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Transactivation of proenkephalin gene by HTLV-1 tax_1 protein in glial cells: involvement of Fos/Jun complex at an AP-1 element in the proenkephalin gene promoter

William Fu[1], Sanjiv R Shah[2,6], Hao Jiang[2,7], Dana C Hilt[2,8], Harish PG Dave[3,4] and Jay B Joshi[1,5]

[1]Laboratories of Molecular Biology/Gene Regulation Research and [3]Molecular Hematology Research, Veterans Administration Medical Center, Washington DC 20422; [2]Department of Neurology, University of Maryland School of Medicine, Baltimore MD 21201; Departments of [4]Hematology/Oncology and [5]Microbiology and Immunology, George Washington University School of Medicine and Health Sciences, Washington DC 20037, USA

The human T-cell lymphotropic virus type 1 (HTLV-1), an etiologic agent for adult T-cell leukemia, is strongly associated with tropical spastic paraparesis, a chronic neurological disease. The HTLV-1 genome encodes a protein, tax_1, an autoregulator of enhanced viral RNA transcription, that also transactivates/represses certain cellular gene promoters. Enkephalins are opioid peptides that function as neurotransmitters and neuroimmunomodulators. We earlier reported that the proenkephalin gene is transactivated by tax_1 protein in glial cells. The nucleotide sequence upstream of −190 base pairs in the proenkephalin gene promoter is necessary for maximal transactivation by tax_1 while the sequence downstream of −190 bp confers modest activation by tax_1. We investigated the cellular transcription factors in tax_1 expressing glial cells that associate with the proenkephalin promoter and herein demonstrate the enhanced interaction and involvement of c-Fos/c-Jun proteins in the complexes formed at the AP-1 site. The HTLV-1 tax_1 expressing stable glial cell lines produced functional tax_1 protein that increased the expression of endogenous proenkephalin gene. The comparative electrophoretic mobility shift and 'supershift' analysis using specific antibodies indicated the enhanced presence of c-Fos and c-Jun proteins in the DNA : protein complex formed at the AP-1 site. The c-Fos protein expression significantly increased in the tax_1 expressing glial cells. The tax_1 induced c-Fos protein levels and the concurrently increased association of c-Fos/c-Jun transcription factors at the AP-1 site imply a strong functional significance in the activation of proenkephalin gene expression in tax_1 expressing glial cells.

Keywords: transcription factors; proenkephalin; HTLV-1 Tax_1; glial; Fos; Jun

Introduction

The human T-cell lymphotropic virus type 1 (HTLV-1) is an etiologic agent of a malignant $CD4^+$ adult T-cell leukemia/lymphoma (ATL/ATLL; Poiesz *et al*, 1980; Yoshida *et al*, 1982) and is strongly implicated as a causative agent of tropical spastic paraparesis (TSP) or HTLV-1 associated myelopathy (HAM), a chronic neurological disorder causing progressive paralysis of the lower limbs (Osame *et al*, 1986). HTLV-1 has been isolated from mononuclear cell cultures of peripheral blood and cerebrospinal fluid (CSF) of TSP/HAM patients (Hirose *et al*, 1986; Jacobson *et al*, 1988). High levels of specific antibodies to the major proteins of HTLV-1 have been detected in the sera and CSF of TSP/HAM patients (Gessain *et al*, 1985; Rodgers-Johnson *et al*, 1988). The HTLV-1 genome encodes a nonstructural 40 kD protein, tax_1, the translational product of a doubly spliced

Correspondence: JB Joshi
[6]Present address: Laboratory of Biochemical Genetics, NHLBI, Bethesda MD 20892; [7]Section on Growth Factors, NICHD, Bethesda MD 20892; [8]Neuroscience Product Development, Amgen Inc., Thousand Oaks CA 91320, USA
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subgenomic mRNA (reviewed in Green and Chen, 1990). Tax_1 strongly stimulates transcription of the viral genome by transactivation of the 5′ long terminal repeat (LTR) promoter (Sodroski *et al*, 1985; Felber *et al*, 1985; Rosen *et al*, 1985; Seiki *et al*, 1986; Paskalis *et al*, 1986). The tax_1 protein also transactivates certain heterologous promoters including IL-2, IL-2 receptor α, vimentin, TGF-β1 and globin genes (Siekevitz *et al*, 1987; Inoue *et al*, 1986; Lilienbaum *et al*, 1990; Kim *et al*, 1990; Fox *et al*, 1989) and transrepresses the β-polymerase gene promoter (Jeang *et al*, 1990). Transgenic mice expressing HTLV-1 LTR driven tax_1 gene develop neurofibromata (Hinrichs *et al*, 1987) as well as Sjögren's syndrome (Green *et al*, 1989), a disease of presumed autoimmune etiology.

Enkephalins are well characterized endogenous opioid peptides (Hughes *et al*, 1975) that function as neurotransmitters in synapses of the central nervous system, as neuromodulators and circulating neurohormones. The proenkephalin gene codes for a precursor polypeptide called preproenkephalin or proenkephalin which is proteolytically cleaved to yield functional enkephalin peptides. The proenkephalin gene is expressed in many tissues and cell types including lymphocytes (Zurawski *et al*, 1986; Rosen *et al*, 1989) and enkephalins have also been implicated as neuroimmunomodulators (Weigent *et al*, 1990). In view of enkephalin's diverse actions, there is considerable interest in understanding the regulation and expression of the proenkephalin gene in normal and pathobiological conditions. We earlier reported that the proenkephalin gene is transactivated by the HTLV-1 tax_1 protein in glial cells (Joshi and Dave, 1992). Promoter deletion analysis indicated that the nucleotide sequence upstream of base pair (bp) -190 with respect to the transcription start site in the proenkephalin gene is necessary for maximal transactivation by tax_1. The nucleotide sequence within the -190 bp region consistently retained the ability to confer the minimal activation by tax_1 in the glial cells. The molecular mechanism(s) involving transacting factors and DNA interaction in the tax_1-mediated proenkephalin gene activation in glial cells is not known. Molecular mechanism of tax_1 action on cellular gene promoters is highly pleiotropic in nature and varies from one gene to another with respect to the *cis-acting* elements required for the activation/repression by the tax_1 protein. The tax_1 protein is believed not to bind directly to DNA, but is presumed to interact with other cellular transcription factors. In this report, using glial cell lines stably expressing HTLV-1 tax_1 protein and electrophoretic mobility shift assays, we demonstrate the specific and significantly increased Fos/Jun protein : DNA interaction at the AP-1 *cis-acting* element in the proenkephalin gene promoter in tax_1 expressing cells.

Results

Establishment and characterization of HTLV-1 tax_1 expressing glial cell lines

In order to investigate the HTLV-1 tax_1-mediated interaction, if any, of cellular transcription factors with the proenkephalin gene promoter sequences in glial cells, we established glial cell cultures stably expressing HTLV-1 tax_1 protein. C6 glioma cells were transfected with pMAXneo plasmid DNA which expresses HTLV-1 tax_1 protein and confers antibiotic neomycin resistance to the transfected cells (Paskalis *et al*, 1986; Ohtani *et al*, 1987). In order to minimize the possible plasmid integration site bias affecting tax_1 expression, several individual neomycin resistant colonies were picked and grown as independent clonal cultures and were termed C6tx-1, C6tx-3 etc., while the rest of the neomycin resistant colonies were pooled and propagated as C6tx-P cells. The functional activity of tax_1 expressed in C6tx cells was tested in transient expression assays by transfection with HTLV-1 LTR CAT DNA which expresses a bacterial chloramphenicol acetyltransferase (CAT) reporter gene under the transcriptional control of HTLV-1 LTR promoter. The same cells were co-transfected with pCMVβgal DNA which expresses a bacterial β-galactosidase reporter gene under the transcriptional control of CMV (Cytomegalovirus) promoter. The specific CAT activity was divided by the specific β-galactosidase activity to obtain CAT values normalized for variations in transfection efficiency. For comparison analysis the basal CAT expression value in the control C6 cells was considered as relative CAT activity 1. The basal CAT activity of plasmid HTLV-1 LTR CAT was higher by 4- to 10-fold in C6tx-P, C6tx-1, C6tx-3 and C6tx-8 cells than in control C6 cells (Figure 1). These results demonstrate that C6tx cells produce functional tax_1 protein that transactivates HTLV-1 LTR promoter.

