MOLECULAR AND CELLULAR BIOLOGY, May 1996, p. 2174–2182 Vol. 16, No. 5
0270-7306/96/$04.00+0

Genetic Characterization of Transactivation of the Human T-Cell Leukemia Virus Type 1 Promoter: Binding of Tax to Tax-Responsive Element 1 Is Mediated by the Cyclic AMP-Responsive Members of the CREB/ATF Family of Transcription Factors

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Received 20 November 1995/Returned for modification 12 January 1996/Accepted 23 February 1996

To achieve a better understanding of the mechanism of transactivation by Tax of human T-cell leukemia virus type 1 Tax-responsive element 1 (TRE-1), we developed a genetic approach with *Saccharomyces cerevisiae*. We constructed a yeast reporter strain containing the *lacZ* gene under the control of the *CYC1* promoter associated with three copies of TRE-1. Expression of either the cyclic AMP response element-binding protein (CREB) or CREB fused to the GAL4 activation domain (GAD) in this strain did not modify the expression of the reporter gene. Tax alone was also inactive. However, expression of the reporter gene was induced by coexpression of Tax and CREB. This effect was stronger with the GAD-CREB fusion protein. Analysis of different CREB mutants with this genetic system indicated that the C-terminal 92 amino acid residues, which include the basic domain and the leucine zipper, are necessary and sufficient to mediate transactivation by Tax. To identify cellular proteins binding to TRE-1 in a Tax-dependent manner, this strain was also used to screen a library of human cDNAs fused to GAD. Of five positive clones isolated from 0.75×10^6 yeast colonies, four were members of the CREB/activating transcription factor (ATF) family: CREB, two isoforms of the cyclic AMP-responsive element modulator (CREM), and ATF-1. Interestingly, these three proteins can be phosphorylated by protein kinase A and thus form a particular subgroup within the CREB/ATF family. Expression of ATF-2 in *S. cerevisiae* did not activate TRE-1 in the presence of Tax. This shows that in a eukaryotic nucleus, Tax specifically interacts with the basic domain-leucine zipper region of ATF-1, CREB, and CREM. The fifth clone identified in this screening corresponded to the Ku autoantigen p70 subunit. When fused to GAD, the C-terminal region of Ku was able to activate transcription via TRE-1 but this activation was not dependent on Tax.

The Tax protein encoded by human T-cell leukemia virus type 1 (HTLV-1) stimulates transcription of the integrated provirus (19, 25, 48). This viral protein also modifies the expression of several cellular genes. In particular, in the promoter of the c-*fos* gene, Tax activates several enhancer sequences, including the serum response element (SRE) motif (3, 22). Activation of this latter sequence element results from binding of Tax to $p67^{srf}$ (23, 55). Several genes coding for cytokines are activated via κB sites, and Tax is a potent inducer of the nuclear translocation of the NF-κB/Rel factors (5, 35, 38, 49). Repression by Tax of transcription of the gene coding for β-polymerase is mediated via a binding site for basic domain-helix-loop-helix factors by a poorly understood mechanism (36, 56). From these observations, it is clear that Tax can interfere with different cellular regulatory pathways, making it a very interesting model of a transcriptional regulator.

In the case of the HTLV-1 provirus, Tax induces the enhancer activity of an imperfect 21-bp repeat. Following the nomenclature suggested by Marriott et al. (40), this sequence will hereafter be called Tax-responsive element 1 (TRE-1). This sequence element can be divided into three perfectly conserved domains named A, B, and C (26, 44). The central B domain (5′-TGACG-3′) corresponds to the consensus sequence for the cyclic AMP response element-binding protein/activating transcription factor (CREB/ATF) family. In vitro, Tax can increase binding to DNAs of various basic domain-leucine zipper (B-ZIP) factors by facilitating their dimerization (21, 57). It has also been shown that Tax binds to HTLV-1 TRE-1 in a sequence-specific manner via cellular factors (6). The cellular B-ZIP factor CREB permits such binding of Tax to TRE-1 (54, 61). Interestingly, it has been shown by selection amplification experiments that the sequence specificity of the Tax-CREB complex is greater than that of CREB alone, indicating that the sequences flanking the CREB binding site are important for establishment of the ternary complex (45). Careful quantitative studies have determined that Tax accelerates both dimerization of CREB and binding of the CREB dimer to DNA (4).

