Utilization of Signal Transduction Pathway by the Human T-Cell Leukemia Virus Type I Transcriptional Activator *tax*

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The human T-cell leukemia virus type I (HTLV-I) *trans*-activator (*tax*)-inducible enhancer was localized to three copies of 21-base-pair repeats within the long terminal repeat. Interestingly, the TGACG motif found in the center of the 21-base-pair *tax*-responsive element (TRE) is also present in the cyclic AMP (cAMP)-responsive elements (CREs) and activating transcription factor (ATF)-binding sites. In this study, we demonstrate that the three TRE-binding proteins, TREB-1, TREB-2, and TREB-3, also bind to various CREs and ATF-binding sites and that the TREs can confer upon a heterologous promoter responsiveness to various inducing agents, including *tax*, cAMP, and E1a. Furthermore, the transcriptional activation of the HTLV-I promoter by *tax* can be inhibited by several protein kinase inhibitors, including sangivamycin. Our results indicate that the TREs, CREs, and ATF-binding sites are similar *cis*-acting elements and further suggest (i) that the transcriptional activation of the HTLV-I promoter by *tax* involves the action of a protein kinase and (ii) that induction by *tax*, cAMP, and E1a might be mediated by distinct factors or kinases.

Human T-cell leukemia virus type I (HTLV-I) has been identified as the etiological agent of adult T-cell leukemia/lymphoma (reviewed in references 54 and 56) and appears to be involved in certain chronic neurological diseases (23, 41, 42, 53). HTLV-I encodes a 40-kilodalton (kDa) nuclear oncoprotein (38), *tax1*, that acts in *trans* to increase the rate of transcription from the HTLV-I promoter in many different cell types (14, 47, 50, 51). The HTLV-I long terminal repeat (LTR) contains three copies of a 21-base-pair (bp) repeat (hereafter called *tax*-responsive element or TRE). These elements are involved both in basal-level expression and in *tax*-mediated induction and are sufficient to confer *tax*-inducibility upon heterologous promoters (7, 40, 43, 48). *tax* was also shown to activate several other cellular and viral genes whose *tax*-responsive regions do not share sequence homology with the HTLV-I TRE (4, 30, 34, 35, 44, 49). This finding indicates the lack of a specific target sequence element that is required exclusively for *tax* action. Recent results indicate that *tax* does not interact directly with TRE, but rather that host-cell proteins mediate *tax* action (1, 39, 52).

Many genes that are transcriptionally regulated by cyclic AMP (cAMP) contain a conserved sequence in the 5' flanking region that is identical or similar to the palindromic octamer sequence TGACGTCA (37). This sequence has been termed the cAMP-responsive element (CRE) (37). A 43-kDa nuclear protein (CREB) that binds to the CRE in the rat somatostatin promoter has been purified from rat cells (36, 55). Another 43- to 47-kDa protein, the activating transcription factor (ATF; 17, 29), binds to a similar sequence (TGACGT motif) that is found in many adenovirus early promoters. The ATF-binding site, in addition to the TATA element, also appears to be involved in E1a-mediated *trans* activation (28, 45; reviewed in references 5 and 25). Several laboratories have suggested that CREs and ATF-binding sites are similar *cis*-acting elements and that ATF and CREB are highly related or identical (12, 19, 31, 32, 45). The TGACG motif found in the CREs and in ATF-binding

sites is also present in the center of the 21-bp TRE (Fig. 1). Recently, we have identified and either partially or completely purified three nuclear proteins that interact with the 21-bp TREs within the HTLV-I LTR (52). Interestingly, the molecular size (35 to 43 kDa) of the TRE-binding protein-1, TREB-1 (52), is similar to those of CREB (38 to 43 kDa; 11, 36, 55) and ATF (43 and 47 kDa; 17, 22, 24). The possibility that TREB-1, CREB, and ATF may be identical or similar factors is intriguing.

In this study, we demonstrate that the TRE-binding proteins, TREB-1, TREB-2, and TREB-3, also bind effectively to both CREs and ATF-binding sites and that the TREs can confer upon heterologous promoters responsiveness to *tax*, cAMP, and E1a. Furthermore, the transcriptional activation of the HTLV-I promoter by *tax* can be inhibited by several protein kinase inhibitors, including sangivamycin. Our results indicate that the TREs, CREs, and ATF-binding sites are similar *cis*-acting elements and further suggest (i) that the transcriptional activation of the HTLV-I promoter by *tax* involves the action of a protein kinase and (ii) that induction by *tax*, cAMP, and E1a might be mediated by distinct factors or kinases.