We also investigated the effect of tax_1 on the endogenous proenkephalin gene in tax_1 expressing cells. Total RNA from these cells was purified and Northern blot hybridizations were performed using a rat proenkephalin cDNA probe (Figure 2a). The same blots were then re-hybridized with housekeeping gene cyclophilin cDNA probe (Figure 2b) to ascertain the integrity, equal loading and transfer of the total RNA and to investigate the specificity, if any, of tax_1 action on the proenkephalin gene. The proenkephalin and cyclophilin mRNA hybridization signals were quantitated and are shown in Figure 2c. The steady-state proenkephalin mRNA levels were 2.3-, 3.5-, 9.1- and 1.5-fold higher in C6tx-P, C6tx-1, C6tx-3 and C6tx-8 cells, respectively, than in control C6 cells. The steady-state levels of cyclophilin mRNA remained constant or varied insignificantly between C6 and C6tx cells reiterating the integrity, equal loading and transfer

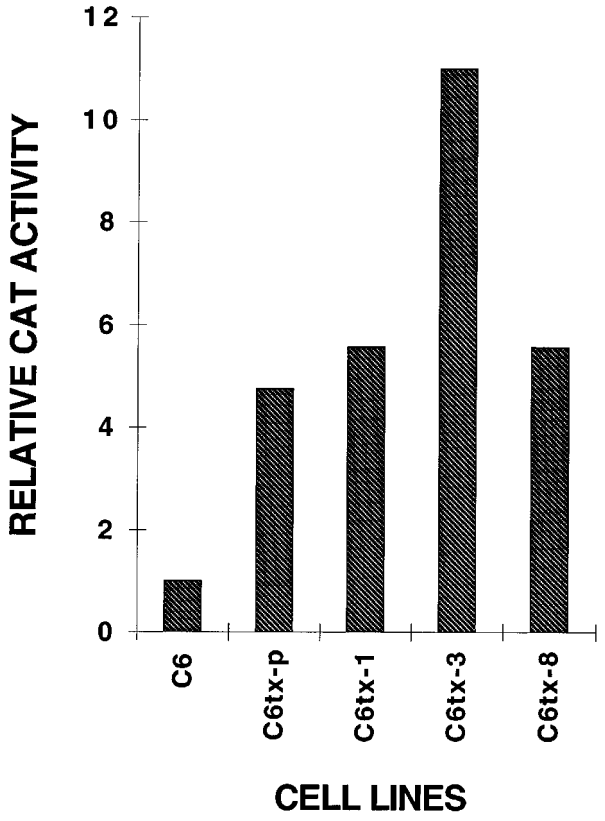

Figure 1 Transiently expressed relative CAT activity of pHTLV-1 LTR CAT plasmid in C6 and C6tx cells. The C6tx cell lines which express tax_1 protein were established by stably transfecting C6 glioma cells with pMAXneo plasmid DNA as described in Materials and methods section. These cells were co-transfected with pHTLV-1 LTR CAT and pCMVβgal DNAs and 24 h later CAT and β-galactosidase activities were assayed. Values represent a mean of three independent experiments carried out using duplicate plates in each transfection and CAT activity in each sample was assayed multiple times. The specific CAT activity was divided by the specific β-galactosidase activity to obtain CAT values normalized for variations in transfection efficiency. For comparison analysis the basal CAT value expressed in the control C6 cells was considered as relative CAT activity 1.

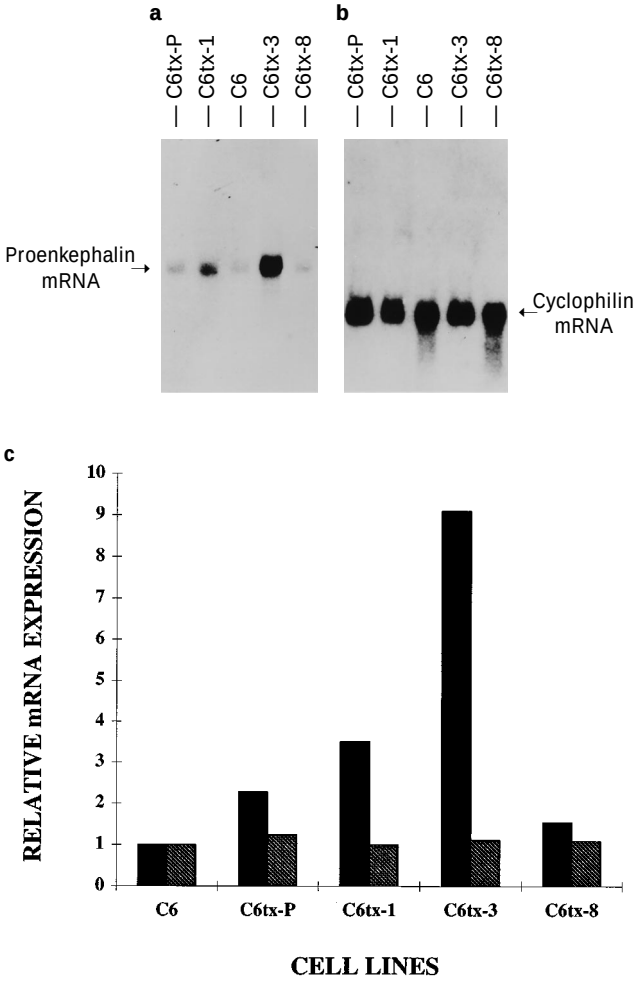

Figure 2 Northern blot analysis of Proenkephalin (panel a) and Cyclophilin (panel b) mRNAs. Total cellular RNA (10 μg) from control (C6) and tax_1-expressing (C6tx) cells in each lane was electrophoresed, blotted and hybridized first with ^{32}P-labeled proenkephalin and rehybridized later with ^{32}P-labeled cyclophilin cDNA probes. Representative autoradiograph shows the hybridization signals for each mRNA indicated by arrows. The hybridized signals were quantitated using AMBIS 4000 Optical Image Analyzer. The proenkephalin (solid bars) and cyclophilin mRNA (hatched bars) levels are expressed relative to the steady-state mRNA levels observed in C6 control cells (Panel c).

of total RNAs in each lane. Moreover, these results suggest the specificity of tax_1 action on proenkephalin gene in glial cells. These results further indicate that tax_1, alone or in concert with cellular transcription factor(s), up-regulates endogenous proenkephalin gene expression in tax_1 expressing C6 glioma cells.

Formation of a specific DNA : protein complex at the AP-1 site

In our earlier report (Joshi and Dave, 1992), we demonstrated that the nucleotide sequence downstream of -190 bp of the proenkephalin promoter consistently retained the ability to minimally transactivate the proenkephalin gene by HTLV-1 tax_1 protein in glial cells. The nucleotide sequences in the promoter are highly conserved between rat and human proenkephalin genes with complete identity in the region encompassing several well-characterized regulatory elements (Joshi and Sabol, 1991; Rosen *et al*, 1984). The proenkephalin promoter contains the ENKCRE-2 element (bp -92 to -86), which is absolutely required for basal transcription of the gene (Comb *et al*, 1988) and harbors an AP-1 transcription factor binding site within its motif. To investigate the role of AP-1 *cis-acting* element in the tax_1-mediated transactivation of proenkephalin gene promoter, we performed electrophoretic mobility shift (EMS) analysis using nuclear extracts prepared from tax_1 expressing glial

cells. From several transfected glial cell lines stably expressing functional HTLV-1 tax_1 protein, established and characterized as described above, we chose the best representative clonal cell line C6tx-3 and pooled clonal cell line C6tx-P for these experiments. The expression of tax_1 in these cells was further confirmed by Western immunoblot analysis using specific anti-tax_1 monoclonal antibody, TAb172 (Duvall *et al*, 1995) (Figure 3). EMS analyses were performed using radiolabeled AP-1 consensus double-stranded oligonucleotide probe and nuclear extracts prepared from control and tax_1 expressing glial cells. A distinct DNA : protein complex formed by interaction of protein(s) with AP-1 motif was observed in nuclear extracts from control C6 as well as from tax_1 expressing C6tx-P and C6tx-3 glial cells (Figure 4, Wt lanes). The DNA : protein complexes formed using nuclear extracts from tax_1 expressing C6 cells were consistently retarded and exhibited slightly slower electrophoretic mobility than the complexes formed using nuclear extracts from control C6 cells. This may be due to the possible presence of an additional protein(s) in tax_1 expressing cells that can directly or indirectly interact and contribute to the DNA : protein complex formation at the AP-1 site. Additional post-translational modifications such as phosphorylation of c-Fos and/or c-Jun proteins to facilitate the transcriptionally enhanced DNA : protein complex formation in tax_1 expressing glial cells, may also influence the electrophoretic mobility of the complex in this analysis.

