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Citation	Journal Of General Virology, 2003, v. 84 n. 12, p. 3203-3214
Issued Date	2003
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Transcriptional activation of immediate–early gene *ETR101* by human T-cell leukaemia virus type I Tax

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Human T-cell leukaemia virus type I (HTLV-I) Tax regulates viral and cellular gene expression through interactions with multiple cellular transcription pathways. This study describes the finding of immediate–early gene *ETR101* expression in HTLV-I-infected cells and its regulation by Tax. *ETR101* was persistently expressed in HTLV-I-infected cells but not in HTLV-I uninfected cells. Expression of *ETR101* was dependent upon Tax expression in the inducible Tax-expressing cell line JPX-9 and also in Jurkat cells transiently transfected with Tax-expressing vectors. Tax transactivated the *ETR101* gene promoter in a transient transfection assay. A series of deletion and mutation analyses of the *ETR101* gene promoter indicated that a 35 bp region immediately upstream of the TATA-box sequence, which contains a consensus cAMP response element (CRE) and a G+C-rich sequence, is the critical responsive element for Tax activation. Site-directed mutagenesis analysis of the 35 bp region suggested that both the consensus CRE motif and its upstream G+C-rich sequence were critical for Tax transactivation. Electrophoretic mobility shift analysis (EMSA) using the 35 bp sequence as probe showed the formation of a specific protein–DNA complex in HTLV-I-infected cell lines. EMSA with specific antibodies confirmed that the CREB transcription factor was responsible for formation of this specific protein–DNA complex. These results suggested that Tax directly transactivated *ETR101* gene expression, mainly through a CRE sequence via the CREB transcription pathway.

Received 10 April 2003
Accepted 3 July 2003

INTRODUCTION

Human T-cell leukaemia virus type I (HTLV-I) is a retrovirus that is closely associated with various human diseases, including adult T-cell leukaemia and HTLV-I-associated myelopathy/tropical spastic paraparesis (Poiesz *et al.*, 1981; Yoshida *et al.*, 1982; Gessain *et al.*, 1985; Osame *et al.*, 1986). The pathogeneses of these diseases are still not well understood but it has been shown that dysregulated expression of cellular genes in HTLV-I-infected cells may play a critical role in the development of HTLV-I-associated diseases (Yoshida, 1996, 2001). Unlike acute leukaemia viruses of animal origin, HTLV-I has no typical oncogene derived from the cellular genome but it encodes a 40 kDa nuclear phosphoprotein, Tax, which is encoded from the 3′ end region of the HTLV-I genome. Tax can stimulate HTLV-I gene expression through interactions with three imperfect 21 bp repeats in the U3 region of the HTLV-I long terminal repeat (LTR) (Fujisawa *et al.*, 1985; Shimotohno *et al.*, 1986; Sodroski *et al.*, 1984). To activate the HTLV-I LTR, Tax requires at least two copies of the 21 bp repeat, with each 21 bp repeat containing a central 8 bp off-consensus cAMP response element (CRE) (Suzuki *et al.*, 1993; Zhao & Giam, 1992). Each off-consensus CRE is flanked by a G+C-rich region critical for Tax transactivation of HTLV-I transcription. Previous studies suggested that Tax activates the HTLV-I LTR by physical interaction with the CREB/ATF protein family members, which bind specifically to the 21 bp repeats (Zhao & Giam, 1992). In addition to transactivating the HTLV-I LTR, Tax can also activate many cellular genes that are normally controlled by external stimuli, including genes for cytokines, cytokine receptors, growth factors and an array of immediate–early genes (IEGs), such as c-*fos*, c-*myc*, *egr-1*/*krox-24*, *egr-2*/*krox-20* and *TR3*/*nur77* (Alexandre *et al.*, 1991; Ballard *et al.*, 1988; Chen *et al.*, 1997, 1998; Duyao *et al.*, 1992; Fujii *et al.*,

1988, 1991; Inoue *et al.*, 1986; Nagata *et al.*, 1989; Trejo *et al.*, 1997). Thus, HTLV-I Tax can functionally mimic extracellular stimuli by interacting with cellular components that are involved in the transduction of the activating signal from the cell membrane to nuclear target genes.

IEGs are rapidly and transiently expressed during the early stage of induction by growth factors or other stimuli, leading to growth arrest or stimulation. Tax has been shown to transactivate IEGs through various cellular transcription factors, including CREB/ATF protein family members, NF-κB, serum response element (SRE)-binding factor $p67^{SRE}$, activation protein 1 (AP-1) and Sp1 (Fujii *et al.*, 1992; Hirai *et al.*, 1992; Leung & Nabel, 1988; Liu *et al.*, 1999; Low *et al.*, 1994). Thus, the aberrant cell growth through the activation of IEGs by Tax could be an initial step in the development of HTLV-I-related diseases.

During the screening of Tax-regulated cellular genes by DNA microarray analysis, the *ETR101* (early TPA response) gene was found to be strongly expressed in Tax-expressing cells (unpublished data). *ETR101* was cloned originally from activated T-lymphocytes treated with cycloheximide, a TPA-treated promyelocytic cell line HL-60 and serum-stimulated BALB/c 3T3 fibroblasts (Charles *et al.*, 1990; Coleclough *et al.*, 1990; Shimizu *et al.*, 1991). *ETR101* has also been called *pip92* and *chx1*. *ETR101* is one of the IEGs that is expressed immediate–early upon induction by TPA in the human myeloid leukaemia cell line HL-60 (Shimizu *et al.*, 1991). *ETR101* is rapidly and transiently induced by stimulation with serum and growth factors in fibroblasts (Charles *et al.*, 1990). In addition to cell growth, *ETR101* is also induced during neuronal differentiation by treatment with nerve growth factor or upon membrane depolarization in PC12 pheochromocytoma cells (Charles *et al.*, 1990). Based on the gene structure and deduced amino acid composition, ETR101 contains multiple phosphorylation sites, suggesting possible post-translational activation by protein kinase C due to its inducibility by TPA. ETR101 also contains nuclear localization signals and has been proven to be selectively expressed in the nucleus as a fusion protein when tagged with green fluorescent protein, suggesting that ETR101 might be a nuclear protein (Chung *et al.*, 2000). Thus, it has been believed that ETR101 is a putative transcription factor that mediates the expression of other cellular genes.

In present study, we report the transcription activation of *ETR101* by HTLV-I Tax and analyse in detail the regulatory mechanism in transactivation.

METHODS

Materials. All oligonucleotides were synthesized by DNA Technology. pCMV, pCMV-Tax and Tax mutant plasmids, M7, M22 and M47, were obtained from W. Greene (Smith & Greene, 1990). The reporter plasmid pE1b-luc, which contains a basal E1b (adenovirus) promoter sequence, was constructed in our laboratory and has been described elsewhere (Liu *et al.*, 1999).