MATERIALS AND METHODS

Plasmids. The plasmids pT₃CAT and pT₃CAT were described previously (52). The plasmids pHTLV₁CAT (named pU3R-I in reference 50) and pFOSCAT (names pFC700 in reference 15) contain the HTLV-I LTR (positions -354 to +263) and the *c-fos* promoter (positions -700 to +42) linked to the chloramphenicol acetyltransferase (CAT) reporter gene, respectively. pHTLV₁CAT and the HTLV-I *tax* expression plasmid, pHTax₁ (named pHTat₁ in reference 43), were obtained from C. Rosen (Roche Institute of Molecular Biology, Nutley, N.J.). The E1a expression plasmid pE1a was obtained from J. Logan and T. E. Shenk (Princeton University, Princeton, N.J.).

Cells, DNA transfection, and CAT assay. CV-1 cells and NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. Clone 33 Jurkat cells, C8166 HTLV-I-transformed T cells (obtained from R. Gallo and F. Wong-Staal, National Institutes of

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		Positions
HTLV-I/TRE	A G G C C C T G A C G T C T C C C C T T G	
Fibronectin/CRE	C C C C C G T G A C G T C A C C C G G G A	-179 to -159
Somatostatin/CRE	T C T C T C T G A C G T C A G C C A A G G	-35 to -55
cFos/CRE	A G C C C G T G A C G T T T A C A C T C A	-68 to -48
E3/ATF	A C C C T G T G A C G A A A G C C G C C C	-48 to -68
E4/ATF	A A A A A T G A C G T A A C G G T T A A	-39 to -59

FIG. 1. Sequence homology among the TREs, CREs, and the ATF-binding sites.

Health, Bethesda, Md.), and K562 erythroleukemia cells (obtained from D.-E. Young, The Rockefeller University, New York, N.Y.) were grown in RPMI medium supplemented with 10% fetal bovine serum plus $4 \times 10^{-5}\%$ beta-mercaptoethanol. DNA transfection, using a modified DEAE-dextran method, and CAT assays were performed as described previously (52).

Gel retardation assay and oligonucleotides. The gel retardation assays were performed as described previously (52). The TRE oligonucleotides TRE (previously named WT), tre (previously named MT-4), TTT, and ttt were as described previously (52).

CRE_{fn} is a 36-bp monomeric CRE oligonucleotide derived from the fibronectin promoter (positions -187 to -152):

5'-CCCACAGTCCCCGTGACGTACCCGGGAGCCGGGGATCC-3'
3'-CCTAGGGGGTGTCAGGGGGCACTGCAGTGGGCCCTCGGGCCC-5'

CC_{FN} is a 38-bp dimerized fibronectin CRE oligonucleotide:

5'-GTGACGTACCCGGGATCCCCCGTGACGTACCCGGGATCCCC-3'
3'-AGGGGGCACTGCAGTGGGCCCTAGGGGGCACTGCAGTGGGCCCT-5'

cc_{fn} is a 32-bp dimerized fibronectin mutant CRE oligonucleotide:

5'-GTGTACCCGGGATCCCCCGTGACCCGGGATCCCC-3'
3'-AGGGGGCACTGGGGCCCTAGGGGGCACTGGGGCCCT-5'

CRE_{som} is a 32-bp monomeric CRE oligonucleotide derived from the rat somatostatin promoter (positions -60 to -29):

5'-CGCCTCCTTGGCTGACGTACAGAGAGAGTTGGATCC-3'
3'-CCTAGGGGGAGGAACCACTGCAGTCTCTCTCAAA-5'

CRE_{fos} is a 24-bp monomeric CRE oligonucleotide derived from the *c-fos* promoter (positions -71 to -48):

5'-GATCTTGAGCCCGTGACGTTTACACTCA-3'
3'-AACTCGGGCACTGCAAAATGTGAGTCTAG-5'

ATF_{E3} is a 26-bp monomeric ATF oligonucleotide derived from the adenovirus type 5 (Ad5) E3 promoter (positions -69 to -44):

5'-GATCCGGGCGGCTTTCGTACAGGGTGCGG-3'
3'-GCCCGCGAAAGCAGTGTCCACGCCCTAG-5'

ATF_{E4} is a 28-bp monomeric ATF oligonucleotide derived from the Ad5 E4 promoter (positions -61 to -35):

5'-GATCCTTTAACCCTTACGTCATTTTTAGTCG-3'
3'-GAAATTGGCAATGCAGTAAAAATCAGCCTAG-5'