We next examined the nucleotide sequence specificity of DNA : protein complex formation at the AP-1 site by using a mutant motif in EMS analysis. The mutant oligonucleotide probe was engineered by a 'CA'→'TG' substitution in the AP-1 core motif (entire 'wild-type' nucleotide sequence is stated in Materials and methods). This change completely abolished the DNA : protein complex formation (Figure 4, M lanes) suggesting the requirement of CA nucleotides in the AP-1 motif and also the specificity of AP-1 motif in the DNA : protein complexes formed using C6tx and C6 cell nuclear extracts.

The sequence specificity of DNA : protein complex formation at the AP-1 site was further confirmed by competition experiments using an unlabeled (cold) wild-type AP-1 oligonucleotide probe. In these experiments, varying molar concentrations of excess unlabeled, double-stranded wild-type AP-1 oligonucleotides were added along with the radiolabeled AP-1 oligonucleotide probe to the EMS reactions as 'cold' competitor for the DNA binding motif. The DNA : protein complex forma-

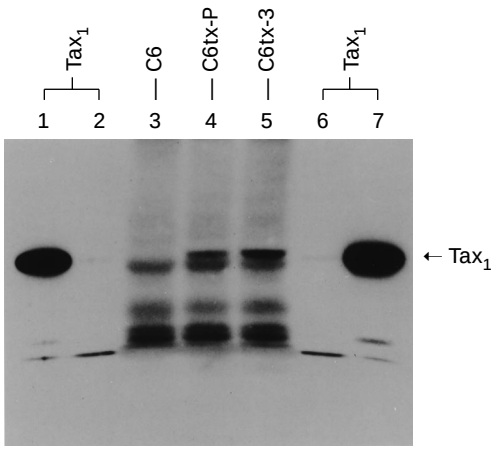

Figure 3 Western immunoblot analysis of HTLV-1 tax_1 protein in tax_1-expressing glial cells. 250 μg of nuclear proteins from indicated cells were loaded in each lane and immunoblot analysis using specific antibody to tax_1 was performed as described in Materials and methods. 10 ng (lanes 2 and 6) and 50 ng (lanes 1 and 7) of highly purified tax_1 protein was used as the positive control and its position is indicated by arrow.

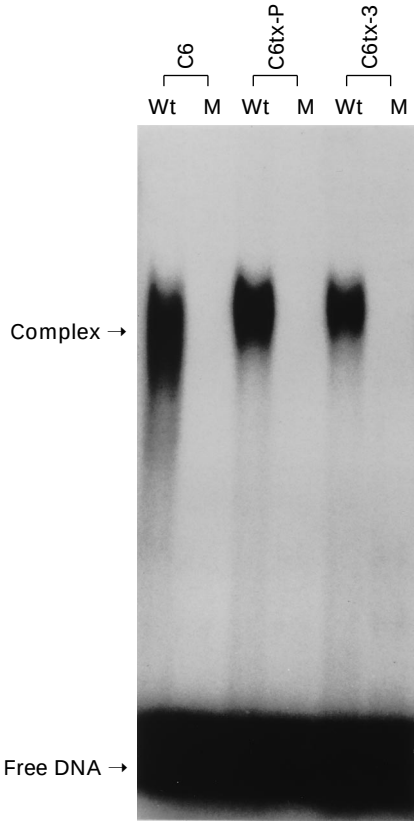

Figure 4 Formation of the DNA/protein complex at the AP-1 site. EMS analysis reactions were performed using 5 μg of nuclear extracts prepared from control C6 and tax_1 expressing C6tx-P and C6tx-3 glial cells and ^{32}P-labeled AP-1 consensus (Wt) or mutant (M) double stranded oligonucleotides probes as described in Materials and methods. The reaction mixtures were subjected to nondenaturing gel electrophoresis and the complexes were detected by autoradiography. DNA : protein complex formed at AP-1 site is indicated by an upper arrow and the unbound free DNA probe is indicated by the lower arrow.

tion at AP-1 site was significantly inhibited (Figure 5, lane 10X) and gradually abolished in the presence of increasing molar concentrations of unlabeled competitor oligonucleotides, (Figure 5, lanes 50X and 100X) further reiterating the nucleotide sequence specificity of the complex formation at the AP-1 site in control C6 and tax_1 expressing C6tx-P and C6tx-3 glial cells.

Presence of c-Fos and c-Jun proteins in the DNA : protein complex at the AP-1 site

It is well established that c-Fos, FosB, Fra1 and Fra2 constitute the family of Fos transcription factors and together with members of the Jun family consisting of the c-Jun, Jun-B and Jun-D form the AP-1 transcription complex (Curran and Vogt, 1992). It is plausible that c-Fos and c-Jun, the most commonly associated proteins in the AP-1 transcription complex, could also be associated with complex formation at the AP-1 site in control C6 and tax_1 expressing C6tx-P and C6tx-3 glial cells. To identify the presence of c-Fos and c-Jun transcription factors in DNA : protein complexes, a specific polyclonal antibody to c-Fos, c-Jun or control pre-immune serum was added to EMS reactions. The electrophoretic mobility of the DNA : protein complex formed at AP-1 site using nuclear extracts from C6, C6tx-P and C6tx-3 cells was further retarded or the 'band' supershifted to the higher position in the gel in the presence of c-Fos (Figure 6) and c-Jun (Figure 7) antibodies, whereas the control pre-immune serum did not affect the migration of the complex and a supershift was not observed. These results indicate the presence of c-Fos and c-Jun proteins in the supershifted bands. The signal intensity of supershifted complexes formed using nuclear extracts from tax_1 expressing C6tx-P and C6tx-3 cells was consistently higher than that observed using the nuclear extracts from control C6 cells suggesting increased binding of c-Fos/c-Jun proteins at the AP-1 site in the tax_1 expressing cells. The supershift EMS analysis using 10-fold diluted nuclear extracts from above mentioned samples also consistently revealed the same differences in the relative intensities of signals (data not shown). The DNA : protein : antibody complexes ('supershifted' complex) formed at the AP-1 site using nuclear extracts from tax_1 expressing C6tx-P and C6tx-3 cells were always found to be modestly retarded and exhibited slightly slower electrophoretic mobility than similar complexes formed using nuclear extracts from control C6 cells. This may be

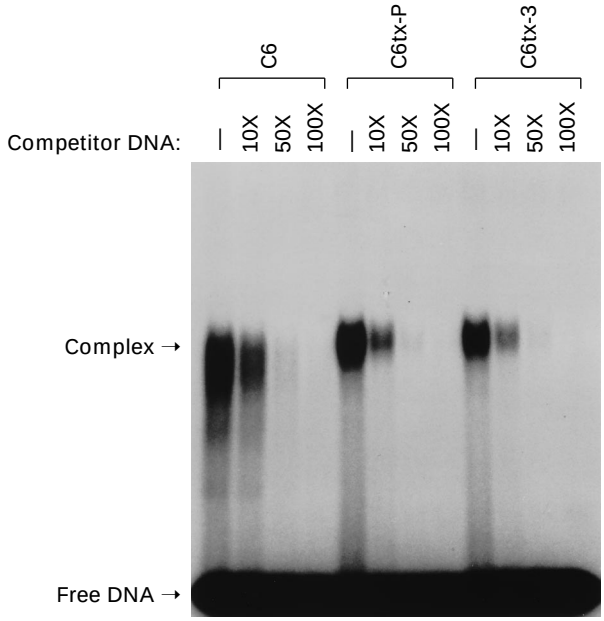

Figure 5 Specificity of the DNA : protein complex formation at the AP-1 site in competition EMS analysis. A 10X, 50X and 100X molar excess of unlabeled AP-1 consensus double stranded oligonucleotides were added as competitors along with ^{32}P-labeled AP-1 oligonucleotides in the reaction mixtures containing 5 μg of nuclear extracts from C6, C6tx-P and C6tx-3 glial cells. The reaction mixtures were subjected to nondenaturing gel electrophoresis and the complexes were detected by autoradiography. DNA : protein complex formed at AP-1 site is indicated by an upper arrow and the unbound free DNA probe is indicated by the lower arrow.