Cell culture. The human T-cell leukaemia cell line Jurkat (clone E6-1) and the acute lymphoblastic leukaemia cell line Molt-4 were obtained from ATCC. HTLV-I-transformed T-cell lines C8166-45 and MT-2 were obtained through the NIH AIDS Research and Reference Reagent Program (Harada *et al.*, 1985; Salahuddin *et al.*, 1983). All these cells were grown in RPMI 1640 medium supplemented with 10 % FCS at 37 °C under 5 % CO_2. The Tax-inducible JPX-9 and control JPX/M cell lines were maintained under the same conditions. Expression of biologically active Tax or a non-functional Tax mutant was induced by the addition of $CdCl_2$ to a 10 μM final concentration (Nagata *et al.*, 1989).

Plasmids. The 1430 bp (−1393 to +37) fragment in the 5′-flanking region of the human *ETR101* gene was obtained by PCR using human MT-2 genomic DNA as template. The primers used for PCR were 5′-AGCTAAGCTTCGGACAACGGGCTCGCTCA-AC-3′ (reverse, +18 to +37) and 5′-AGCTCTCGAGCACCCGGCT-GGAATGCGAGTCA-3′ (forward, −1393 to −1372). The amplified PCR product was digested with *Xho*I/*Hin*dIII and cloned into pGL2-Basic (Promega), which resulted in pGL2_ETR-1393. For the construction of various length DNA fragments from the *ETR101* gene promoter, PCR was performed using pGL2_ETR-1393 as template. PCR products were cloned into the *Xho*I/*Hin*dIII sites of pGL2-Basic. The oligonucleotides containing potential Tax responsive element (TRE) sequences (see Fig. 3B) in the *ETR101* gene promoter were synthesized, annealed and cloned into the *Xma*I/*Xho*I sites of pE1b-luc. Likewise, the oligonucleotides with various mutations (see Fig. 5) were cloned into pE1b-luc. pGL2_ETR-1393 was used as template to generate the *ETR101* gene promoter carrying specific point mutations, created using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Nucleotide sequences of all constructs were confirmed by DNA sequencing.

Cell transfection and assay of reporter gene activity. Transfection was performed using the cationic lipid method, as described previously (Liu *et al.*, 1999). The pSV-β-galactosidase plasmid (Promega) was included in each transfection experiment to normalize transfection efficiency. Luciferase activity was assayed using the Steady-Glo Luciferase Assay reagent (Promega) and measured in a luminometer (Wallac 1420 Victor^2, EG&G Wallac). Luciferase activity in individual samples was normalized on β-galactosidase activity measured using the β-Galactosidase Enzyme Assay system (Promega).

Real-time quantitative PCR. Primers for the *ETR101* gene were designed with the Lasergene software package DNASTAT (forward, 5′-CTTCGGGTGGGAGCGGGGACTGAT-3′; reverse, 5′-GGGCA-CGCTGGAACCTGGGAACC-3′). Cytoplasmic RNAs were collected at different time-points after transfection in Jurkat cells and purified using the RNeasy Midi kit (Qiagen). Real-time PCR was performed as described previously (Fink *et al.*, 2001). Advance *Taq* Plus DNA polymerase (Clontech) and SYBR Green I Fluorophore (Molecular Probes) were used in a two-temperature cycling, consisting of a denaturation step of 95 °C for 15 s and an annealing/extension step of 68 °C for 30 s. The predicted size and optimal concentration of primers were checked prior to routine use. To normalize the input load of cDNA among samples, 18S rRNA was quantified in a separated real-time PCR analysis and used as an endogenous standard (Fink *et al.*, 2001). For the quantitative analysis of individual genes, four independent cDNA samples were prepared from each culture and each of the cDNA samples was tested in duplicate.

Northern and Western blot analysis. For Northern blot analysis, cytoplasmic RNA was prepared from different cell lines using the RNeasy Midi kit. RNA (5 μg) was separated in a 1 % formaldehyde/agarose gel and transferred to a PVDF-N membrane (Millipore) using vacuum transfer. The probes for *ETR101*, *tax* and *GAPDH* were prepared from IMAGE clone 187164 (UK HGMP Resource

Centre), pCMV-Tax and pHcGAP, respectively, and labelled with the Strip-EZ DNA Random-Primed StripAble DNA Synthesis and Removal kit (Ambion). Hybridization was performed as described previously (Chen *et al.*, 1997). For Western blot analysis, 10 μg protein was subjected to electrophoresis on a 12 % denaturing polyacrylamide gel and transferred to a PVDF-P membrane (Millipore) using a semi-dry blotting system (Owl Separation system). Anti-Tax antibodies (Lt-4 and TAXY-8) were obtained from Y. Tanaka (Tanaka *et al.*, 1991). Protein detection was performed with the ECL Western Blotting Detection system (Amersham Pharmacia Biotech).

Electrophoretic mobility shift analysis (EMSA). Nuclear extracts of Jurkat, Molt-4, C8166-45 and MT-2 cells were prepared according to an established protocol (Liu *et al.*, 1999). Double-stranded oligonucleotide ETR-p35 (sequence shown in Fig. 5A) was end-labelled with [α-^{32}P]dCTP (800 Ci per mmol) and DNA polymerase Klenow fragment (Invitrogen) at room temperature for 30 min. Then, ^{32}P-labelled ETR-p35 was purified with the MicroSpin G-25 column (Amersham Pharmacia Biotech). Binding reactions were performed by incubating 4 μg nuclear extract with 2 μg poly(dI-dC) and 1 μg BSA in 14·5 μl binding buffer (12 mM HEPES-KOH, pH 7·9, 60 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, 12 % glycerol and 0·2 % Nonidet P-40) on ice for 30 min. Then, approximately 26 000 c.p.m. ^{32}P-ETR-p35 was added and incubated at room temperature for 20 min. The binding reaction mixture was resolved on a 5 % non-denaturing polyacrylamide gel (40 : 1 acrylamide/bisacrylamide) at 4 °C for 2·5 h in 0·25 × TBE and developed on a Packard InstantImager (Packard Instrument). For competition analysis, a 50-fold molar excess of cold probe was added to the binding reaction mixture and incubated on ice for 30 min before addition of ^{32}P-ETR-p35. The probes containing the consensus binding sites for AP-1, AP-2, AP-3, CREB, NF-κB, NF1/CRF, Sp-1 and Oct-1 were from Stratagene. For EMSA, nuclear extracts were pre-incubated in the binding buffer on ice for 30 min with 1 μg poly(dI-dC), 1 μg BSA and 1 pmol double-stranded ETR-p35-mu5. Then, 26 000 c.p.m. ^{32}P-ETR-p35 was added and incubated at room temperature for 20 min prior to addition of 1 μg specific antibody. Reaction mixtures were incubated at 37 °C for 30 min and subjected to electrophoresis and detection. Antibodies for c-Jun (N), JunB (N-17), JunD (329), c-Fos (4), ATF-1 (FI-1), ATF-2 (C-19), ATF-3 (C-19) and ATF-4 (C-20) were from Santa Cruz Biotechnology and the antibody for CREB was from Upstate Biotechnology.