AA_{E4} is a 47-bp dimerized Ad5 E4 ATF oligonucleotide:

5'-GATCCTTTAACCCTTACGTCATTTTTAACCCTTACGTCATTTTTAGTCG-3'
3'-GAAATTGGCAATGCAGTAAAAATGGCAATGCAGTAAAAATCAGCCTAG-5'

cAMP induction and protein kinase inhibitor experiments. For cAMP induction experiments, 1 μ M 8-bromoadenosine-3':5'-cyclic monophosphate (8-Br-cAMP) (Sigma) plus 0.5 μ M 3-isobutyl-1-methyl-xanthine (IMX; Sigma) or 25 μ M forskolin (Sigma) plus 0.5 μ M IMX was added to the medium at 20 h posttransfection for 20 h. For kinase inhibition experiments, protein kinase inhibitors were added to the medium during both 8-h DNA transfection and 48-h posttransfection periods. H-7 and HA1004 were purchased from Seikagaku American Inc.; sphingosine was purchased from Sigma Inc.; sangivamycin (65346-L/15) was obtained from M. Suffness (National Institutes of Health, Bethesda, Md.).

RESULTS

TRE-binding proteins also bind effectively to CREs and to ATF-binding sites. We have previously identified three distinct TRE-binding proteins, named TREB-1, TREB-2, and TREB-3, that formed specific protein-DNA complexes (B1, B2, and B3, respectively) with the TRE oligonucleotides (52). Competition experiments were performed to examine whether CREs and ATF-binding sites are recognized by the same factors that bind to the HTLV-I TRE. These experiments examined the ability of the various synthetic oligonucleotides containing monomeric and multimeric CREs or ATF-binding sites to inhibit formation of the protein-DNA complexes (B1, B2, and B3) detected in gel retardation assay. A gel retardation assay using a 0.5 M KCl step-eluted phosphocellulose fraction is shown in Fig. 2A. TREB-1 and TREB-2 in this fraction (52) formed the specific complexes B1 and B2, respectively, with a radiolabeled monomeric TRE oligonucleotide probe (lane 1). The HTLV-I TRE (TRE; positions -254 to -230), the fibronectin CRE (CRE_{fn}; positions -187 to -152), the somatostatin CRE (CRE_{som}; positions -60 to -29), the *c-fos* CRE (CRE_{fos}; positions -71 to -48), and the Ad5 E3 (ATF_{E3}; positions -69 to -44) and E4 (ATF_{E4}; positions -61 to -35) ATF-binding sites effectively inhibited both TREB-1 and TREB-2 binding to the TRE oligonucleotide probe (lanes 2 through 4 and 8 through 22), while a mutant TRE oligonucleotide (tre) did not (lanes 5 through 7). The relative TREB-1 affinities for the various binding sites were as follows: CRE_{fn} > CRE_{som}, TRE > ATF_{E3}, ATF_{E4}, and CRE_{fos} (Fig. 2A). Under the conditions tested, all sites except CRE_{fos} (Fig. 2A, lanes 14 through 16) showed similar TREB-2 binding affinities (Fig. 2A). This result demonstrates that TREB-1 and TREB-2 also bind to various CREs and ATF-binding sites.

A separate gel retardation experiment using the 0.1 M phosphocellulose flow-through fraction is shown in Fig. 2B. The TREB-3 in this fraction (52) formed a specific complex (B3) with the radiolabeled triple TRE oligonucleotide probe (Fig. 2B, lane 1). We have shown previously that TREB-3 bound more avidly to a multimerized TRE than to a single-copy TRE (52). Interestingly, as found for the triple TRE oligonucleotide (52), dimerized fibronectin CRE and dimerized E4 ATF-binding sites also competed effectively for the TREB-3 binding (Fig. 2B, lanes 2 through 4, 8 through 10, and 14 through 16), while monomeric fibronectin or somatostatin CRE showed less efficient competition (data not shown). The trimerized mutant TRE and the dimerized fibronectin mutant CRE did not compete with TREB-3 binding (Fig. 2B, lanes 5 through 7 and 11 through 13). It is interesting to note that TREB-3 showed a higher affinity for the monomeric fibronectin CRE than for the monomeric TRE (data not shown). This may be due to the existence of two copies of the TGACG motif (in inverted orientation) in

