           | GAGGCTGAGGCTGCTGCTCACTG         | −1790 to −1799       |
| CREB-DN-F       | CTGGCTGAGGCTGCTGCTCACTG         | −1381 to −1400       |
| CREB-DN-R       | ACAATCTCTGGGAGGGCTG             |                |
| EgrU            | ACCGAGTCTCGGCCGCGGAGGGCTGATGC  |                |
| EgrD            | ATGCCGACCTTGAGGCAATTCATCTGCGAG |                |

<sup>a</sup>Shown are the oligonucleotide positions, where +1 is the transcription start site of the ZNF268 gene.

<sup>b</sup>Nucleotides in **bold** are the mutated binding sites of the corresponding base.

**FIGURE 9.** ChIP assay shows that both Tax and CREB-1 are bound to the CRE region of the 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. Shown is PCR amplification of DNA precipitated with anti-Tax (lanes 2, 3, and 7), anti-phosho-CREB-1 (lanes 4 and 5), and anti-GFP (lane 8) by using primers for the ZNF268 promoter, PECS11 and PECA, flanking the CRE (+594 to +925) (A). PCR amplification of DNA precipitated without any antibody (lane 6) was used as the negative control. The input lane (lane 1) shows product of PCR amplification of chromatin DNA prior to immunoprecipitation. PCR with primers PU and PDT1A for an upstream region (∼1790 to −1381) (B) of the ZNF268 promoter was done as a negative control.

**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 α. 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 CREs and are regulated by signals that elevate the cellular cAMP level (22). However, the ability of Tax to regulate transcription via the CRE site is context-specific, since at many other CRE-binding sites, where Tax-CREB 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 an HTLV-1-infected cell line and cells stably expressing 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 the 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 the ZNF268 gene, ZNF268a, ZNF268b1, and ZNF268b2, due to alternative splicing (32). Since Tax represses the ZNF268 promoter, all three ZNF268 products are probably 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 previous findings, it is reasonable to suggest that Tax represses ZNF268 gene expression through the CREB/ATF pathway. Indeed, EMSA and ChIP assays confirmed the binding of both Tax and CREB-1 to the ZNF268 CRE site in vitro and in vivo, respec-
Tax Represses ZNF268 Expression through the CREB/ATF Pathway

Recently, these results suggest the formation of a 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 are transcription factors (such as c-fos, c-jun, c-myc, egfr-I, and egfr-2), cytokines (such as IL-1α, IL-2, IL-4, IL-6, and IL-8), and cell cycle regulators (such as cyclin D1 and cyclin D2) (33). Recently, however, several prototypic transcription activators, such as p53, E2F, and 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 a repressor, as shown recently by several studies describing its repressive activity on genes such as c-fos, c-jun, c-myc, egfr-I, and egfr-2. These results suggest the formation of a 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 are transcription factors (such as c-fos, c-jun, c-myc, egfr-I, and egfr-2), cytokines (such as IL-1α, IL-2, IL-4, IL-6, and IL-8), and cell cycle regulators (such as cyclin D1 and cyclin D2) (33). Recently, however, several prototypic transcription activators, such as p53, E2F, and 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 a repressor, as shown recently by several studies describing its repressive activity on genes such as c-fos, c-jun, c-myc, egfr-I, and egfr-2. These results suggest the formation of a 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.

Acknowledgments—The Tax, M22, and M47 expression plasmids were kindly gifts from Warner C. Greene (Gladdstone Institute of Virology and Immunology, University of California, San Francisco). The anti-Tax monoclonal antibody (Tab172) was a kind gift from John N. Brady (NCI, National Institutes of Health). We thank Dr. Xue Zhang for experimental technology.

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, 6476–6480
2. Poiesz, B. J., Busscetti, F. W., Mier, J. W., Woods, A. M., and Gallo, R. C. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6815–6819
3. Robek, M. D., and Ratner, L. (1999) J. Virol. 73, 4856–4865
4. Grassmann, R., Berchtold, S., Radant, I., Alt, M., Fleckenstein, B., Srodoski, J. G., Hestipline, W. A., and Ramstedt, U. (1992) J. Virol. 66, 4570–4575
5. Napolitano, M., Modi, W. S., Cevario, S. I., Gnarra, J. R., Seuanez, H. N., and Leonard, W. J. (1991) J. Biol. Chem. 266, 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, 7981–7987
7. Brauweiler, A., Garrus, J. E., Reed, J. C., and Nyborg, J. K. (1997) Virology 231, 135–140
8. Zhao, L. J., and Giam, C. Z. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7070–7074
9. Zhao, L. J., and Giam, C. Z. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11445–11449
10. Suzuki, T., Fujisawa, J. L., Toita, M., and Yoshida, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 610–614
11. Alexandre, C., and Verrier, B. (1991) Oncogene 6, 543–551
12. Fujii, M., Niki, T., Mori, T., Matsuda, T., Matsui, M., Nomura, N., and Seiki, M. (1991) Oncogene 6, 1023–1029
13. Fuji, M., Tsuchiya, H., Chuhuo, T., Akizawa, T., and Seiki, M. (1992) Genes Dev. 6, 2066–2076
14. Trejo, S. R., Fahl, W. E., and Ratner, L. (1996) J. Biol. Chem. 271, 14584–14590
15. Fu, W., Shah, S. R., Jiang, H., Hilt, D. C., Dave, H. P., and Joshi, J. B. (1997) J. Neurovirol. 3, 16–27
16. Joshi, J. B., and Dave, H. P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 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, 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, 981–989
19. Hoshino, H., Tanaka, H., Shimotohno, K., Miwa, M., Akatsuka, T., and Odaka, T. (1995) Virology 217, 223–226
20. Kibler, K. V., and Jeang, K. T. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1071–1075
21. Yoshikawa, J. U., Hanaoka, M., Nakai, M., Matsumoto, T., and Hinuma, Y. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 306–310
22. Aszner, I., Scavich-Krampen, Y., and Aboud, M. (2004) Retrovirology 1, 20
23. Sun, Y., Gou, D., Sun, Y., Gao, L., Chow, L. M., Huang, J., Feng, Y. D., Jiang, D. H., and Li, W. X. (2001) Biochim. Biophys. Acta 1518, 306–310
24. Gebelein, B., and Urrutia, R. (2001) Mol. Cell. Biol. 21, 928–939
25. Sun, Y., Gou, D. M., Liu, H., Peng, X., and Li, W. X. (2003) J. Biol. Chem. 278, 127–131
26. Gou, 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, 24623–24636
Tax Represses ZNF268 Expression through the CREB/ATF Pathway

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, 2123–2131
29. Smith, M. R., and Greene, W. C. (1990) Genes Dev. 4, 1875–1885
30. Nguyen, L. Q., Kopp, P., Martinson, F., Stanfield, K., Roth, S. I., and Jameson, J. L. (2000) Mol. Endocrinol. 14, 1448–1461
31. Pathak, S. K., Bhattacharyya, A., Pathak, S., Basak, C., Mandal, D., Kundu, M., and Basu, J. (2004) J. Biol. Chem. 279, 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, 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, 29394–29399
35. Cox, J. M., Sloan, L. S., and Schepartz, A. (1995) Chem. Biol. 2, 819–826