Despite what has been demonstrated in vitro, it has remained unclear whether transactivation in vivo depends on binding of Tax to TRE-1 or merely results from increased binding of CREB/ATF factors to this sequence element. The precise nature of the members of the CREB/ATF family that allows transactivation of TRE-1 by Tax also remains to be precisely established. Since Tax induces the activity of TRE-1 in a wide variety of mammalian cell lines, in vivo studies are difficult to interpret because of the active endogenous factors. To circumvent this problem, an experimental system was developed with *Saccharomyces cerevisiae*. TRE-1, either wild type or mutated, was cloned upstream of a yeast minimal promoter fused to the β-galactosidase reporter gene. These constructs were introduced into the yeast genome by homologous recombination. In this context, TRE-1 did not stimulate expression of the reporter gene, indicating that no active yeast factors bind to this sequence. Under these conditions, either Tax or CREB expressed in *trans* was unable, by itself, to activate TRE-1. By

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contrast, their coexpression in the strain bearing wild-type TRE-1 led to marked activation of the β-galactosidase gene. This experimental approach was used both to delineate the domains within both Tax and CREB that allow assembly of the ternary complex in vivo and to screen a library of human cDNAs for factors that allow association of Tax with TRE-1. The results of these experiments show that Tax interacts with TRE-1 through a region overlapping the B-ZIP domain of the factors CREB, the cyclic AMP-responsive element modulator (CREM), and ATF-1. ATF-2, another member of the same family of transcription factors, lacked the capacity to cooperate with Tax. These experiments also indicate that the Ku autoantigen p70 protein binds to TRE-1.

MATERIALS AND METHODS

Plasmids. Plasmid pRSX0RLacZ contains a minimal *CYC1* promoter fused to *lacZ*. This plasmid integrates a reporter gene into the yeast genome by homologous recombination at the *HIS3* locus. This construct was made by inserting an *Sca*I restriction fragment of pLGΔ312-178 (31) between the *Eco*RV and *Sma*I restriction sites of pRS303 (50). Plasmids pRSABC3RLacZ and pRSMBM3RLacZ were obtained by inserting three copies of a double-stranded oligonucleotide corresponding to the most proximal 21-bp repeat of the HTLV-1 promoter, either wild type (ABC) or mutated in domains A and C (MBM), into the *Xho*I restriction site of pRSX0RLacZ (44). The three repeats were in the same orientation as in the HTLV-1 promoter with respect to the *CYC1* minimal promoter. Plasmid pLG-ABC3LacZ (2μm *URA3*) was obtained by inserting the ABC3 motif into the *Xho*I restriction site of pLGΔ312-178 (31).