FIG. 2. Competition experiments of the TRE-binding proteins. (A) The binding activities of TREB-1 (complex B1) and TREB-2 (complex B2) were tested by a gel retardation assay using a radiolabeled monomeric TRE oligonucleotide probe. The DNA competitors used in the competition experiments were: TRE, a 25-bp wild-type TRE oligonucleotide; tre, a mutant TRE oligonucleotide; CRE_{fn}, a fibronectin CRE oligonucleotide (positions -187 to -152); CRE_{som}, a rat somatostatin CRE oligonucleotide (positions -60 to -29); CRE_{fos}, a *c-fos* CRE oligonucleotide (positions -71 to -48); ATF_{E3}, an oligonucleotide containing the Ad5 E3 ATF-binding site (positions -69 to -44); and ATF_{E4}, an oligonucleotide containing the Ad5 E4 ATF-binding site (positions -61 to -35). (B) The binding activity of TREB-3 (complex B3) was tested by a gel retardation assay using a radiolabeled triple TRE oligonucleotide as the DNA probe. The DNA competitors were: TTT, trimerized wild-type TRE oligonucleotide; ttT, trimerized mutant TRE oligonucleotide; CC_{FN}, dimerized wild-type fibronectin CRE oligonucleotide; cc_{fn}, dimerized mutant CRE oligonucleotide; and AA_{E4}, dimerized wild-type Ad5 E4 ATF-binding site.

the fibronectin CRE. This and the above results demonstrate that the TRE-binding proteins, TREB-1, TREB-2, and TREB-3, also bind effectively to both CREs and ATF-binding sites and support the hypothesis that TREs, CREs, and ATF-binding sites are functionally similar, if not identical, *cis*-acting elements.

***tax*-inducible HTLV-I promoter is inducible by cAMP and E1a; and cAMP-inducible *c-fos* promoter is inducible by *tax* and E1a.** If one assumes that the TREs, CREs, and ATF-binding sites are recognized by common factors (TREB-1/CREB/ATF, TREB-2, and TREB-3), then any of the TREs, CREs, or ATF-binding sites should be responsive to all three different signals, including *tax*, cAMP, and E1a. Experiments were performed to examine the possible effect of E1a and cAMP on a *tax*-inducible HTLV-I promoter. The HTLV-I promoter (positions -354 to +263) containing three TREs was fused to a reporter gene encoding the bacterial enzyme CAT. This construct, pHTLV₁CAT, was cotransfected with a *tax* expression vector (pHtax₁), an E1a expression vector (pE1a), or with salmon sperm DNA as a negative control. The plasmids were cotransfected into CV-1 cells, and the induction of CAT expression was examined by enzymatic CAT assay at 48 h posttransfection (see Materials and Methods). The result of the CAT assay is shown in Fig. 3. Consistent with the results obtained by Chen et al. (8), pHTLV₁CAT expression was induced strongly by the pHtax₁ plasmid (Fig. 3, lane 2) and weakly by the pE1a plasmid (lane 3). The cAMP inducibility of pHTLV₁CAT was examined by treating pHTLV₁CAT-transfected CV-1 cells with either the cAMP analog 8-Br-cAMP in the presence of the phosphodiesterase inhibitor, IMX, or the adenylate cyclase activator, forskolin, plus IMX. As shown in Fig. 3, an increase in CAT activity was observed when the cells were

treated with either 8-Br-cAMP plus IMX or forskolin plus IMX (lanes 4 and 5). This demonstrates that HTLV-I promoter activity is inducible by the second messenger, cAMP.

The cAMP-inducible *c-fos* promoter was examined for *tax* or E1a responsiveness. The cAMP inducibility of the *c-fos*

FIG. 3. CAT assays of the induction of HTLV-I and *c-fos* promoter expression by various inducing agents. CV-1 cells were transfected with 4 μ g of either the HTLV-I promoter-CAT construct, pHTLV₁CAT (lanes 1 through 5), or the *c-fos* promoter-CAT construct, pFOSCAT (lanes 6 through 10). The inducers were: 4 μ g of pHtax₁ (lanes 2 and 7), 4 μ g of pE1a (lanes 3 and 8), 25 μ M forskolin plus 0.5 μ M IMX (lanes 4 and 9), 1 μ M 8-Br-cAMP plus 0.5 μ M IMX (lanes 5 and 10), or 4 μ g of salmon sperm DNA as control (lanes 1 and 6).