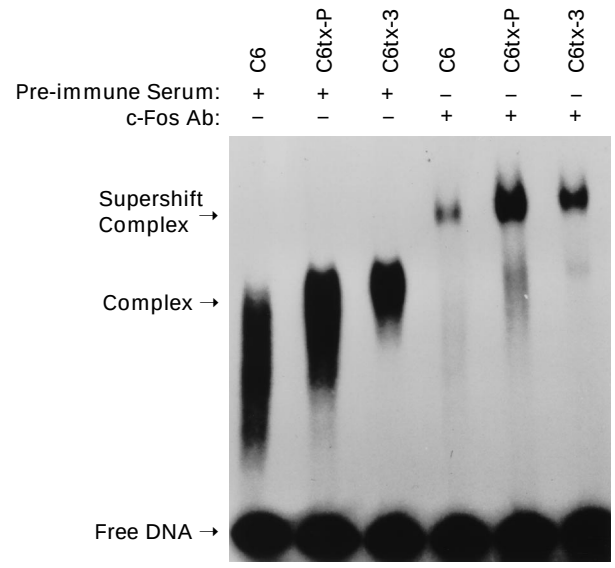

Figure 6 Presence of c-Fos in the protein complex binding to AP-1 motif in EMS supershift analysis. After the DNA : protein complexes were formed by incubating radiolabeled AP-1 consensus oligonucleotides and the nuclear extracts from C6, C6tx-P and C6tx-3, control (pre-immune) serum or specific polyclonal antibodies to the c-Fos protein were added to the mixtures and immune complexes were allowed to form as described in Materials and methods. The DNA : protein and supershifted immune complexes were resolved by electrophoresis and autoradiography. The positions of free DNA, DNA : protein complex and of supershifted DNA : protein : antibody complexes are indicated by arrows.

due to the possible presence of an additional protein(s) in tax_1 expressing cells that contribute to c-Fos/c-Jun proteins : DNA complex formation at the AP-1 site. To test whether tax_1 protein is present in this complex, supershift EMS analysis using specific monoclonal antibody to tax_1 was performed. The anti-tax_1 antibody, monoclonal antibody TAb172, used in these experiments was previously utilized to demonstrate the involvement of tax_1 in the transactivation of HTLV-1 tax_1-responsive-21-base-pair repeats in *in vitro* transactivation assay (Duvall *et al*, 1995). Our experiments did not reveal any supershifted complex (data not shown) indicating the absence of tax_1 protein in the c-Fos/c-Jun proteins : DNA complex formed in the tax_1 expressing cells. However, it is possible that the tax_1 protein may be present in the DNA : protein complex formation but the antibody TAb172 may not recognize the epitope(s) available in tax_1 for efficient antigen-antibody reaction. Taken together, the results of these experiments clearly indicate the increased binding and the involvement of c-Fos and c-Jun proteins in the complexes formed at the AP-1 site in tax_1 expressing cells. Moreover, the results further suggest the possible presence of an additional transcription factor(s), interacting with c-Fos and/or c-Jun proteins at the AP-1 motif in tax_1 expressing glial cells.

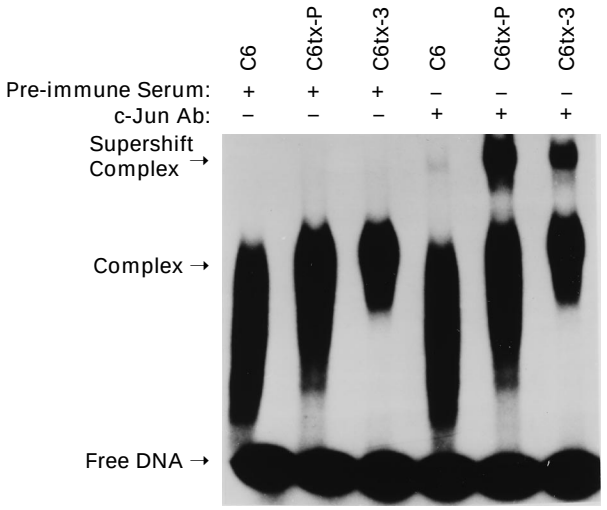

Figure 7 Presence of c-Jun in the protein complex binding to AP-1 motif in EMS supershift analysis. After the DNA : protein complexes were formed by incubating radiolabeled AP-1 consensus oligonucleotides and the nuclear extracts from C6, C6tx-P and C6tx-3, control (pre-immune) serum or specific polyclonal antibodies to the c-Jun protein were added to the mixtures and immune complexes were allowed to form as described in Materials and methods. The DNA : protein and supershifted immune complexes were resolved by electrophoresis and autoradiography. The positions of free DNA, DNA : protein complex and of supershifted DNA : protein : antibody complexes are indicated by arrows. The autoradiograph was deliberately overexposed to detect and document the DNA : protein : antibody complex formation in the control C6 cells.

Expression of c-Fos and c-Jun proteins in the tax_1 expressing glial cells

Our results of the experiments using EMS and 'supershift' assays indicated a quantitative increase of the c-Fos/c-Jun complex formation at the AP-1 site using nuclear extracts from the tax_1 expressing glial cells. To test whether tax_1 affects the levels of constitutively expressed c-Fos and/or c-Jun proteins in glial cells, we compared the c-Fos and c-Jun protein expression in C6tx and control cells using Western immunoblot analysis. The c-Fos protein 'band' was nearly undetectable in control C6 cells while significantly higher c-Fos protein levels were observed in samples from C6tx-P and C6tx-3 cells (Figure 8a). Densitometric quantitation of autora-

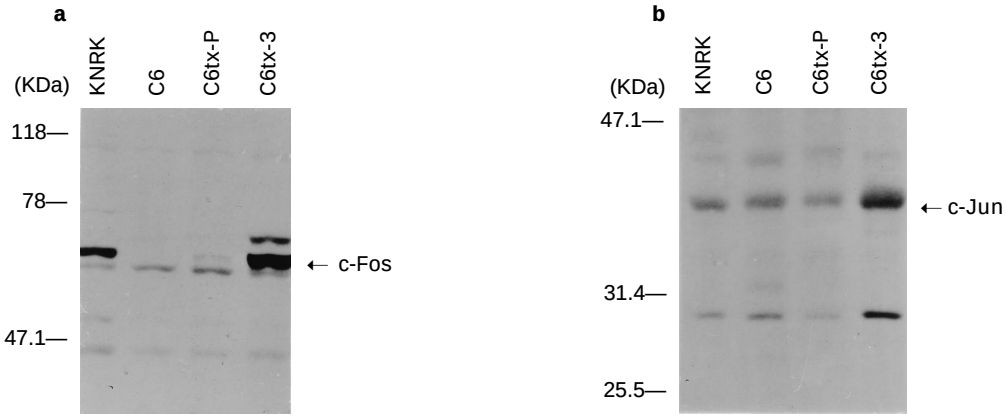

Figure 8 Western immunoblot analysis of c-Fos and c-Jun protein expression in tax_1-expressing glial cells. 75 μg of nuclear proteins from indicated cells were loaded in each lane and immunoblot analysis using specific antibodies to c-Fos (panel a) or c-Jun (panel b) was performed as described in Materials and methods. The nuclear extracts prepared from phorbol ester-treated rat KNRK cells were used as positive controls for detection of c-Fos and c-Jun proteins and the positions of these proteins and of molecular weight protein markers are indicated by arrows.

diographs from at least three independent experiments showed that the c-Fos protein levels were 7- and 50-fold higher in C6tx-P and C6tx-3 cells, respectively, than those observed in the control C6 cells. Contrary to this, the c-Jun protein 'band' was easily detectable in the control cells and the protein levels varied insignificantly (Figure 8b, lane C6tx-P) or were marginally increased by about 2-fold in the tax_1 expressing C6tx-3 cells (Figure 8b). These results suggest that the tax_1 protein significantly induces the c-Fos protein expression in tax_1 expressing glial cells, in part, to facilitate the functionally and quantitatively enhanced c-Fos/c-Jun complex formation at the AP-1 site in the proenkephalin gene promoter.