Yeast vectors expressing Tax and CREB and bearing either the *URA3* or *LEU2* selection marker were constructed as follows. The first series of vectors were derivatives of pMD1 (*CEN6 LEU2*). This vector (kindly provided by R. Bernards through the courtesy of M. Billaud) is similar to pPC62 (15) but contains a different polylinker. The Tax cDNA, as a *Hin*dIII-*Bst*Y1 restriction fragment of pSGT (6), was inserted between the *Hin*dIII and *Bgl*II restriction sites of pMD1 to give pMD1-Tax. Plasmids pMD1-Tax/M21, pMD1-Tax/M23, pMD1-Tax/M26, pMD1-Tax/M30, pMD1-Tax/M32, pMD1-Tax/M35, pMD1-Tax/M41, and pMD1-Tax/M47, expressing the corresponding Tax point mutations (52), were constructed by inserting the *Mlu*I-*Sma*I restriction fragments of pGAL4-Tax/M21, pGAL4-Tax/M23, pGAL4-Tax/M26, pGAL4-Tax/M30, pGAL4-Tax/M32, pGAL4-Tax/M35, pGAL4-Tax/M41, and pGAL4-Tax/M47 (13) between the *Mlu*I and *Sma*I restriction sites of pMD1-Tax. Plasmid pMD1-CREB was created by inserting the rat CREB cDNA between the *Hin*dIII and *Bgl*II restriction sites of pMD1. The CREB cDNA was obtained by PCR with pSG-CREB (kindly provided by P. Sassone-Corsi) (20) and specific primers. The 5′ primer generated a *Hin*dIII restriction site upstream of the AUG codon. A second series of vectors bearing the *URA3* selection marker was constructed. Plasmids pYU-Tax and pYU-CREB were obtained by inserting the *Xho*I-*Bam*HI restriction fragments of pMD1-Tax and pMD1-CREB, respectively, between the *Xho*I and *Bam*HI restriction sites of pRS316 (*CEN6 URA3*) (50). Plasmid pYT-CREB, bearing the *TRP1* selection marker, was obtained by inserting the *Xho*I-*Bam*HI restriction fragment of pMD1-CREB between the *Xho*I and *Bam*HI restriction sites of pRS314 (*CEN6 TRP1*) (50).

Vectors producing proteins fused to the GAL4 DNA-binding domain (GB) and the GAL4 activation domain (GAD) are derivatives of pGBT9 (2μm *TRP1*) and pGAD424 (2μm *LEU2*), respectively (Clontech). A DNA fragment corresponding to the Tax coding sequence was generated by PCR amplification with plasmid pSG-Tax (16) and appropriate primers. The 5′ primer created an in-frame *Eco*RI restriction site which preceded the second codon of Tax. The PCR product cut by *Eco*RI and *Bam*HI restriction enzymes was inserted between the *Eco*RI and *Bam*HI restriction sites of pGB-T9 and pGAD424, giving plasmids pGB-Tax and pGAD-Tax, respectively. pGB-TaxM9, pGB-TaxM18, and pGB-TaxM47 were generated by the same procedure, with plasmids pGAL4-TaxM9, pGAL4-TaxM18, and pGAL4-TaxM47 (13). Plasmid pTax-GB is similar to pGB-Tax, except that GB is fused to the C terminus of Tax. A fragment including the Tax coding sequence was prepared by PCR from plasmid pMD1-Tax and cut by *Hin*dIII and *Nsi*I restriction enzymes. Another fragment including GB was generated by PCR from pGBT9 and cut by *Nsi*I and *Eco*RV restriction enzymes. The in-frame *Nsi*I restriction sites were created by the primers used in the amplification reactions. These two fragments were inserted between the *Hin*dIII and *Eco*RV restriction sites of pGBT9. In this construct, the last codon of Tax is fused to the third codon of GB and the two coding sequences are separated by two codons (Met and His). Plasmid pTax-GAD, expressing Tax fused to GAD at its C terminus, was created by a similar procedure. The fragment corresponding to GAD was prepared by PCR from pGAD424, and insertion of both coding sequences was done between the *Hin*dIII and *Eco*RV restriction sites of this latter vector. pGB-CREB and pGAD-CREB were generated by inserting the CREB coding sequence between the *Eco*RI and *Bam*HI restriction sites of pGBT9 and pGAD424, respectively. The fragment including the CREB coding sequence was obtained by PCR from plasmid pSG-CREB by using a 5′ primer creating an *Eco*RI site, which preceded the initiation codon of CREB. pGAD-ATF2 was generated by inserting a *Bam*HI restriction fragment of pSG424-ATF2 (39) (kindly provided by M. R. Green through the courtesy of C. Kédinger) into the *Bam*HI restriction site of pGAD424.