FIG. 4. CAT assays of pT₃CAT expression by various inducing agents. (A) Schematic representation of the pT₃CAT and pt₃CAT constructs. pT₃CAT (or pt₃CAT) plasmid contains trimerized wild-type (or mutant) TRE oligonucleotide cloned 5' to the enhancerless SV40 early promoter linking to the reporter CAT gene. (B) CAT assay of the inducible expression of pT₃CAT by *tax*. NIH 3T3 cells were cotransfected with 4 μ g of the *tax*-expressing plasmid, pHtax₁, plus 4 μ g of either pT₃CAT or pt₃CAT plasmid. (C) CAT assay of the inducible expression of pT₃CAT by cAMP and E1a. K562 cells were transfected with 10 μ g of either pT₃CAT or pt₃CAT plasmid. The inducers used were as described in the legend to Fig. 3.

promoter using the pFOSCAT plasmid was confirmed (15) and is shown in Fig. 3 (lanes 9 and 10). Similarly, CV-1 cells were cotransfected with pFOSCAT plus pHtax₁, pFOSCAT plus pE1a, or pFOSCAT plus salmon sperm DNA, and CAT assays were performed 48 h later. Indeed, pFOSCAT expression was induced when cotransfected with either *tax* (Fig. 3, lane 7) or E1a (lane 8). This result demonstrates that the cAMP-inducible *c-fos* promoter is also inducible by *tax* and E1a. Similar results were also reported recently by others (16, 46).

HTLV-I TRE is responsive to multiple inducing agents. To definitively prove that the TRE element alone is sufficient to confer upon heterologous promoters responsiveness to *tax*, E1a, or cAMP, the plasmids shown in Fig. 4A were constructed. pT₃CAT was constructed by inserting a synthetic triple TRE oligonucleotide, TTT, into a pSVCAT plasmid which lacks an enhancer element but contains an intact simian virus 40 (SV40) early promoter region (GC-rich 21-bp repeats, the TATA element, and the transcriptional initiation sites). A mutant version of pT₃CAT, the pt₃CAT, was constructed by using a synthetic mutant triple TRE oligonucleotide, tt₃, with a 3-bp deletion from each of the three TREs. The inducibility of the wild-type (pT₃CAT) and mutant (pt₃CAT) plasmids was examined by cotransfecting them with the pHtax₁ plasmid into NIH 3T3 cells. Indeed, pT₃CAT, but not pt₃CAT, expression was induced in the presence of *tax* (Fig. 4B, lanes 1 and 2). The *tax* inducibility of pT₃CAT was also observed in CV-1, K562, Jurkat, and

C8166 cells (data not shown). This result demonstrates that the synthetic triple TRE oligonucleotide is sufficient to confer *tax* inducibility to a heterologous promoter and that the 3-bp deletion mutation disrupting the TGacGTCT consensus disrupts not only the formation of the three sequence-specific protein-DNA complexes (B1, B2, and B3) in vitro (Fig. 2A and B; 52), but also the expression of the promoter in vivo. Inducibility of pT₃CAT expression by E1a was tested by cotransfecting K562 cells with the wild-type (pT₃CAT) or mutant (pt₃CAT) plasmid plus the pE1a plasmid. Inducibility of pT₃CAT expression by cAMP was tested by treating the pT₃CAT- or pt₃CAT-transfected K562 cells with the cAMP analog or inducer as described above. As shown in Fig. 4C, 8-Br-cAMP plus IMX or forskolin plus IMX activated CAT expression from the wild-type pT₃CAT construct (lanes 1 and 3) but not from the mutant pt₃CAT construct (lanes 2 and 4). Similarly, E1a induced significantly pT₃CAT expression (lane 5) but much less efficiently pt₃CAT expression (lane 6). These results demonstrate that a triple TRE oligonucleotide is sufficient to confer upon a heterologous SV40 promoter responsiveness to *tax*, cAMP, and E1a.

***tax*-mediated transcriptional activation is inhibited by protein kinase inhibitors, including sangivamycin.** The responsiveness of the HTLV-I TRE to both the cAMP analog (8-Br-cAMP) and an adenylate cyclase activator (forskolin) suggested that *tax* may act through a protein kinase which in turn activates the TRE-binding proteins. We therefore tested the effect of several protein kinase inhibitors on the *trans* activation of the HTLV-I promoter by *tax*. CV-1 cells were cotransfected with the reporter plasmid pHTLV₁CAT plus the *tax* expression plasmid pHtax₁ in the presence of increasing amounts of the protein kinase inhibitor sangivamycin

FIG. 5. Inhibition of *tax*-mediated activation by the protein kinase inhibitor sangivamycin. CV-1 cells were cotransfected with pHTLV₁CAT plus pHtax₁ (left) or with pRSVCAT (right) plasmid in the presence of increasing amounts of the protein kinase inhibitor sangivamycin.