Sequence analysis. A multiple sequence alignment was performed with the program CLUSTALX (Jeanmougin *et al.*, 1998) and the output format was processed further with the program GENEDOC. Scanning of possible transcription-binding sites was performed by searching against the TRANSFEC database (Wingender *et al.*, 2000).

RESULTS

Expression of *ETR101* in HTLV-I-infected and Tax-expressing cells

Northern blot analysis was performed to analyse *ETR101* expression in HTLV-I uninfected Molt-4 cells or HTLV-I-transformed MT-2 and C8166-45 cells. Our results showed that high levels of *ETR101* expression were detected in both MT-2 and C8166-45 cell lines, which constitutively express Tax, but not in Molt-4 cells (Fig. 1A). The effect of Tax on *ETR101* expression was also examined in Tax-inducible cell lines. JPX-9, a derivative of Jurkat cells, contains an inducible Tax cDNA under the control of the metallothionein promoter. JPX/M cells are counterparts of JPX-9 cells, which contain a functionally inactive Tax point mutant (a single arginine insertion at residue 62) (Nagata *et al.*, 1989; Neuveut *et al.*, 1998). Expression of *tax* and its mutants can be induced with 10 μM $CdCl_2$. As shown in Fig. 1, induction of *tax* and mutant *tax* expression by $CdCl_2$ was readily detected after 4 h of incubation in JPX-9 and JPX/M cells. In parallel, expression of the *ETR101* gene was undetectable until 4 h of incubation and kept increasing at the similar time lag as that of *tax* expression in JPX-9 cells, but not in mutant *tax* expression JPX/M cells. This result showed that Tax expression is responsible for the increase in *ETR101* expression observed in HTLV-I-transformed cells.

To further investigate the dependence of *ETR101* expression upon Tax, Tax-expressing pCMV-Tax or the empty vector pCMV were transiently transfected into Jurkat cells. Cytoplasmic RNA was collected at different time-points after transfection. Real-time PCR and Western blotting were performed to detect the expressions of the *ETR101* gene and *tax* after transfection (Fig. 1B). In cells transfected with pCMV-Tax, real-time PCR data showed that *tax* transcripts were readily detected within 3 h and reached a peak at 6 h after transfection. Expression of *tax* was detectable at 3 h and reached the highest level within 18 h. In accordance with this expression pattern, expression of *ETR101* transcripts consistently increased for 12 h to around 2·8-fold higher than that observed in cells transfected with pCMV. This result indicated that the expression of the *ETR101* gene is directly associated with the expression of *tax* in HTLV-I-transformed cells.

Structure of the *ETR101* gene promoter and its responsiveness to Tax transactivation

To analyse the regulatory mechanisms of *ETR101* gene expression, the genomic sequences of the 5′-flanking region of both human and mouse *ETR101* were aligned with CLUSTALX. As shown in Fig. 2, 1430 bp of 5′ upstream sequence of the human *ETR101* gene showed 52 % sequence similarity with the mouse counterpart. Further sequences upstream had little similarity (data not shown). Therefore, it is highly plausible that the regulatory elements of *ETR101* gene expression were situated in the 1430 bp region (the transcription start site was numbered as +1). The 1430 bp sequences were analysed with TRANSFEC to search for potential binding site(s) for transcription regulation (Wingender *et al.*, 2000). Several potential TREs were noted, including two Ets motifs (positions −1276/−1271 and −1218/−1213), two CArG-like motifs (positions −1232/−1223 and −1180/−1171), two NF-κB motifs (positions −212/−203 and −120/−111) and one CRE (position −78/−71). The 1430 bp (−1393 to +37) *ETR101* gene promoter sequence was amplified from MT-2 cells and cloned into pGL2-Basic (pGL2_ETR-1393). *ETR101* gene promoter activity was analysed by co-transfecting pGL2_ETR-1393 with pCMV-Tax into Jurkat cells. An 8·3-fold increase in promoter activity upon Tax expression was observed, indicating strong activation of the *ETR101* gene promoter by Tax (Fig. 3A).