Another series of expression vectors was constructed from plasmid pYUMD1Δ (*CEN6 URA3*). This latter construct was created as follows. Plasmid pMD1, digested by restriction enzymes *Hin*dIII and *Sal*I, was filled in and ligated to itself, giving pMD1Δ. The *Xho*I-*Bam*HI restriction fragment of pMD1Δ, which includes the *ADC* promoter, a polylinker, and the *ADC* terminator, was inserted between the *Xho*I and *Bam*HI restriction sites of pRS316 (50). pYU-GADCREB was constructed by inserting the *Hin*dIII-*Bgl*II restriction fragment of pGAD-CREB between the *Hin*dIII and *Bgl*II restriction sites of pYUMD1Δ. Plasmid pYU-ATF1 was obtained by inserting the ATF-1 cDNA generated by reverse transcription-PCR from mRNA of Jurkat cells between the *Sma*I and *Bgl*II restriction sites of pYUMD1Δ. Plasmid pYU-ATF2 was created by inserting the *Bam*HI restriction fragment of pSG424-ATF2 into the *Bgl*II restriction site of pYUMD1Δ. The constructs expressing various C-terminal portions of CREB and ATF-2 are also derivatives of pYUMD1Δ. Fragments including sequences coding for the C-terminal 92 and 59 amino acid residues of CREB were generated by PCR from pSG-CREB and cut with the *Sma*I and *Bgl*II restriction enzymes. These fragments were inserted between the *Sma*I and *Bgl*II restriction sites of pYUMD1Δ, giving plasmids pYU-CREBBZ92 and pYU-CREBBZ59. Plasmids pYU-ATF2BZ190 and pYU-ATF2BZ92 were generated by the same procedure from pSG424-ATF2 and pYUMD1Δ. The various plasmid constructs were systematically verified by sequencing.

Yeast strains. Yeast cells were grown on rich medium (1% yeast extract, 2% peptone, 2% dextrose [YPD]) or appropriate selective minimal media at 30°C. All transformations were carried out with a variation of the lithium acetate method (28). Insertion into the yeast genome of the reporter constructs including the HTLV-1 21-bp repeat upstream of the *CYC1* promoter fused to *lacZ* was performed by homologous recombination. Plasmids pRSX0RLacZ, pRSABC 3RLacZ, and pRSMBM3RLacZ were linearized by digestion with *Pst*I. The *Pst*I restriction site is located within the *HIS3* gene. *S. cerevisiae* S150-2B (*MAT***a** *ura3-52 leu2-3,112 trp1-289 his3*Δ *gal2*) (kindly provided by J. Broach through the courtesy of S. Gasser and E. Gilson) was transformed by these linearized plasmids, and transformants were selected on histidine-negative synthetic medium (0.67% yeast nitrogen base, 2% dextrose [SD]). Integrations were confirmed by Southern blot and PCR analysis of yeast genomic DNA. One clone resulting from integration of each construct was selected, giving strains RH4X0, RH8-ABC3, and RH14-MBM3. These strains were further used for transformation by the Tax and CREB expression vectors. The two-hybrid analysis was performed with the *S. cerevisiae* HF7c reporter strain [*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2*::*GAL1-HIS3 URA3*::(*GAL4* 17-mers)$_3$-*CYC1-lacZ*] (Clontech). Strain HF7ΔURALacZ (*MAT***a** *ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2*::*GAL1-HIS3*) was derived from HF7c as follows. Strain HF7c was transformed with the pCL1 plasmid coding for the full-length *GAL4* gene (2μm *LEU2*) (Clontech). Cells (10^8) were plated on SD medium without Leu or His and complemented with uracil (50 μg/ml) and 5-fluoro-orotic acid (0.1%). 5-Fluoro-orotic acid was used for positive selection of *ura3* auxotrophic mutants (8). Twenty-six colonies resistant to 5-fluoro-orotic acid were obtained. These colonies lost both the *URA3* selection marker and the *GAL4-lacZ* reporter gene (white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] in the presence of the pCL1 plasmid). One yeast colony was selected and cured of the pCL1 vector by growth in YPD, giving strain HF7ΔURALacZ, which was auxotrophic for Ura, Leu, and His. This strain contains a functional *GAL1-HIS3* reporter gene. HF7ΔURALacZ was further used for transformation by the pLG-ABC3LacZ reporter plasmid and the Tax and CREB expression vectors.