(Fig. 5 left; 33). Interestingly, *tax*-induced CAT activity was reduced significantly in the presence of 1×10^{-5} or 2×10^{-5} M sangivamycin (Fig. 5 left; data not shown). The observed inhibitory effect of the protein kinase inhibitor was not due to a direct inhibition of CAT enzymatic activity because as high as 5×10^{-5} M sangivamycin did not inhibit the constitutive CAT expression from the Rous sarcoma virus LTR (Fig. 5 right). Similar inhibitory effects were observed, albeit with higher effective concentrations, by two additional protein kinase inhibitors, H-7 (2×10^{-5} to 4×10^{-5} M) and sphingosine (6×10^{-5} to 8×10^{-5} M) (data not shown). On the other hand, the protein kinase A inhibitor HA1004 did not inhibit *tax* action (data not shown). These data suggest, but do not prove, that a protein kinase is involved in *tax*-mediated activation.

DISCUSSION

The HTLV-I transcriptional activator *tax* activates its own promoter through three copies of 21-bp TREs (7, 47, 50, 51). Interestingly, the TGACG motif found in the CREs and in the ATF-binding sites is also present in the center of the 21-bp TREs. CREs and ATF-binding sites can confer upon heterologous and cognate promoters responsiveness to cAMP (37) and E1a (28, 45; reviewed in references 5 and 25), respectively. In this report, we have studied (i) the functional similarity among the TREs, CREs, and ATF-binding sites and their interacting factors and (ii) the possible utilization of signal transduction pathway(s) by *tax*.

We have demonstrated that the TRE-binding proteins, TREB-1, TREB-2, and TREB-3, also bind to the CREs and ATF-binding sites. Recently, several laboratories have suggested that CREs and ATF-binding sites are similar elements, with a TGACG motif, and that ATF and CRE-binding protein (CREB) are the same (12, 19, 31, 32, 45). Our past and present work demonstrates (i) that the molecular size of TREB-1 (35

to 43 kDa; 52) is similar to those of ATF (43 and 47 kDa; 17, 22, 24) and CREB (38 to 43 kDa; 36, 55), (ii) that TREB-1 binds to both the CREs and ATF-binding sites (Fig. 2A; 52), and (iii) that the affinity-purified TREB-1 can interact with the TATA-binding factor (TFIID) on the Ad5 E4 promoter in a fashion similar to that reported for ATF (52). This result further supports the notion that TREB-1, ATF, and CREB are one and the same. It may be noted, however, that TREB-1 was purified by using an affinity matrix (HTLV-I promoter sequence) distinct from that (E4 or somatostatin promoter sequences) employed for ATF (17) or CREB (55). We have also shown previously that TREB-1 stimulates transcription from the HTLV-I promoter, indicating the functional relevance of this factor and its promoter interactions (52).

We have shown here that TREB-2 and TREB-3 also bind to both the CREs and ATF-binding sites (Fig. 2). In addition to the TREB-1/CREB/ATF factor, several laboratories have reported other factors that bind to the CRE (21, 27) and another 65- to 72-kDa protein (termed EivF) that binds to the ATF-binding site (10). It is interesting to note that the CREB binding to CRE is not cAMP inducible, but another CRE-binding factor which showed a cAMP-inducible binding property has been identified (27). It is possible that TREB-2 and TREB-3 are identical to some of these recently identified factors.

It has been demonstrated that the HTLV-I promoter can be activated by E1a in addition to *tax* (8). In this study we further demonstrate that the HTLV-I promoter is also inducible by cAMP and that the cAMP-inducible *c-fos* promoter can also be *trans* activated by *tax* and E1a. We have demonstrated that three different inducers, including *tax*, cAMP, and E1a, can activate promoters via the HTLV-I TREs. Sassone-Corsi has also shown recently that the adenovirus E2a ATF-binding site is responsive to either E1a or cAMP (45). Together with the results that TREs, CREs, and ATF-binding sites share a significant sequence homology and that the same factors, TREB-1, TREB-2, and TREB-3, bind to these three elements (Fig. 2), these observations indicate that the TREs, CREs, and ATF-binding sites are highly related *cis*-acting elements. Several other laboratories have also suggested that both CREs and ATF-binding sites are similar, and that their interacting factors (CREB and ATF) are related if not identical (12, 19, 31, 32, 45).

After examining more closely the sequences between TREs of the HTLV-I LTR and CREs of the somatostatin and fibronectin gene promoters, a striking and extensive sequence homology emerged: as shown in Fig. 1, 12 of 14 bp (86%) of the core sequences between 21-bp TREs and CREs are the same. After examining the sequences among the known TREs, CREs, and ATF-binding sites from various genes, the consensus appeared to be the 14-bp sequence -(C/A)(T/G)(C/G)TGACGT(C/A)(T/A)(C/G)YY-, while the absolutely invariable sequences appeared to be the sequence -TGACG- (Table 1).