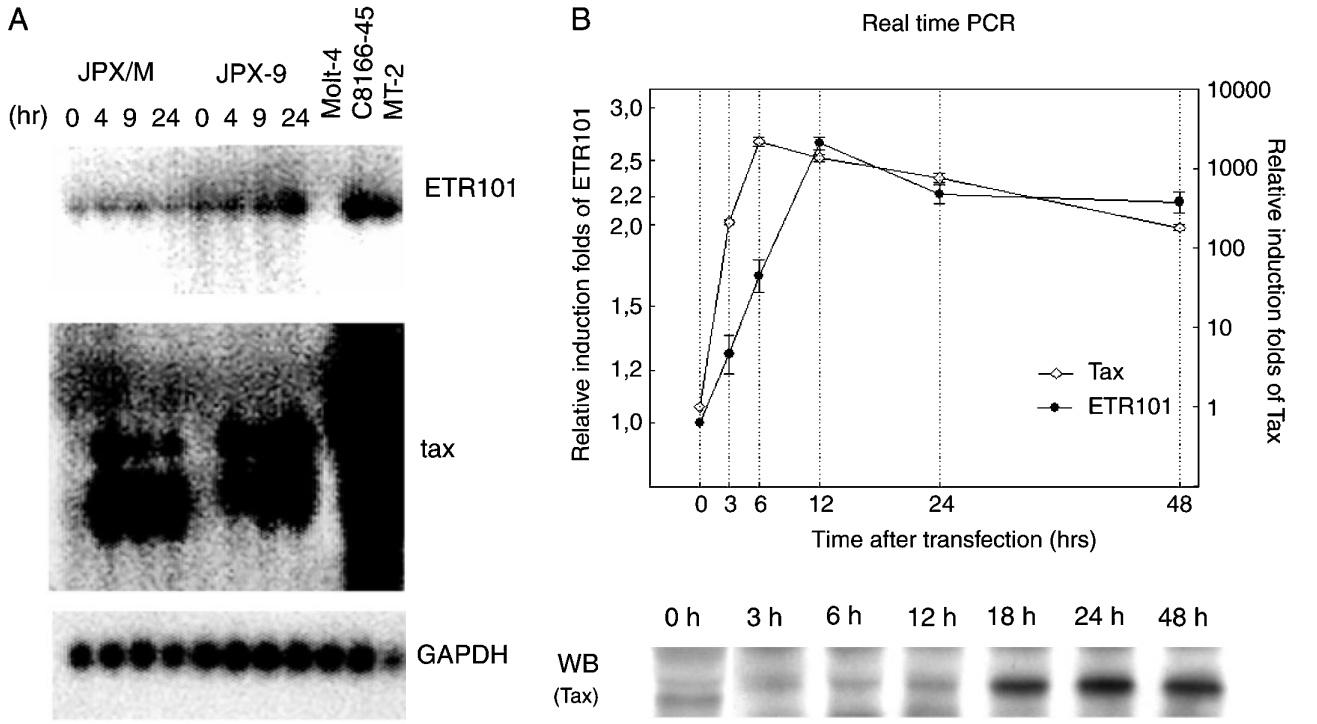

Fig. 1. Transactivation of *ETR101* expression in HTLV-I-transformed cell lines and Tax-expressing cells. (A) Northern blotting analysis of *ETR101*-, *tax*-, and *GAPDH*-specific transcription expression in HTLV-I-transformed cell lines and Tax-inducible expression cell lines. Total RNA (5 μg) from HTLV-I uninfected Molt-4- and HTLV-I-transformed C8166-45 and MT-2 cells was separated in a 1% formaldehyde/agarose gel. The JPX-9 and JPX/M cells stably transfected with *tax* and mutant *tax* genes under the control of inducible promoters were analysed after *tax* induction with 10 μM $CdCl_2$ at the intervals indicated. Expression of the *GAPDH* gene was analysed for standardization purposes. (B) Real-time PCR and Western blot (WB) analysis for the expression of ETR101 and Tax in Jurkat cells transiently transfected with control pCMV and Tax-expressing pCMV-Tax plasmids. The ratio of ETR101 and Tax expression in cells separately transfected with pCMV-Tax to pCMV were calculated at each time-point as fold induction. Cell lysate (10 μg) from pCMV-Tax-transfected Jurkat cells at each time point was analysed for Tax expression by Western blotting using Tax-specific Lt-4 antibody.

Mapping of TRE in the *ETR101* gene promoter

To identify the region necessary for the response to Tax transactivation, we cloned various fragments of the 5′-flanking region of the *ETR101* gene upstream from the transcription initiation site into luciferase reporter vector pGL2-Basic. These constructs were transiently co-transfected with pCMV-Tax or pCMV and their luciferase activities were determined. As shown in Fig. 3(A), the luciferase activity of pGL2_ETR-1393 increased by around 8·3-fold compared with that of cells co-transfected with pCMV. After deletion of both Ets and CArG-like motifs, progressive deletions of pGL2_ETR-679 and pGL2_ETR-311 show no significant decrease in luciferase activities and ratio of induction (8·3-fold and 12·3-fold, respectively). Further deletion of two possible NF-κB-binding motifs (pGL2_ETR-110) resulted in around 50% reduction in promoter activity, but the induction ratio kept to 11-fold. Further deletion of an additional 42 bp (pGL2_ETR-68) totally abolished responsiveness to Tax transactivation. These results indicated that the essential sequence motifs in response to Tax transactivation on the *ETR101* gene promoter are situated between positions −310 and −68. The region at −110/−68 may contribute mainly to Tax transactivation.

To define further the region responsive to Tax transactivation in the *ETR101* promoter, the representative fragments in the region −310/−68 were cloned into the pE1b-luc reporter plasmid and placed directly in front of the basal adenovirus E1b TATA-box sequence (Liu *et al.*, 1999). As shown in Fig. 3(B), the −310/−175 region (as represented by −310/−265, −280/−235, −240/−195 and −220/−175 fragments) was not responsive to Tax. The −140/−100 region of the *ETR101* gene promoter, which includes the second potential NF-κB-binding motif, can be activated 4·2-fold by Tax. The −98/−64 region resulted in an 11-fold increase in its activity in response to Tax, which is equivalent to pGL2_ETR-110 in response to Tax (Fig. 3B). This result indicated that the region at positions −140/−64,

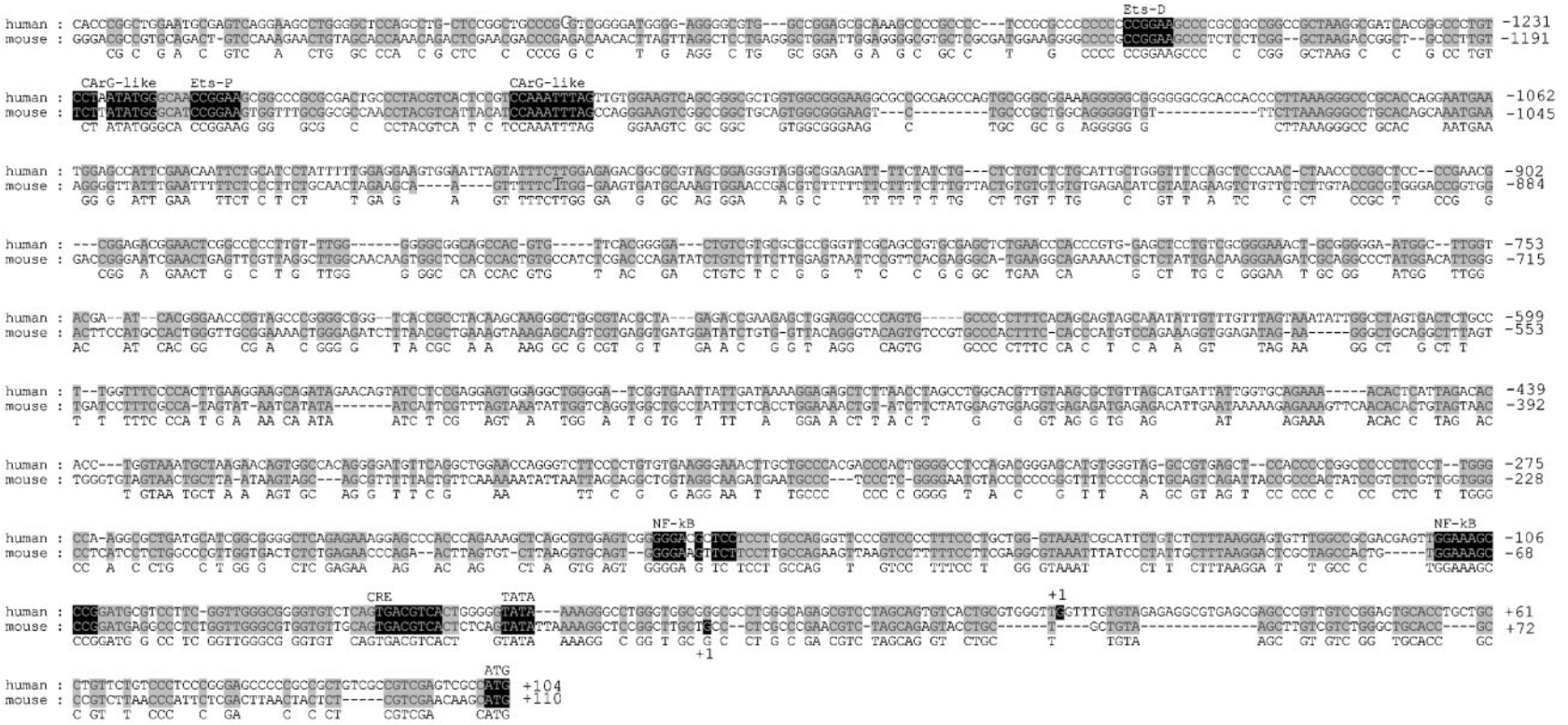

Fig. 2. Sequence alignment of human and mouse *ETR101* gene promoters. Numbers referred to are relative to the transcription start site at +1. Sequence alignment was performed using CLUSTALX and the output format was processed further with GENEDOC.