Screening of the library of human cDNAs fused to GAD. The RH8-ABC3 reporter strain carrying plasmid pYU-Tax was transformed with a library of human cDNAs fused to GAD (18). The cDNAs were prepared with mRNA from Epstein-Barr virus-transformed human peripheral blood lymphocytes and inserted into λ-ACT (18). After transformation, yeast cells were plated on SD medium complemented with Trp (SD minus His, Ura, and Leu) at a density of 10×10^3 to 20×10^3/20-cm^2 dish. Plates were incubated at 30°C for 4 days. A total of 7.5×10^5 transformants were obtained and assayed on filters for production of β-galactosidase. Blue colonies were isolated, and the β-galactosidase assay was repeated. To test whether expression of β-galactosidase was dependent on the plasmid expressing the GAD-cDNA fusion protein, yeast cells were cured of this plasmid by growth in SD medium complemented with leucine and several colonies were tested for β-galactosidase activity and leucine requirement. Plasmids of transformants that lost β-galactosidase expression together with the leucine requirement were recovered and transformed into *Escherichia coli* XL1-Blue by electroporation as previously reported (33). pACT-cDNA plasmids amplified in bacteria were retransformed into the various reporter strains and analyzed by sequencing.

β-Galactosidase assays. For the β-galactosidase filter assay, yeast colonies were grown on appropriate SD medium and transferred to nitrocellulose filters (BAS45; Schleicher & Schüll). For screening of the cDNA library, colonies were grown on filters for 24 h over SD medium. The filters were immersed in liquid nitrogen for 1 min to permeabilize the cells, air dried for 2 to 3 min, and then laid on Whatman 3MM paper moistened with Z buffer containing β-mercaptoetha-