In principle, *tax*, cAMP, and E1a could all act in the same signal transduction pathway, which in turn impinges directly or indirectly upon a single (common) transcription factor; one or both of the other factors could mediate the basal-level expression or possibly other induction pathways. Alternatively, *tax*, cAMP, and E1a could act in different signal transduction pathways, each of which then impinges specifically upon a distinct TRE-binding factor. It appears that the relative extent of induction by the three inducers (*tax*, cAMP, and E1a) varied in different cell lines (Fig. 3, 4B, and 4C; data not shown). In addition, the HTLV-I TRE was *tax* or E1a responsive in every cell line tested, but cAMP

TABLE 1. Compilation of TRE-CRE-ATF sequences

Promoter ^a	Sequence														Agreement	Position
HTLV-I LTR	C	G	T	T	G	A	C	G	A	C	A	A	C	C	11	-100 to -87
	C	C	C	T	G	A	C	G	T	G	T	C	C	C	12	-199 to -186
	C	T	C	T	G	A	C	G	T	C	T	C	C	C	14	-248 to -235
HTLV-II LTR	C	T	C	T	G	A	C	G	A	T	T	A	C	C	11	-86 to -73
	C	C	C	T	G	A	C	G	T	C	C	C	T	C	12	-179 to -166
	C	T	C	T	G	A	C	G	T	C	T	C	C	C	14	-219 to -206
Bovine leukemia virus LTR	T	G	C	T	G	A	C	G	G	C	A	G	C	T	12	-128 to -115
	A	G	C	T	G	A	C	G	T	C	T	C	T	G	13	-150 to -163
Somatostatin	C	T	C	T	G	A	C	G	T	C	A	G	C	C	14	-38 to -51
Fibronectin	C	C	G	T	G	A	C	G	T	C	A	C	C	C	13	-176 to -163
<i>c-fos</i>	C	C	G	T	G	A	C	G	T	T	T	A	C	A	10	-69 to -52
	T	G	C	T	G	A	C	G	C	A	G	A	T	G	9	-300 to -287
Enkephalin	A	G	C	T	G	A	C	G	C	A	G	G	C	C	12	-83 to -96
hCG	C	C	A	T	G	A	C	G	T	C	A	A	T	T	11	-114 to -127
	C	C	A	T	G	A	C	G	T	C	A	A	T	T	11	-132 to -145
TH	C	T	T	T	G	A	C	G	T	C	A	G	C	C	13	-36 to -49
PEPCK	C	T	C	T	G	A	C	G	T	A	A	G	G	G	12	-80 to -93
VIP	C	T	G	T	G	A	C	G	T	C	T	T	T	C	13	-67 to -80
E1a	T	T	G	T	G	A	C	G	T	G	G	C	G	C	10	-438 to -425
	G	G	G	T	G	A	C	G	T	A	G	T	A	G	9	-406 to -393
	A	A	G	T	G	A	C	G	T	T	T	T	T	G	10	-332 to -319
E2a	A	G	A	T	G	A	C	G	T	A	G	T	T	T	11	-77 to -64
E3	C	T	G	T	G	A	C	G	A	A	A	G	C	C	13	-52 to -65
E4	A	A	A	T	G	A	C	G	T	A	A	C	G	G	10	-41 to -54
	A	A	G	T	G	A	C	G	A	T	T	T	G	A	8	-147 to -134
	A	A	G	T	G	A	C	G	T	A	A	C	G	T	12	-171 to -158
	G	G	G	T	G	A	C	G	T	A	G	G	T	T	12	-237 to -224
	T	T	G	T	G	A	C	G	T	G	G	C	G	C	10	-269 to -256
Frequency of consensus (base[s])	22	18	22	28	28	28	28	28	21	21	20	17	21	20		
Summary																
A	7	4	4	0	0	28	0	0	4	9	11	6	1	2		
G	2	8	11	0	28	0	0	28	1	3	7	7	6	6		
C	15	6	11	0	0	0	28	0	2	12	1	10	12	14		
T	4	10	2	28	0	0	0	0	21	4	9	5	9	6		
TRE-CRE-ATF consensus																
	C	T	C							C	T	C				
	A	G	G	T	G	A	C	G	T						Y	Y
										A	A	G				