Discussion

Our previous report showed that the proenkephalin gene is transactivated by the HTLV-1 tax_1 protein in glial cells (Joshi and Dave, 1992) and the nucleotide sequence downstream of -190 bp consistently retained the ability to minimally transactivate the proenkephalin gene promoter. In order to understand the molecular mechanism(s) of the tax_1-mediated activation of the proenkephalin gene, we investigated the cellular transcription factors in tax_1 expressing glial cells that associate with the proenkephalin promoter conferring the minimal transactivation. In this study, we characterize and demonstrate the significantly enhanced interaction and involvement of c-Fos/c-Jun proteins in the complexes formed at the AP-1 site. The HTLV-1 tax_1 expressing stable cell lines used in these experiments produced functional tax_1 protein as evidenced by its ability to transactivate both the HTLV-1 LTR and proenkephalin gene promoters. The tax_1 produced in these cells also specifically transactivated the endogenous proenkephalin gene expression but not the expression of a housekeeping gene, cyclophilin, emphasizing the cogency and the utility of the cell lines in studying tax_1-mediated transregulation of the proenkephalin gene.

The comparative EMS and 'supershift' analysis using specific antibodies and nuclear extracts from normal and tax_1 expressing glial cells clearly indicated the enhanced presence of c-Fos and c-Jun proteins in the DNA : protein complex formed at the AP-1 site (Figures 6 and 7). The complexes formed using nuclear extracts from the tax_1 expressing C6tx cells consistently exhibited modestly retarded electrophoretic mobility compared to similar complexes formed using nuclear extracts from the control C6 cells. This may be due to the possible presence of additional transcription factors associated with the primary complex formed at the AP-1 site. The transacting factors interacting at the AP-1 site predominantly represent the protein products of the Fos and Jun gene families that become active transcription factors by forming Fos/Jun heterodimers or Jun/Jun homodimers at a specific cognate *cis-acting* element (Vogt and Bos, 1990). These AP-1 transcription factors constitute a subfamily within a larger family of bZIP proteins whose members form a variety of dimeric complexes with each other via leucine-zipper domains. For example, c-Fos can heterodimerize with ATF-4, but not with ATF-2 and ATF-3 (Hai and Curran, 1991) and c-Jun can form dimers with ATF-2, ATF-3 and ATF-4, but not with ATF-1 and CREB (Hai and Curran, 1991). c-Ets1 can also functionally associate with the c-Fos/c-Jun primary complex (Logan *et al*, 1996) and recently, the novel bZIP protein B-ATF, from Epstein-Barr virus stimulated human B-cells, was shown to efficiently interact with c-Jun/c-Fos proteins at an AP-1 site (Dorsey *et al*, 1995). Previously ATF-2 and ATFa have been identified as the targets for the adenoviral transactivator protein E1A (van Dam *et al*, 1993; Chatton *et al*, 1993) and HTLV-1 tax_1 (Montagne *et al*, 1990). Our results do not rule out the conceivably specific and/or combinatorial effect of tax_1-influenced posttranslational modification of both pre-existing and newly synthesized transcription factors that may associate with the primary complex formed at the AP-1 site in tax_1 expressing glial cells, contributing to the apparent retarded mobility of the complex. Phosphorylation of serine residues located within the transactivation domain of c-Jun potentiates its ability to activate transcription as either a homodimer or a heterodimer with c-Fos (Deng and Karin, 1994). Additionally, phosphorylation may augment c-Jun transcriptional activity through recruitment of CBP, a protein that binds to another bZIP transcription factor, phospho-CREB, that is activated by protein kinase A (Kwok *et al*, 1994; Arias *et al*, 1994). Likewise, phosphorylation of c-Fos leading to functional enhancement of transcriptional activity is well documented (Abate *et al*, 1991; Karin, 1995).

The c-Fos/c-Jun mediated proenkephalin gene regulation by various physiological modulators in brain is a focus of intensive investigations and it has been adequately demonstrated that the AP-1 transcription factors can functionally up- or down-regulate proenkephalin gene expression (Sonnenberg *et al*, 1989; Kobierski *et al*, 1991). The HTLV-1 tax_1 protein is known to transactivate the c-*fos* promoter (Fujii *et al*, 1988) as well as to enhance the expression of c-*fos* gene (Fujii *et al*, 1991). Our results demonstrate the significantly higher expression of c-Fos protein in the tax_1 expressing glial cells (Figure 8a). Therefore, it is conceivable that the tax_1 induced c-Fos protein levels and the concurrently increased association of c-Fos/c-Jun transcription factors at the AP-1 site imply a strong functional significance in the activation of the proenkephalin gene expression in the tax_1 expressing glial cells.

The action of HTLV-1 tax_1 protein on its own viral LTR promoter and on targeted cellular gene promoters is highly pleiotropic in nature and does not mandate a requirement for a unique consensus *cis-acting* element. Moreover, tax_1 does not bind directly to DNA, but exerts its effect by recruiting cellular transcription factors for its action. The tax_1 protein autoregulates viral RNA transcription by interacting at multiple tax_1 responsive *cis-acting elements* (TREs) in the HTLV-1 LTR promoter (Reviewed in Gitlin *et al*, 1993). Our earlier report demonstrated that nucleotide sequences between −190 bp and −437 bp in the proenkephalin gene promoter were required for maximal transactivation and that the sequences downstream of −190 bp consistently retained a modest level of transactivation by the tax_1 protein (Joshi and Dave, 1992). Hence, it is possible that the AP-1 *cis-acting* element located within the well-characterized ENKCRE-2 (Comb *et al*, 1988) motif (−92 to −86 bp) alone may be sufficient to confer the tax_1-mediated modest transactivation. This proximal element may also further co-operate with the relatively stronger, albeit different, distal element in potentiating the maximal activation of the proenkephalin gene in the glial cells. Recently, Low *et al* (1994) identified a cyclic AMP-responsive AP-1 element (−92 to −86 bp) as the region in the proenkephalin gene responsive to tax_1 in normal, concanavalin A, cyclic AMP and 12-o-tetradecanoylphorbol-13-acetate treated Jurkat T-lymphocytes and showed the involvement of ATF-3 but not of CREB, c-Fos, c-Jun, JunB and JunD in tax_1 dependent activation in murine teratocarcinoma F9 cells. However, earlier Kadison *et al* (1990) had demonstrated that a plasmid containing proenkephalin gene sequences from bp −193 to +70 bp (ENKAT or pENKAT-12) was unresponsive to tax_1 in wild-type S49 thymoma cells.

These differences observed in glial, thymoma, Jurkat T-lymphocytes and F9 teratocarcinoma cells suggest that the molecular mechanism(s) of tax_1-mediated transactivation of proenkephalin gene may be cell-specific in recruiting the cellular transcription factor(s). This, in turn, may dictate the differential use of one or multiple cognate cis-acting element(s) for gene activation/repression in the given cell type. Indeed, recently Wigdahl and his colleagues highlighted such differences between Jurkat T-lymphocytes and glial cells and identified and characterized the glial cell-specific DNA-protein complexes formed with the HTLV-1 LTR 21 bp enhancer element that is required for tax_1-mediated activation of viral RNA transcription (Tillman *et al*, 1994; Wessner *et al*, 1995). The ability of HTLV-1 to engineer selective interplay with diverse cell-type specific transcription factors could be crucial to differentially infecting neural or immune cells, further underscoring the complexities involved in the pathogenesis of HTLV-1 associated disorders.