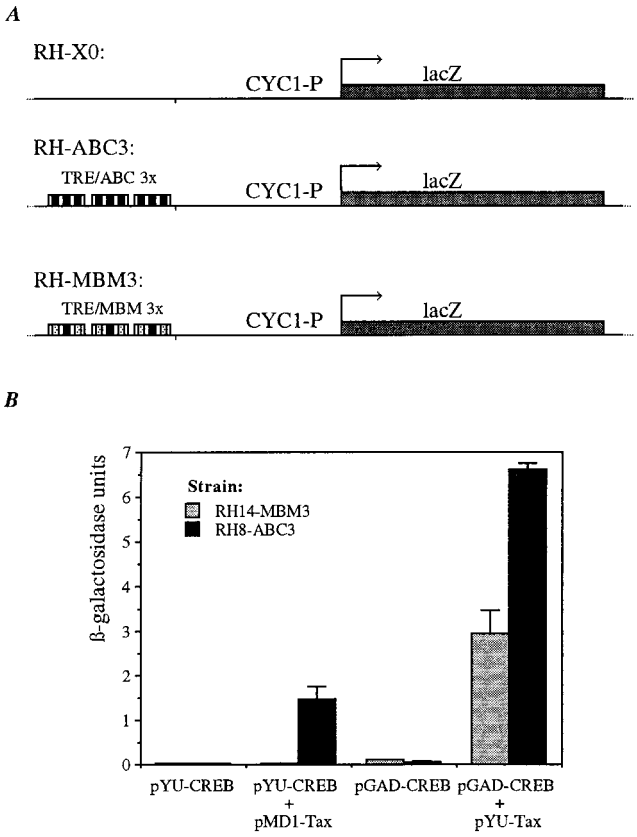

FIG. 1. (A) Structures of the reporter constructions used to construct strains RH-X0, RH-ABC3, and RH-MBM3. TRE-1, either wild type (ABC) or mutated in domains A and C (MBM) (44), was cloned as three tandem repeats in the *Xho*I site of plasmid pRSX0RLacZ. This sequence element is upstream of the *CYC1* promoter (CYC1-P) fused to the *lacZ* gene. (B) Quantification of β-galactosidase activity in strains RH8-ABC3 and RH14-MBM3 transformed by plasmids expressing CREB and Tax. Strains RH8-ABC3 and RH14-MBM3 were transformed by plasmids pYU-CREB, pYU-CREB plus pMD1-Tax, pGAD-CREB, and pGAD-CREB plus pYU-Tax as indicated below the graph. β-Galactosidase activity was measured with a liquid culture assay. The bars represent β-galactosidase activity in Miller units. Measurements were done on two independent transformants for each combination of plasmids, and the mean values are shown.

nol and X-Gal (2.5 ml/90-mm-diameter filter; 21 ml/20-cm^2 filter) (11). Assays were performed at 30°C, and the appearance of blue colonies was periodically checked. Four independent colonies were analyzed for each test. For β-galactosidase quantitation by liquid culture assay, 2-ml cultures were grown in the appropriate selective media to an optical density at 600 nm of 1.2 to 1.8. Cells were harvested and permeabilized as previously described (30). *o*-Nitrophenyl-β-D-galactopyranoside was used as the substrate, and assays were performed at 30°C. β-Galactosidase activities are expressed in Miller units, which were calculated from the following equation: 1,000 × optical density at 420 nm/time of incubation (minutes) × optical density at 600 nm × volume of culture (milliliters) (43). Each β-galactosidase activity determination was done in duplicate.

RESULTS

Cooperative activation of TRE-1 by CREB and Tax. The TRE-1 sequence corresponding to the most proximal 21-bp repeat of the HTLV-1 promoter was inserted as three tandem repeats upstream of the *CYC1* promoter fused to the *E. coli lacZ* gene (Fig. 1A). This construct was made with a plasmid which includes the *HIS3* selection marker. A similar construct was made with TRE-1 mutated in domains A and C (mutation MBM) (44). These constructs were introduced into the yeast genome by homologous recombination at the *HIS3* locus. This was done with the parental plasmid and the two derivatives

TABLE 1. Results of the β-galactosidase filter assay performed with reporter strains RH4-X0, RH14-MBM3, and RH8-ABC3 transformed with plasmids expressing Tax and CREB

Plasmid(s)	Color[a] of strain:		
	RH4-X0	RH14-MBM3	RH8-ABC3
None	W	W	W
pYU-Tax	W	W	W
pMD1-Tax	W	W	W
pYU-CREB	W	W	W
pMD1-CREB	W	W	W
pMD1-Tax + pYU-CREB	W	W	BB
pGAD-CREB	W	W/B	W/B
pGAD-CREB + pYU-Tax	W	BB	BBB

[a] Coloration of colonies was scored as follows: W, white after incubation for 24 h; W/B, blue after incubation for 24 h; BB, blue after incubation for 2 h; BBB, blue after incubation for 1 h.

containing three copies of TRE-1 which were either the wild type or mutated. The efficiency of the recombination event was monitored by Southern blot and PCR analyses, and one clone for each plasmid was selected, giving strains RH4-X0 (no TRE-1), RH8-ABC3 (three copies of wild-type TRE-1), and RH14-MBM3 (three copies of TRE-1 mutated in domains A and C). None of the three strains expressed detectable β-galactosidase activity (Table 1). Transformation of these strains by plasmids expressing either Tax or CREB did not activate expression of the reporter gene (Table 1 and Fig. 1B). However, coexpression of Tax and CREB in strain RH8-ABC3 clearly activated production of β-galactosidase. This effect was not observed in the two other strains. That CREB did not activate transcription via TRE-1 in the absence of Tax could be due to its inability either to bind to DNA or to activate transcription. To investigate this point more precisely, CREB was fused to GAD. Transformation of strains RH8-ABC3 and RH14-MBM3 by a plasmid expressing the GAD-CREB fusion protein led to weak expression of the reporter gene. Cotransformation with a Tax-expressing construct markedly increased the levels of expressed β-galactosidase (Table 1 and Fig. 1B). This effect was more pronounced with wild-type TRE-1 than with its mutated form. These results show that CREB binds poorly to TRE-1 by itself and that Tax increases this binding.