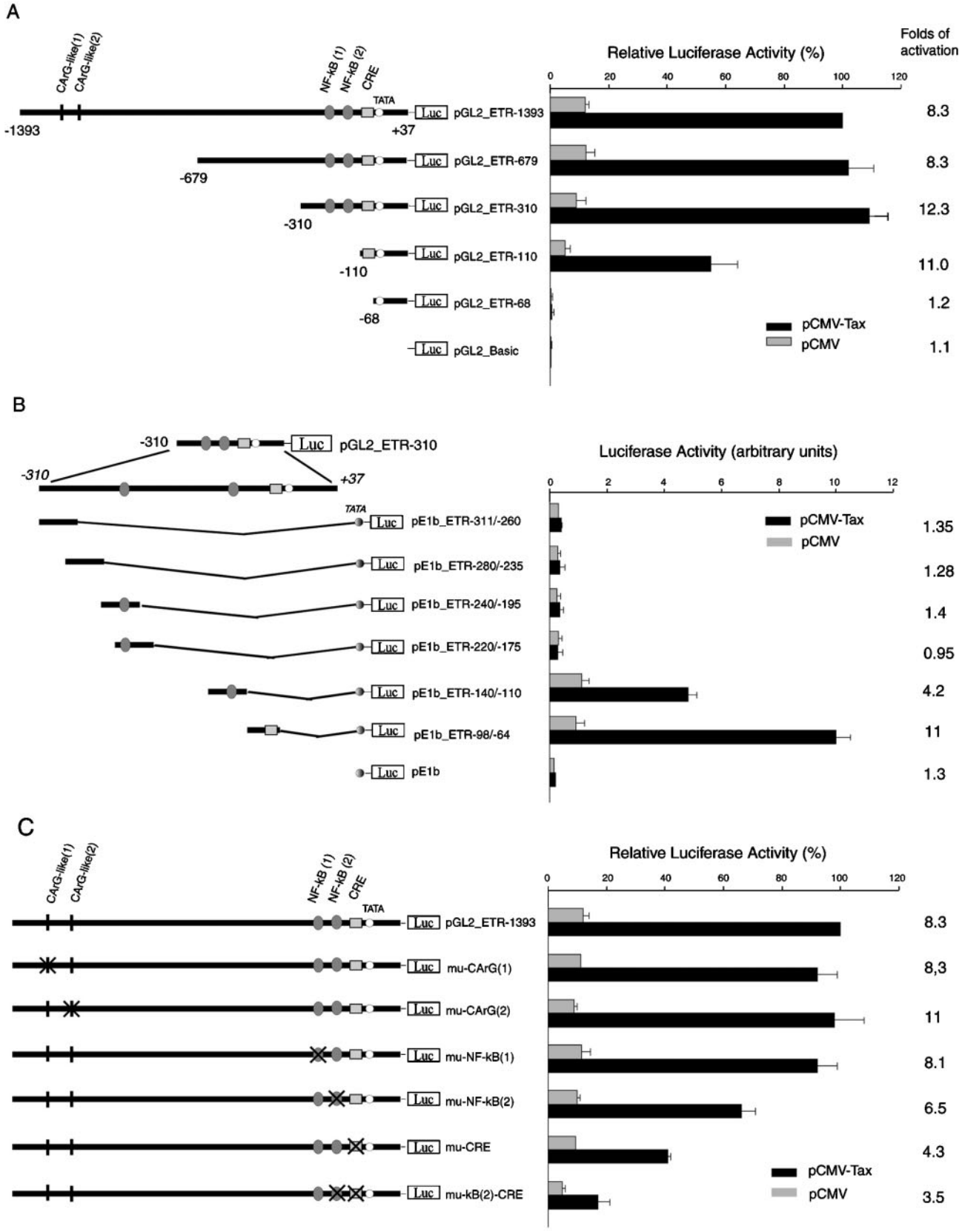
A
CArG-like(1)
CArG-like(2)
NF-kB (1)
NF-kB (2)
CRE
TATA
-1393
+37
-679
-310
-110
-68
Luc
pGL2_ETR-1393
pGL2_ETR-679
pGL2_ETR-310
pGL2_ETR-110
pGL2_ETR-68
pGL2_Basic
Relative Luciferase Activity (%)
Folds of activation
0
20
40
60
80
100
120
8.3
8.3
12.3
11.0
1.2
1.1
pCMV-Tax
pCMV
B
pGL2_ETR-310
pE1b_ETR-311/-260
pE1b_ETR-280/-235
pE1b_ETR-240/-195
pE1b_ETR-220/-175
pE1b_ETR-140/-110
pE1b_ETR-98/-64
pE1b
Luciferase Activity (arbitrary units)
0
2
4
6
8
10
12
1.35
1.28
1.4
0.95
4.2
11
1.3
C
mu-CArG(1)
mu-CArG(2)
mu-NF-kB(1)
mu-NF-kB(2)
mu-CRE
mu-kB(2)-CRE
8.3
8,3
11
8.1
6.5
4.3
3.5

Fig. 3. Mapping of the TRE sequence in the *ETR101* promoter region. Diagrams of different deletion constructs are shown (A). Locations of possible functional elements are labelled at the corresponding positions and are indicated on top of the sequence. The luciferase reporter vectors containing various lengths of the 5′ end region of the *ETR101* gene were co-transfected together with pCMV-Tax (black bars) or the control pCMV (white bars) plasmids in Jurkat cells. (B) Analysis of transcription activation of putative TRE sequences on the *ETR101* promoter in a heterologous minimal promoter. The different double-stranded oligonucleotides corresponding to positions −310 to −64 upstream of the transcription initiation site of the *ETR101* promoter sequence were constructed into the immediate upstream region of the minimal adenovirus E1b promoter at the *Xma*I/*Xho*I sites of pE1b-luc. All reporter vectors were co-transfected with pCMV-Tax (black bars) or the control pCMV (white bars) plasmids in Jurkat cells. (C) Mutational analysis of each putative TRE on Tax transactivation of *ETR101* promoter activity. Mutated sites are shown (X); constructs were named according to the positions of mutations. Luciferase activities were quantified and normalized by β-galactosidase activity. pGL2_ETR-1393 with pCMV-Tax was given a value of 100 % and the activities of the other transfection were adjusted relative to this activity. Bars represent the means ± SE of at least three replicates.

harbouring potential NF-κB and CRE motifs, is involved in the Tax activation of the *ETR101* gene promoter.

Point mutation studies were performed to further confirm the critical TREs in the *ETR101* promoter. Mutants of each potential TRE on the *ETR101* promoter were generated and analysed in transient transfection with pCMV-Tax. Mutations of each CArG-like element and the first putative NF-κB-binding motif did not affect Tax activation of the *ETR101* gene promoter. These results are line with our deletion analysis. Mutation of the second putative NF-κB-binding motif resulted in a 15 % reduction in the ratio of Tax activation, from 8·3- to 6·2-fold. Mutation of the potential CRE motif reduced the ratio of Tax activation from 8·3- to 4·3-fold (48 % reduction). Double mutations of these two sites further lowered the induction ratio to 3·5-fold (59 % reduction) (Fig. 3C). These data suggested that the CRE and one of the putative NF-κB-binding elements may be involved in Tax activation in the *ETR101* promoter and that CRE is more prominent in response to Tax transactivation.