Materials and methods

Cell culture, plasmid DNAs, transient and stable transfections

C6 rat glioma cells (from American Type Culture Collection, Rockville, MD) were cultured at 37°C in 175 or 75 cm^2 flasks containing 90% DME medium (GIBCO BRL/Life Technologies) supplemented with 20 mM Hepes, pH 7.3 and 10% fetal calf serum (HyClone Laboratories) in humidified, 5% CO_2 incubator. Plasmid pU3RCAT (pHTLV-1 LTR CAT) was the kind gift of Dr K-T Jeang. pCMVβgal plasmid was kindly provided by Dr Rong Fong Shen and the proenkephalin cDNA plasmid pYSEC1 was kindly supplied by Dr SL Sabol. All plasmids used in transfection assays were banded twice by CsCl-ethidium bromide density gradient centrifugation. Transfections were performed essentially as described (Joshi and Dave, 1992) with minor modifications. For transient transfections, the cells were seeded at 3×10^5 cells per 100 mm dish 48 h prior to transfection. $CaPO_4$/DNA precipitates, generally containing 15 μg pHTLV-1 LTR CAT and 5 μg pCMVβgal DNAs were prepared as described (Gorman *et al*, 1982) and applied to the cells and the cells incubated for 18 h in the growth conditions described above. At the end of this incubation, the monolayer cells were washed twice with DMEM, fresh growth medium was added and the cells were harvested 24 h later for chloramphenicol acetyltransferase (CAT) and β-galactosidase enzyme assays. For establishing the stably transfected cell line expressing HTLV-1 tax_1 protein, 1×10^5 C6 glioma cells/100 mm dish were transfected with 5 μg pMAXneo plasmid (Paskalis *et al*, 1986; Ohtani *et al*, 1987) which contains entire tax_1 coding gene (open reading frame IV of the pX region, Sodroski *et al*, 1985) of HTLV-1 isolated from ATL patient and bacterial neomycin (G418) gene cartridge. After 48 h, the cells were transferred to medium supplemented with 725 μg/ml G418 (Geneticin, GIBCO BRL/Life Technologies) and grown for 2 weeks under continuous G418 selection. At the end of 2 weeks, in order to minimize the possible plasmid integration site bias, several individual G418 resistant colonies were picked and grown as independent clonal cultures while the rest of the G418 resistant colonies were pooled and propagated in G418 containing medium.

Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays

Cells were harvested in phosphate-buffered saline and lysed by four cycles of freezing and thawing, centrifuged at $12\,000 \times g$ for 2 min and the supernatants assayed for protein content by the BCA (Pierce Chemicals) reagent. A portion of the lysate was immediately used to determine β-galactosidase activity and the rest of the lysate was heated at 60°C for 10 min to inactivate endogenous deacetylase

activity (Mercola *et al*, 1985), centrifuged at 12 000 × *g* for 2 min and the supernatants were re-assayed for protein content using the BCA (Pierce Chemicals) reagent in 96-well plates. CAT and *β*-galactosidase assays were performed as described previously (Jiang *et al*, 1993). Briefly, the CAT assay reaction mixture included cell extract, 133 *μ*M chloramphenicol (Boehringer Mannheim), 0.1 *μ*Ci [^{3}H]acetyl-CoA(Amersham), 66 *μ*M acetyl-CoA(P-L Biochemicals), and 250 mM Tris.Cl pH 7.8. The reaction mix was incubated at 37°C for 6 h, extracted with ethyl acetate, back-extracted with 250 mM Tris.Cl pH 7.8 buffer in order to remove residual [^{3}H]acetyl-CoA or degraded products, if any, contaminating the ethyl acetate layer, dried and radioactivity was counted in scintillation counter. The *β*-galactosidase activity was assayed in 96-well plates using modification of previously described (Miller, 1972) method. The specific CAT activity (counts/min/h of CAT assay reaction/mg protein) was divided by the specific *β*-galactosidase activity (ODA_{420} nm/h/mg protein) to obtain a relative CAT activity normalized for variations in transfection efficiency.

RNA isolation and Northern blot analyses
Confluent C6 and C6tx cells were lysed and sonicated in 4 M guanidinium thiocyanate solution (Chirgwin *et al*, 1979). Total RNA was purified by centrifugation through a cushion of 5.7 M CsCl and 0.1 M EDTA at 20°C (usually a 2 ml cushion in a Beckman SW55 Ti rotor at 42 000 r.p.m. for 12–14 h), followed by phenol/chloroform extraction and ethanol precipitation. Northern blot analysis of proenkephalin and cyclophilin mRNAs was performed according to standard methods (Sambrook *et al*, 1989) with 6% formaldehyde-1% agarose gels and capillary transfer to Nytran membranes (Schleicher and Schuell, Keene, NH). Blots were hybridized with the ^{32}P-labeled (random primed) 930 bp Sac I-Sma I fragment of the rat proenkephalin cDNA clone pYSEC1 as described (Joshi and Sabol, 1991). Same blots were re-hybridized with random primed ^{32}P-labeled 712 bp EcoRI fragment of the rat cyclophilin (house-keeping gene) cDNA clone pCyp (Lad *et al*, 1991). The autoradiograms were quantitated by densitometry using AMBIS 4000 optical image analysis system.

Oligonucleotides and radiolabeling
AP-1 consensus and mutant double-stranded (ds) oligonucleotides used in electrophoretic mobility shift analysis were purchased from Santa Cruz Biotechnology. The AP-1 core motif is underlined in the following sqeuence: 5′-CGCTTGATGACTCAGCCGGAA-3′. The proenkephalin gene's AP-1 regulatory sequence is functionally indentical which adheres to the AP-1 consensus and binds Fos-Jun heterodimers (Morgan and Curran, 1991). The mutant was engineered by 'CA'→'TG' substitution in the AP-1 core motif. The wild-type and mutant ds oligonucleotides used as probes in electrophoretic mobility shift assays were end-labeled (phosphorylated) with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) using polynucleotide kinase (Boehringer Mannheim) following manufacturer's protocol. The unincorporated [γ-^{32}P]ATPs were removed from the reaction mixture using Sephadex G-25 quick spin columns (Boehringer Mannheim), and the radioactivity counted using a liquid scintillation counter.

Nuclear extracts, electrophoretic mobility shift (EMS) and supershift analysis
Nuclear extracts used in EMS analysis were prepared from rapidly growing sub-confluent cultures of normal C6 and tax_1 expressing stable C6 glial cell lines following previously described procedure (Dignam *et al*, 1983). Protein content in the nuclear extracts was determined using BCA reagent kit (Pierce Chemicals). The typical EMS analysis reaction mixture of 20 *μ*l volume contained 5×10^4 c.p.m. of [^{32}P] labeled ds oligonucleotides, 5 *μ*g nuclear extract, 10 mM Tris.Cl (pH 7.5), 75 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 3.5% (v/v) glycerol, 1 *μ*g of poly(dI : dC) and 100 *μ*g of bovine serum albumin. After incubation for 30 min at room temperature, the mixtures were loaded on a 6% polyacrylamide gel and electrophoresis was performed in a 0.5X Tris-borate (pH 8.0) buffer at 200 V at 4°C (gels were pre-run for 1 h at 200 V). The gels were then dried prior to autoradiography. In the competition experiments, varying concentrations of excess unlabeled (cold) competitor ds oligonucleotides were added along with the radiolabeled analogous oligonucleotide probe to the EMS reactions as a competitor for the binding motif. For supershift analysis, the EMS reaction was performed as described above and 2 *μ*l of specific rabbit polyclonal antiserum to c-Fos and c-Jun (100 *μ*g/0.1 ml, Santa Cruz Biotechnology) or control rabbit pre-immune antiserum (100 *μ*g/0.1 ml) for gel shift assays were added to the reactions. The reactions were incubated overnight at 4°C and complexes resolved by polyacrylamide gel electrophoresis. The nuclear extracts prepared from phorbol-ester treated rat KNRK cells used in the Western immunoblot analysis were purchased from Santa Cruz Biotech.