A direct protein-protein interaction between Tax and CREB was also investigated by using the two-hybrid system. Both proteins were fused to either GB or GAD. It has been reported that fusion of the N-terminal domain of Tax with glutathione *S*-transferase impairs its interaction with CREB (1). As a consequence, GB and GAD were fused to the C terminus of Tax. We failed to detect an interaction between Tax and CREB in this assay, whatever the combination tested (Table 2). This was also the case when GB and GAD were fused to the N terminus of Tax (data not shown). This negative result could be due to the absence of expression of CREB and Tax fused to GB and GAD. To eliminate this possibility, the *lacZ* gene under the control of *GAL4* sites, which is integrated in strain HF7c, was removed (see Materials and Methods). The resulting strain was transformed with a plasmid including the *lacZ* gene under the control of three copies of wild-type TRE-1. In this strain, Tax-CREB cooperation was tested on both the *GAL1* upstream activating sequence and TRE-1 (Table 3). The results of this experiment unambiguously show that CREB and Tax fused to GB and GAD cause activation via TRE-1, whereas they do not form a complex on the *GAL1* upstream activating sequence (Table 3). This indicates that the negative result

TABLE 2. Analysis by the two-hybrid system of the Tax-CREB interaction with reporter strain HF7c

Plasmid 1 (GB)	Plasmid 2 (GAD)	Growth[a] on His-negative SD medium	Colony color[b]
pGBT9	pGAD424	−	W
pGB-CREB	pGAD424	−	W
pGBT9	pTax-GAD	−	W
pGB-CREB	pTax-GAD	−	W
pTax-GB	pGAD424	−	W
pGBT9	pGAD-CREB	−	W
pTax-GB	pGAD-CREB	−	W
pTax-GB	pTax-GAD	+	B
pTax-GB	pGAD-Tax	+	B
pGB-Tax	pTax-GAD	+	BB
pGB-Tax	pGAD-Tax	+	BB
pGB-Tax	pMD1-Tax	+	B

[a] −, no growth; +, growth.
[b] Coloration of colonies was scored as follows: W, white after incubation for 24 h; B, blue after incubation for 4 h; BB, blue after incubation for 2 h.

obtained with the two-hybrid assay is not due to absence of expression of the fusion proteins in *S. cerevisiae.*

With the two-hybrid system, Tax was found to interact with itself. A cooperative effect was observed between Tax fused to GB and Tax fused to GAD (Tables 2 and 3). This effect was observed when GB and GAD were fused to either the N terminus or the C terminus of Tax. Tax fused to GB and wild-type Tax also cooperated (Table 2). This shows that Tax activates transcription in *S. cerevisiae* when it indirectly binds to DNA. Hence, the transcriptional activation observed with the Tax-CREB combination in strain RH8-ABC3 is probably due to Tax. Fusion of CREB with an activation domain could cause an increased effect. Collectively, these experiments show that TRE-1 is an active element in the formation of the Tax-CREB complex. At intracellular concentrations of these two factors, their association is not observed in the absence of TRE-1. Domains A and C of the 21-bp element are important for assembly of this ternary complex. When they are mutated, the activation observed with CREB and Tax is abrogated and that resulting from the GAD-CREB-Tax combination is reduced.