Tax transactivation of *ETR101* promoter activity is dependent mainly on the CREB pathway

Contribution of CRE and NF-κB elements in Tax-activated *ETR101* expression was characterized further using Tax mutants in transfection analyses. pGL2_ETR-1393 was co-transfected with wild-type Tax and Tax mutant plasmids M7, M22 and M47. The mutation within a zinc finger domain of Tax (M7), which abolishes transactivation of both CREB/ATF and NF-κB pathways, totally abolished the Tax effect on the *ETR101* promoter. Tax mutant M22 ($T^{130}S$ and $L^{131}A$), which activates the CREB-dependent pathway but not the NF-κB-dependent pathway, activated pGL2_ETR-1393 reporter activity to approximately 82 % of wild-type. On the other hand, the M47 mutant, which is defective in the CREB pathway but is fully active in the NF-κB pathway, showed only 18 % of activity compared with wild-type Tax when co-transfected with the *ETR101* promoter (Fig. 4). These results strongly suggested that Tax transactivation of the *ETR101* promoter is predominantly dependent on the CREB pathway.

Site-directed mutagenesis of the *cis*-acting elements of the proximal (−98/−64) TRE in a heterologous reporter

Sequence analysis of this 35 bp *ETR101* promoter showed that it contains a consensus CRE sequence (TGACGTCA), which is conserved between human and mouse promoter sequences (Fig. 2). An 8 bp G + C-rich sequence in the 5′ end of this region was also found. This 35 bp TRE was cloned into the pE1b-luc plasmid and a series of mutations were introduced to replace the consensus sequence. Transfection results showed that m5 and m6 both disrupted the CRE motif, rendering the promoter unresponsive to Tax (Fig. 5A). Mutations of the 5′ end G + C-rich sequence (m1 and m2) showed significantly different effects. Mutation of GGC→AAA (m1) essentially abolished Tax responsiveness but mutation of GGG→AAA (m2) reduced activity to

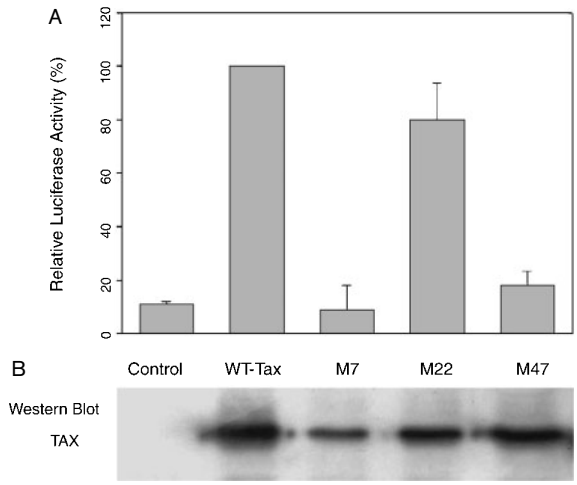

Fig. 4. Differential activation of pGL2_ETR-1393 reporter activity by Tax and its mutants. (A) Jurkat cells were transfected with pGL2_ETR-1393 and expression plasmids for wild-type Tax (WT) and Tax mutants M7, M22 and M47. The percentage of promoter activity was calculated relative to wild-type Tax. (B) Expression of the wild-type and mutant Tax proteins in the transfected cells were confirmed by Western blotting with Lt-4 and TAXY-8 anti-Tax antibodies.

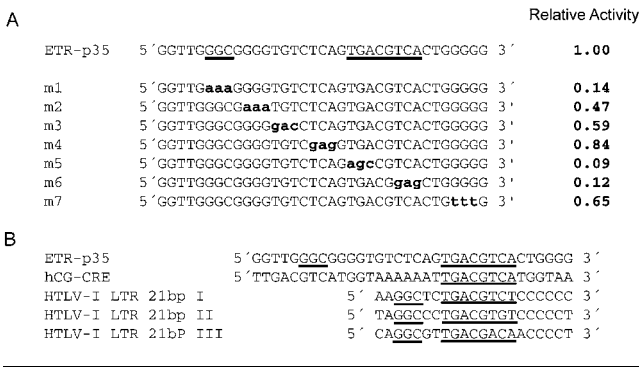

Fig. 5. Site-directed mutagenesis of the 35 bp *ETR101* promoter region in response to Tax transactivation. (A) Illustration of the 35 bp sequence of the *ETR101* promoter (ETR-p35) and its mutants. Mutations are indicated in bold, lowercase letters. Wild-type and mutant *ETR101* promoter reporter vectors were co-transfected with the pCMV-Tax and control pCMV plasmids in Jurkat cells. The activity of wild-type ETR101 35 bp TRE was arbitrarily given the value of 1. The activities of the mutants (m1–m7) were calculated and listed accordingly. (B) Sequence comparison of the *ETR101* 35 bp TRE with the three 21 bp HTLV-I LTR TREs and hCG CRE.

around 47 %. Other mutants showed that the remaining sequence was dispensable for Tax transactivation. These results indicated that CRE and its 5′ G+C-rich sequences were essential for Tax transactivation of the *ETR101* promoter. When compared with three HTLV-I LTR 21 bp TREs, the GGC sequence was also situated upstream of off-consensus CRE (Fig. 5B). Mutation of this GGC sequence in the 21 bp TRE abolished its Tax responsiveness (Fujisawa *et al.*, 1985). The sequence similarity between the 35 bp *ETR101* TRE and HTLV-I LTR 21 bp TRE was not observed in the vicinity of the CRE of human chorionic gonadotropin (hCG), which is highly responsive to cAMP stimulation but not to Tax transactivation (Fig. 5B).