Western immunoblot analysis
For Fos and Jun protein detection, 75 *μ*g of nuclear extract proteins prepared from control and tax_1 expressing glial cells were suspended in 2 × SDS gel loading buffer containing 100 mM Tris.Cl (pH 6.8), 200 mM dithiothreitol, 4% (w/v) sodium dodecyl sulfate (SDS), 0.2% (w/v) bromophenol blue and 20% (v/v) glycerol. The mixtures were heated for 5 min at 95°C, cooled to room temperature and electrophoresed on a 10% SDS-polyacry-

lamide gel in 1 × Tris-glycine (pH 8.3) buffer at 45 V overnight. The gel was electroblotted to a Hybond ECL nitrocellulose membrane (Amersham) for 4 h at 150 mA using transfer buffer (39 mM glycine, 48 mM Tris-base, 20% methanol and 0.037% SDS, pH 8.3) and the membrane air dried. Non-specific binding sites were blocked by incubating the membrane in TBS-T buffer (20 mM Tris-base, 137 mM NaCl and 0.1% Tween-20, pH 7.6) containing 5% (wv) non-fat dried milk for 1 h at room temperature with gentle shaking. The membrane was then washed with TBS-T once for 15 min and twice for 5 min at room temperature. Specific rabbit polyclonal antibodies for immunoblottings of c-Fos and c-Jun (100 μg/0.1 ml, Santa Cruz Biotechnology) were diluted to 1 : 500 in TBS-T and the membrane was incubated in the diluted primary antibodies for 1 h at room temperature followed by the washing in TBS-T buffer as described above. The membrane was then incubated in 1 : 10 000 dilution of horseradish peroxidase (HRP) conjugated anti-rabbit antibody (200 μg/0.5 ml, Santa Cruz Biotechnology) for 45 min at room temperature and washed in TBS-T buffer as described above. The immunoreactive specific proteins were detected by chemiluminescence reagents (Amersham International) as instructed by supplier and autoradiography. Prestained molecular weight marker proteins were purchased from Bio-Rad Laboratories (Melville, New York).

For the detection of tax_1 protein, 250 μg of nuclear extract proteins prepared from control and tax_1 expressing glial cells and 10 ng and 50 ng of highly purified tax_1 protein (a kind gift of Dr Fatah Kashanchi, NIH) as positive control, were electrophoresed on 8% SDS-polyacrylamide gel and transferred to membrane as described above. Specific mouse anti-tax_1 antibody (a kind gift of Dr Fatah Kashanchi, NIH), immunoglobulin G2A-purified monoclonal antibody TAb172 (Duvall *et al*, 1995) was diluted to 1 : 1000 in TBS-T and the membrane was incubated in the diluted primary antibodies for 1 h at room temperature followed by the washing in TBS-T buffer as described above. The membrane was then incubated in 1 : 10 000 dilution of horseradish peroxidase (HRP) conjugated anti-mouse IgG antibody (1 mg/ml, Promega Corporation) for 45 min at room temperature and washed in TBS-T buffer as described above. The immunoreactive specific proteins were detected by chemiluminescence reagents (Amersham International) as instructed by supplier and autoradiography.

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References

Abate C, Marshak DR, Curran T (1991). Fos is phosphorylated by $p34^{cdc2}$, cAMP-dependent protein kinase and protein kinase C at multiple sites clustered within regulatory regions. *Oncogene* **6:** 2179–2185.

Arias J, Alberts AS, Brindle P, Claret FX, Smeal T, Karin M, Feramisco J, Montminy M (1994). Activation of c-AMP and mitogen responsive genes relies on a common nuclear factor. *Nature* **370:** 226–229.

Chatton B, Bocco JL, Gaire M, Hauss C, Reimund B, Goetz J, Kedinger C (1993). Transcriptional activation by the adenovirus larger E1a product is mediated by members of the cellular transcription factor ATF family which can directly associate with E1a. *Mol Cell Biol* **13:** 561–570.

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979). Isolation of biologically active RNA from sources enriched in ribonuclease. *Biochemistry* **18:** 5294–5299.

Comb M, Mermod N, Hyman SE, Pearlberg J, Ross ME, Goodman HM (1988). Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. *EMBO J* **7:** 3793–3805.

Curran T, Vogt PK (1992). Dangerous liaison: Fos and Jun, oncogenic transcription factors. In: *Transcriptional regulation*. McKnight SL, Yamamoto KR (eds). Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, pp 797–831.

Deng T, Karin M (1994). c-Fos transcriptional activity stimulated by H-ras-activated protein kinase distinct from JNK and ERK. *Nature* **371:** 171–175.

Dignam JD, Lebovitz RM, Roeder RG (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acid Res* **11:** 1475–1489.

Dorsey MJ, Tae HJ, Sollenberger KG, Mascarenhas NT, Johansen LM, Taparowsky EJ (1995). B-ATF: a novel human bZIP protein that associates with members of the AP-1 transcription factor family. *Oncogene* **11:** 2255–2265.

Duvall JF, Kashanchi F, Cvekl A, Radonovich MF, Piras G, Brady JN (1995). Transactivation of the human T-cell lymphotropic virus type 1 tax_1-responsive 21-base-pair repeats requires holo-TFIID and TFIIA. *J Virol* **69:** 5077–5086.

Felber BK, Paskalis H, Kleinman-Ewing C, Wong-Staal F, Pavlakis GN (1985). The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. *Science* **229**: 675–679.

Fox HB, Gutman PL, Dave HPG, Cao SX, Mittleman M, Berg PE, Schechter AN (1989). Transactivation of human globin genes by HTLV-1 tax1. *Blood* **74:** 2749–2754.

Fujii M, Sassone-Corsi P, Verma IM (1988). *c-fos* promoter trans-activation by the tax_1 protein of human T-cell leukemia virus type I. *Proc Natl Acad Sci USA* **85:** 8526–8530.

Fujii M, Niki T, Mori T, Matsuda T, Matsui M, Nomura N, Seiki M (1991). HTLV-1 Tax induces expression of various immediate early serum responsive genes. *Oncogene* **6:** 1023–1029.

Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de-The G (1985). Antibodies of human T lymphotropic virus type I in patients with tropical spastic paraparesis. *Lancet* **ii:** 407–410.

Gitlin SD, Dittmer J, Reid RL, Brady J (1993). The molecular biology of human T-cell leukemia viruses. In: '*Frontiers in molecular biology*', Brian Collen (Ed). Oxford Press: pp 159–192.

Gorman CM, Moffat LF, Howard BH (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2:** 1044–1051.

Green JE, Hinrichs SH, Vogel J, Jay G (1989). Exocrinopathy resembling Sjögren's syndrome in HTLV-1 *tax* transgenic mice. *Nature* **341:** 72–74.

Green PL, Chen ISY (1990). Regulation of human T-cell leukemia virus expression. *FASEB J* **4:** 169.

Hai T, Curran T (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc Natl Acad Sci USA* **88:** 3720–3724.

Hinrichs SH, Nerenberg M, Reynolds RK, Khoury G, Jay G (1987). A transgenic mouse model for human neurofibromatosis. *Science* **237:** 1340–1343.

Hirose S, Uemura Y, Fujishita M, Kitagawa T, Yamashita M, Imamura J, Ohtsuki Y, Taguchi H, Miyoshi I. (1986). Isolation of HTLV-1 from cerebrospinal fluid of a patient with myelopathy. *Lancet* **ii:** 397–398.

Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, Morris HR (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature*, **258:** 577–579.

Inoue J, Seiki M, Taniguchi T, Tsuru S, Yoshida M (1986). Induction of interleukin 2 receptor gene expression by $p40^x$ encoded by human T-cell leukemia virus type 1. *EMBO J* **5:** 2883–2888.