^a hCG, Human chorionic gonadotropin; TH, tyrosine hydroxylase; PEPCK, phosphoenolpyruvate carboxykinase; VIP, human vasoactive intestinal polypeptide.

inducibility of the HTLV-I TRE was not observed in several cell lines, including Jurkat, CV-1, and NIH 3T3 cells (data not shown). On the other hand, in a mutant cell line which lacks the cAMP-dependent protein kinase II, E1a is still able to activate adenovirus E2a and E3 promoters (45). These observations do not rule out either of the two possible mechanisms mentioned above, but they do favor the notion that *tax* (E1a) and cAMP do not necessarily act directly upon the same factor. It is interesting to note that the affinity-purified transcription factor AP-1 also bound to the HTLV-I TRE, albeit its binding to the TRE was not easily detectable in nuclear extract (data not shown). Nevertheless, it is still possible that AP-1, which is involved in the phorbol ester induction pathway (3, 6), may also mediate *tax* (or E1a) action. Finally, the flanking sequences, which can be different from element to element, may confer individuality on the response (e.g., by binding different neighboring factors). This study does not define the protein-DNA complexes which contribute to the activation mechanism. However, it

is valuable in defining what may be a group of related but distinct mechanisms. More study is needed in order to delineate the exact role of each of the three identified factors (TREB-1/CREB/ATF, TREB-2, and TREB-3), and possibly the AP-1/*jun* family of proteins (3, 6), in the three different signal transduction pathways.

The fact that the HTLV-I TRE was also induced by cAMP and that *tax*-mediated activation was inhibited by the protein kinase inhibitor sangivamycin (Fig. 5 left) suggests that a protein kinase is involved in *tax*-mediated activation. There is the caveat that the drug (sangivamycin) employed here may have other unreported specific or nonspecific effect(s) (e.g., an effect on the posttranslational modification of the functional *tax* protein) that could lead to the observed results. However, the observed inhibitory effect did not reflect a direct inhibition of CAT enzymatic activity by sangivamycin because it did not inhibit constitutive CAT expression from the Rous sarcoma virus LTR (Fig. 5 right). These results indicate that the observed sangivamycin-me-

diated inhibition of the *tax*-inducible pHTLV₁CAT expression is not caused by an effect on CAT mRNA transport-processing and CAT enzymatic activity or by a toxic effect on cell viability.

Interestingly, sangivamycin is known as a potent protein kinase C inhibitor (33). Indeed, similar inhibitory effects on *tax* action were observed by two additional protein kinase C inhibitors, H-7 (data not shown; 13, 20, 26) and sphingosine (data not shown; 13, 18). Yamamoto et al. (55) suggested that CREB binds to the CRE and stimulates transcription as a dimer and that phosphorylation by protein kinase C promotes dimerization of CREB. In addition, the CRE within the proenkephalin gene promoter can be induced by either cAMP or the phorbol ester, 12-tetradecanoylphorbol-13-acetate (9). The human cDNA encoding CREB was isolated recently by Hoeffler et al. (21). The deduced protein sequence revealed a putative DNA-binding domain which is structurally similar to the corresponding domain in the phorbol ester-responsive AP-1/*jun* protein (3, 6). Finally, the *tax*-responsive regions within other *tax*-inducible promoters (interleukin-2 receptor, granulocyte-macrophage colony-stimulating factor, human immunodeficiency virus type 1, and SV40) which do not share sequence homology with the HTLV-I TRE were also phorbol ester responsive (4, 35, 44, 49). These results suggest the possibility that protein kinase C is involved in *tax* action and that the HTLV-I TREs may also be phorbol ester inducible. However, it is still possible that another nuclear protein kinase (e.g., casein kinase II), rather than protein kinase C or A, mediates *tax* action. Whatever the nature of the responsible kinase, which could also be an intrinsic kinase activity associated with *tax*, it could modify and activate either (i) the *tax* protein itself or (ii) the TRE-binding factor(s) that in turn mediates *tax* action. More studies, using either specific kinase-deficient cell lines or dominant mutants of cloned protein kinase genes, are required for a positive identification of the putative protein kinase involved in *tax* action.

This study provides clear evidence that multiple TRE-binding factors (TREB-1, TREB-2, and TREB-3) interact with CREs and ATF-binding sites, as well as with TREs, and raises questions about the presumed role of CREB (ATF) in mediating the cAMP or E1a induction pathway (29, 36). The fact that the HTLV-I TRE is also E1a responsive suggests that the modes of action of two distinct virus-encoded *trans* activators (*tax* and E1a) may be similar and that adenovirus could potentially act synergistically with HTLV-I in viral pathogenesis.

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