EMSA analysis of a specific DNA–protein complex in the *ETR101* (−98/−64) region

To define the transcription factors specifically bound to the −98/−64 region of the *ETR101* promoter, EMSAs were performed with a ^{32}P-labelled 35 bp sequence (^{32}P-ETR-p35) as the probe (sequence shown in Fig. 5). The results showed that binding of nuclear extracts from Tax-expressing cell lines C8166-45 and MT-2 with the probe resulted in two binding complexes, the slow migrating complex I (Com I) and the faster migrating complex II (Com II) (Fig. 6A). In contrast, only Com I was detected with nuclear extracts from Jurkat and Molt-4 cells. These results suggested that the faster mobility Com II contains the specific nuclear protein(s) to the *ETR101* promoter upon Tax expression and the slower mobility Com I represents a constitutive binding activity irrespective of induction by Tax. To verify the specific DNA-binding motifs involved in the formation of Com I and Com II, a competition analysis was performed with seven mutated ETR-p35 probes (m1 to m7), used as cold probes. The results revealed that Com II was efficiently competed out by the cold probes m1–m4 and m7 but not by the cold probes generated by m5 and m6, in which CRE (TGACGTCA) was disrupted (Fig. 6B). These results demonstrated that the CRE sequence is involved in the formation of Com II. Moreover, Com I was not competed by cold probes m2 and m3, indicating that the sequence covering both m2 and m3 mutations is responsible for the formation of Com I. The 5′ G+C-rich sequence from the human *ETR101* gene (GGGCGGGG) is identical to the consensus Sp1-binding motif. But the homologous region in the mouse *ETR101* promoter showed a single base variation (GGGCGTGG), which disrupts the consensus Sp1 site. The mutation in m1 also disrupted this consensus Sp1 motif, but m1 could compete Com I. These results suggested that the 5′ G+C-rich sequence did not represent a Sp1 motif and the 5′ (GGGC) and 3′ (GGGG) ends of this G+C-rich sequence were functionally separated. To analyse these complexes further, the 22–24 bp cold oligonucleotide probes with consensus DNA-binding motifs (Sp1, NF1/CTF3, AP-1, AP-2, Ap-3, NF-κB, CRE and Oct-1) were used for competition analysis. As shown in Fig. 6(C), Com II was competed away by the CRE or AP-1 probes (Fig. 6C), but Sp1 could compete neither Com I nor Com II. These results indicated that the transcription factor(s) in Com II might belong to CREB/ATF or AP-1 families. But the proteins in Com I were not related to Sp1 transcription factors.

To determine the members of the CREB/ATF and AP-1 transcription factor families that were involved in the formation of inducible Com II, supershift assays with antibodies against different members of the CREB/ATF and AP-1 transcription factors were performed. Only in the presence of anti-CREB antibody did Com II show supershifted mobility (Fig. 6D), indicating that CREB is a component in Com II. Collectively, these results indicated that transcription factor CREB binding with CRE in the *ETR101* promoter contributes to Tax-regulated *ETR101* expression.

DISCUSSION

In this report, we have investigated the expression of *ETR101* in HTLV-I-transformed cells and its regulation by Tax. Our work showed that *ETR101* was constitutively expressed in HTLV-I-transformed cells. The steady state accumulation of *ETR101* mRNA was induced in the Jurkat clone, JPX-9 upon Tax expression and in Jurkat cells transiently transfected with Tax-expressing plasmid. These results suggested that Tax was directly responsible for the constitutive expression of the endogenous *ETR101* gene in cells transformed by HTLV-I.

It has been reported previously that transcriptional activation of *ETR101* by serum growth factors in the NIH 3T3 cell line requires the SRE, which contains both the Ets and

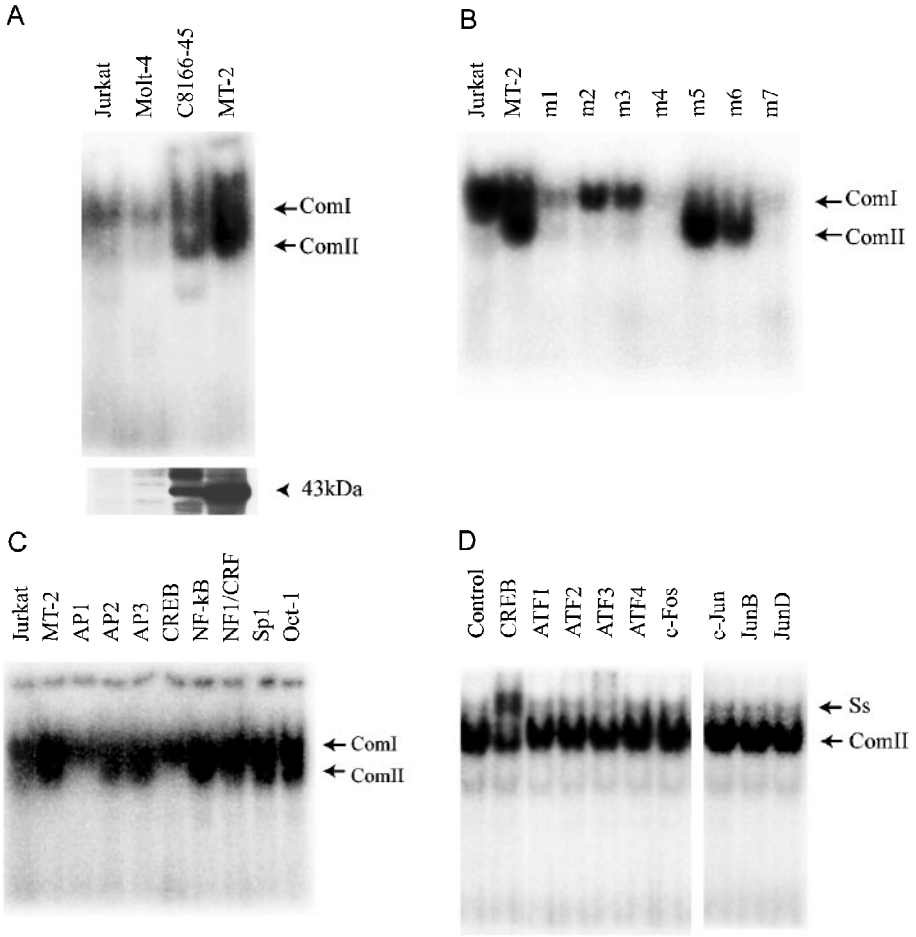

Fig. 6. Gel shift analysis of ETR-p35-binding activity. (A) ^{32}P-ETR-p35 (−98/−64 of the *ETR101* promoter) was incubated with 4 μg nuclear extract from HTLV-I-negative Jurkat and Molt-4 cells and from HTLV-I-transformed C8166-45 and MT-2 cells. Arrows indicate the DNA–protein binding complexes. Presence of Tax in nuclear extracts of C8166-45 and MT-2 was detected with the Tax-specific antibody Lt-4. (B) Competition analysis with different ETR-p35 mutant probes. The 50-fold molar excess of cold ETR-p35 mutant probes m1–m7 were pre-incubated with 4 μg nuclear extract on ice for 30 min before addition of labelled ETR-p35 probe. (C) Competition analysis with 22–24 bp consensus transcription factor-binding probes. The 50-fold molar excess of cold AP-1, AP-2, AP-3, CREB, NF-κB, NF1/CRF, Sp-1 and Oct-1 binding probes were incubated with 4 μg MT-2 nuclear extracts on ice for 30 min before addition of labelled ETR-p35 probe. (D) Supershift analysis. MT-2 nuclear extracts (4 μg) was incubated with 1 μg (1–2 μl) of anti-c-Jun, JunB, JunD, CREB, c-Fos, ATF-1, ATF-2, ATF-3 and ATF-4 antibodies, respectively. As a control, 2 μl PBS was incubated in parallel (lane 1). The supershifted band is indicated as Ss.