Jacobson S, Raine CS, Mingioli ES, McFarlin DE (1988). Isolation of an HTLV-I like retrovirus from patients with tropical spastic paraparesis (TSP) by activation with antibodies to the CD4 complex. *Nature* **331:** 540–543.

Jeang KT, Widen SG, Semmes OJ, Wilson SH (1990). HTLV-1 trans-activator protein, Tax, is a trans-repressor of the human β-polymerase gene. *Science* **247:** 1082–1084.

Jiang H, Shah S, Hilt DC (1993). Organization, sequence and expression of the murine S100β gene. Transcriptional regulation by cell type-specific *cis*-acting regulatory elements. *J Biol Chem* **268:** 20502–20511.

Joshi JB, Sabol SL (1991). Proenkephalin gene expression in C6 rat glioma cells: potentiation of cAMP-dependent transcription by glucocorticoids. *Mol Endocrinol* **5:** 1069–1080.

Joshi JB, Dave HPG (1992). Transactivation of the proenkephalin gene promoter by the tax_1 protein of human T-cell lymphotropic virus type I. *Proc Natl Acad Sci USA* **89:** 1006–1010.

Kadison P, Poteat HT, Klein KM, Faller DV (1990). Role of protein kinase A in *tax* transactivation of the human T-cell leukemia virus type I long terminal repeat. *J Virol* **64:** 2141–2148.

Karin M (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* **270:** 16483–16486.

Kim S, Kehrl JH, Burton J, Tendler CL, Jeang K, Danielpour D, Thevenin C, Kim KY, Sporn MB, Roberts AB (1990). Transactivation of the transforming growth factor β1(TGF-β) gene by human T lymphotropic virus type I Tax: a potential mechanism for the increased production of TGF-β1 in adult T cell leukemia. *J Exp Med* **172:** 121–129.

Kobierski L, Chu HM, Tan Y, Comb M (1991). Cyclic AMP dependent regulation of proenkephalin by JunD and JunB: Positive and negative effects of AP-1 proteins. *Proc Natl Acad USA* **88:** 10222–10226.

Kwok RP, Lundblad JR, Chrivia JC, Richards JP, Bachinger HP, Brennan RG, Roberts SG, Green MR, Goodman RH (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* **370:** 223–226.

Lad RP, Smith MA, Hilt DC (1991). Molecular cloning and regional distribution of rat brain cyclophilin. *Mol Brain Res* **9:** 239–244.

Lilienbaum A, Duc Dodon M, Alexandre C, Gazzolo L, Paulin D (1990). Effect of human T-cell leukemia virus type I tax protein on activation of the human vimentin gene. *J Virol* **64:** 256–263.

Logan SK, Garabedian MJ, Campbell CE, Werb Z (1996). Synergistic transcriptional activation of the tissue inhibitor of metalloproteinases-1 promoter via functional interaction of AP-1 and Ets-1 transcription factors. *J Biol Chem* **271:** 774–782.

Low KG, Chu HM, Schwartz PM, Daniels GM, Melner MH, Comb MJ (1994). Novel interactions between human T-cell leukemia virus type I Tax and activating transcription factor 3 at a cyclic AMP-responsive element. *Mol Cell Biol* **14:** 4958–4974.

Mercola M, Goverman J, Calame K (1985). Immunoglobulin heavy-chain enhancer requires one or more tissue-specific factors. *Science* **227:** 266–270.

Miller JH (1972). Assay of β-galactosidase. In: *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 352–355.

Montagne J, Beraud C, Crenon I, Lombard-Plater G, Gazzolo L, Sergent A, Jalinot P (1990). Tax1 induction of the HTLV-1 21 bp enhancer requires cooperation between two cellular DNA-binding proteins. *EMBO J* **9:** 957–964.

Morgan JI, Curran T (1991). Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*.. *Annu Rev Neurosci* **14:** 421–451.

Ohtani K, Nakamura M, Saito S, Noda T, Ito Y, Sugamura K, Hinuma Y (1987). Identification of two distinct elements in the long terminal repeat of HTLV-I responsible for maximum gene expression. *EMBO J* **6:** 389–395.

Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M (1986). HTLV-I associated myelopathy: a new clinical entity. *Lancet* **i:** 1031–1032.

Paskalis H, Felber BK, Pavlakis GN (1986). Cis-acting sequences responsible for the transcriptional activation of human T-cell leukemia virus type I constitute a conditional enhancer. *Proc Natl Acad Sci USA* **83:** 6558–6562.

Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* **77:** 7415–7419.

Rodgers-Johnson P, Morgan OSC, Mora C, Sarin P, Ceroni M, Piccardo P, Garruto RM, Gibbs CJ Jr., Gajdusek DC (1988). The role of HTLV-1 in tropical spastic paraparesis in Jamaica. *Ann Neurol* **23** (Suppl): s121–s126.

Rosen CA, Sodroski JG, Haseltine WA (1985). Location of cis-acting regulatory sequences in the human T-cell leukemia virus type I long terminal repeat. *Proc Natl Acad Sci USA* **82:** 6502–6506.

Rosen H, Douglass J, Herbert E (1984). Isolation and characterization of the rat proenkephalin gene. *J Biol Chem* **259,** 14309–14313.

Rosen H, Behar O, Abramsky O, Ovadia H (1989). Regulated expression of proenkephalin A in normal lymphocytes. *J Immunol* **143:** 3703–3707.

Sambrook J, Fritsch EF, Maniatis T (1989). Extraction, Purification and Analysis of mRNA from Eukaryotic Cells. In: *Molecular Cloning, A laboratory manual* (2nd edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York pp 7.10–7.87.

Seiki M, Inoue J, Takeda T, Yoshida M (1986). Direct evidence that p40 X of human T-cell leukemia virus type 1 is a transacting transcriptional activator. *EMBO J* **5:** 561–565.

Siekevitz M, Feinberg MB, Holbrook N, Yodoi J, Wong-Staal F, Green WC (1987). Activation of interleukin-2 and interleukin-2 receptor (Tac) promoter expression by the trans-activator (tat) gene product of human T-cell leukemia virus, type I. *Proc Natl Acad Sci USA* **84:** 5389–5393.

Sodroski JG, Rosen CA, Goh WC, Haseltine WA (1985). A transcriptional activator protein encoded by the X-lor region of the human T-cell leukemia virus. *Science* **228:** 1430–1434.

Sonnenberg JL, Rauscher FJ, Morgan JI, Curran T (1989). Regulation of proenkephalin by Fos and Jun. *Science* **246:** 1622–1625.

Tillman M, Wessner R, Wigdahl B (1994). Identification of human T-cell lymphotropic virus type I 21-base pair repeat-specific and glial cell-specific DNA-protein complexes. *J Virol* **68:** 4597–4608.

van Dam H, Duyndam M, Rottier R, Bosch A, de Vries-Smits L, Herrlich P, Zantena A, Angel P, van der Eb AJ (1993). Heterodimer formation of c-Jun and ATF-2 is responsible for induction of c-Jun by the 243 amino acid adenovirus E1A protein. *EMBO J* **12:** 479–487.

Vogt PK, Bos TJ (1990). Jun: oncogene and transcription factor. *Adv Cancer Res* **55:** 1–35.

Weigent DA, Carr DJJ, Blalock JE (1990). Bidirectional communication between the neuroendocrine and immune systems. *Ann NY Acad Sci* **579:** 17–27.

Wessner R, Tillmann-Bogush M, Wigdahl B (1995). Characterization of a glial cell-specific DNA-protein complex formed with the human T cell lymphotropic virus type I (HTLV-1) enhancer. *J NeuroVirol* **1:** 62–77.

Yoshida M, Miyoshi I, Hinuma Y (1982). Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc Natl Acad Sci USA* **79:** 2031–2035.

Zurawski G, Benedik M, Kamb BJ, Abrams JS, Zurawski SM, Lee FD (1986). Activation of mouse T-helper cells induces abundant preproenkephalin mRNA synthesis. *Science* **232:** 772–775.