CArG-like *cis*-acting elements, to form an Elk1–SRF–DNA ternary complex (Latinkic & Lau, 1994; Latinkic *et al.*, 1996). The consensus sequence $CC(A/T)_6GG$, termed the CArG box, is the core sequence in the SRE that is present in the promoters of many IEGs (Johansen & Prywes, 1995). The CArG box also serves as the TRE for transactivation of c-*fos*, *egr-1* and *egr-2* by Tax (Fujii *et al.*, 1992). Deletion of the SRE from the *ETR101* promoter resulted in no significant decrease in promoter activity in Tax transactivation. In our report, specific mutations were also introduced to disrupt both CArG-like elements. Mutations of these two CArG-like elements in the *ETR101* promoter have no effect on Tax transactivation. The results indicated that the SREs in the *ETR101* gene promoter were not responsible for Tax transactivation.

NF-κB defines a family of dimeric transcription factors composed of a combination of NF-κB/Rel family members that are able to mediate Tax transactivation of several cellular genes, including c-*myc* and the IL-2 receptor (Duyao *et al.*, 1992; Leung & Nabel, 1988). Deletion of two putative NF-κB-binding sites (−120/−110 and −214/−200) from the 347 bp fragment (pGL2_ETR-110) resulted in a further 50 % reduction in promoter activity, suggesting

that the NF-κB pathway may contribute to the basal transcription activity of *ETR101*. But this deletion did not change the fold induction of *ETR101* promoter activity by Tax. We performed point mutation analysis to disrupt both putative NF-κB sites separately. Mutations of two putative NF-κB elements has no effect on basal *ETR101* promoter activity in Jurkat cells. It seems that the regulatory elements other than these two putative NF-κB elements in the −310/−110 region may contribute to basal *ETR101* promoter activity. We further analysed the effects of these two mutations on Tax responsiveness. Only the second NF-κB element (−120/−110) has moderate effect on Tax transactivation. Although this NF-κB element can respond to Tax transactivation when cloned into a heterologous E1b basal promoter, analysis using a Tax mutant depleted in NF-κB transduction pathway (M22) has little effect on Tax transactivation of the *ETR101* promoter. This indicated that the NF-κB transduction pathway had very weak or no direct effect on Tax-regulated *ETR101* expression.

Deletion of the 42 bp sequence containing the consensus CRE (TGACGTCA) from the *ETR101* promoter completely abolished its Tax responsiveness, implying the critical element responsive to Tax located to this region. Mutating the consensus CRE sequence results in 60 % reduction in Tax transactivation of the promoter activity. It strongly indicates that the CRE motif was the critical responsive element to Tax in the *ETR101* promoter. This result is further solidified by additional studies with Tax mutant M47, which is defective in the CREB signal transduction pathway. In addition to the consensus CRE sequence, this region contains a 5′ G+C-rich sequence (GGGCGGGG), which is identical to a consensus Sp1-binding motif. But the same region in the mouse *ETR101* promoter has a single base variation (GGGCGTGG) that disrupts the consensus Sp1 site. This sequence variation indicated that this G+C-rich sequence might not function as an Sp1-binding motif in the *ETR101* promoter. A series of site-directed mutagenesis studies of this 35 bp TRE sequence showed that disruption of CRE (m5 and m6) abolished Tax transactivation activity, indicating the responsiveness of this CRE to Tax transactivation. To verify the specific transcription factors mediating the transactivation of *ETR101* by Tax, EMSA was performed with specific antibodies from both AP-1 and CREB/ATP families of proteins. As expected, only anti-CREB antibody supershifted the protein(s) bound to the 35 bp *ETR101* TRE. These results clearly demonstrated that Tax activates the *ETR101* gene promoter via interaction with CREB.

The *ETR101* TRE bears several sequence similarities to three 21 bp HTLV-I LTR TREs. Both structures contain a CRE with 5′ and 3′ G+C-rich sequences in its close vicinity. 5′ GGC sequences were critical for both the *ETR101* 35 bp and the LTR 21 bp TREs, while the 3′ G+C-rich sequences are dispensable to Tax transactivation. It is well recognized that the consensus CRE (TGACGTCA) without specific upstream sequence is not sufficient for Tax responsiveness (Fujisawa *et al.*, 1989). The flanking G+C-rich sequences are critical for the entry of Tax into the complex containing CREB and for the enhanced binding of CREB to the CRE core sequence (Brauweiler *et al.*, 1995; Paca-Uccaralertkun *et al.*, 1994; Yin & Gaynor, 1996). Our EMSA analysis showed that this G+C-rich sequence was functionally separated. The 3′ end of this G+C-rich sequence (GGGG) was engaged in the formation of a specific DNA–protein complex (Com I), although formation of this complex did not depend on Tax expression. It is not known based on our current data which protein is involved in formation of this complex, but Sp1 is not a component, as revealed by competition analysis. From our data, formation of Com I had no effect on Tax-associated formation of Com II via the downstream CRE. The 5′ end of this G+C-rich region (GGC) was not involved in the formation of Com I, but mutation of the GGC sequence (m1) also abolished its response to Tax activation of *ETR101*. Comparing the flanking region of CRE elements between *ETR101* and hCG, this GGC sequence is not present in immediate upstream regions of hCG CRE. This may provide an explanation as to why hCG CRE is not Tax responsive.

We report the finding of transcription activation of the *ETR101* gene by Tax and show a detailed analysis of the TREs in its promoter region. Although the significance of *ETR101* expression in the regulation of cell-cycle progress has not been well investigated, its weak sequence similarity with the c-*jun* oncogene and its expression during G_0/G_1 transition hint to the possible functions in the control of cellular activation and transformation. It is interesting to note that the *ETR101* gene is localized in the same region on chromosome 19 as the human *junB* and *junD* proto-oncogenes (Scott *et al.*, 1994). Translocations involving this region of chromosome 19 have been observed frequently in some cases of acute non-lymphoblastic leukaemia and acute lymphoblastic leukaemia (Mitelman *et al.*, 1990). As documented previously, Tax can induce multiple mesenchymal tumours and leukaemia in transgenic mice (Grossman *et al.*, 1995; Nerenberg *et al.*, 1987) and it can alter the growth properties of rodent fibroblast cells and human T-cells (Nerenberg *et al.*, 1987; Schmitt *et al.*, 1998). Therefore, further analysis of *ETR101* function may provide more information, necessary for better understanding of Tax-induced cellular transformation.

ACKNOWLEDGEMENTS

We are grateful to Warner Greene (San Francisco, CA) for providing Tax-expressing plasmids, Masataka Nakamura (Tokyo, Japan) for providing the JPX-9 and JPX/M cell lines and Yuetsu Tanaka (Kanagawa, Japan) for providing anti-Tax antibodies Lt-4 and TAXY-8. The following reagents were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH): MT-2 from Douglas Richman and C8166-45 from Robert Gallo. L. C. was supported by Arkitekt Holger Hjortenberg og Hustru Dagmar Hjortenbergs Fond. This project is supported by Danish Cancer Society grant 99-215-61 to X. L.

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