Screening for cellular factors binding to HTLV-1 TRE-1 in a Tax-dependent manner. The previous experiments indicated

TABLE 3. Analysis of the Tax-CREB interaction by using strain HF7ΔURALacZ transformed with plasmid pLG-ABC3LacZ

Plasmid 1 (*TRP1*)	Plasmid 2 (*LEU2*)	Growth[a] on His-negative SD medium (*GAL1-HIS3* reporter)	Colony color[b] (*ABC3-lacZ* reporter)
pGBT9	pGAD424	−	W
pGB-CREB	pGAD424	−	W
pGBT9	pTax-GAD	−	W
pGB-CREB	pTax-GAD	−	BBB
pTax-GB	pGAD424	−	W
pGBT9	pGAD-CREB	−	W
pTax-GB	pGAD-CREB	−	BB
pYT-CREB	pMD1Δ	−	W
pRS314	pMD1-Tax	−	W
pYT-CREB	pMD1-Tax	−	BBB
pGB-Tax	pGAD-Tax	+	W
pRS314	pCL1(Gal4)	+	W

[a] −, no growth; +, growth.
[b] Coloration of colonies was scored as follows: W, white after incubation for 12 h; BB, blue after incubation for 2 h; BBB, blue after incubation for 1 h.

TABLE 4. Results of the β-galactosidase filter assay performed with reporter strain RH8-ABC3 and the five positive clones identified by screening of the cDNA library

Clone	Color[a] of strain:	
	RH8-ABC3	RH8-ABC3 (pYU-Tax)
SCL 2.7	W	BB
SCL 2.38	W	BB
SCL 4.1	W	BB
SCL 4.9	W	BB
SCL 4.16	BBB	BBB

[a] Coloration of colonies was scored as follows: W, white after incubation for 24 h; BB, blue after incubation for 2 h; BBB, blue after incubation for 1 h.

that the RH8-ABC3 reporter strain could be used to screen for human proteins whose binding to TRE-1 is stimulated by Tax. Transformation of this strain carrying a plasmid expressing Tax by a construct producing a factor exhibiting properties similar to that of CREB should indeed lead to activation of the reporter gene, and this event could be monitored by examining coloration of the colonies in a β-galactosidase assay. On the basis of the results obtained with CREB, to obtain maximal expression of β-galactosidase, constructs containing cDNAs fused to GAD were used. Strain RH8-ABC3 carrying plasmid pYU-Tax was transformed with a library of human cDNAs fused to GAD (kindly provided by S. Elledge), and colonies were examined for blue coloration. Plasmids present in positive clones were amplified in *E. coli*. Isolated plasmids were reintroduced into strain RH8-ABC3 with or without the Tax-expressing plasmid. Yeast colonies (0.75×10^6) were screened by this method, and five positive clones were identified (Table 4). Four clones caused expression of the reporter gene in a Tax-dependent manner, whereas the activity of another was not modified by Tax. The nature of these clones was determined by sequencing. One (SCL 4.9) corresponded to almost the entire sequence of a human CREB isoform (7) (Fig. 2A). Clones SCL 2.38 and SCL 2.7 corresponded to the C-terminal part of two different CREM isoforms including the first B-ZIP domain (20, 24, 37, 58) (Fig. 2B). The fourth clone (SCL 4.1) corresponded to the C-terminal domain of ATF-1/TREB-36 (32, 60) (Fig. 2C). Thus, all of the clones binding to TRE-1 in a Tax-dependent manner corresponded to members of the CREB/ATF family. The coding sequences of these four clones were not in the same reading frame as that of GAD. The coding sequence of CREB was even inverted with respect to that of GAD. These results show that in addition to CREB, binding of CREM and ATF-1 to TRE-1 can also be induced by Tax. The fifth clone identified in this screening corresponded to the C-terminal moiety of the p70 subunit of the Ku autoantigen (Fig. 2D). In this case, the coding sequence of the p70 subunit of Ku and GAD were in the same reading frame and removal of this latter domain abrogated activation (data not shown). The association of the p70 subunit of Ku with TRE-1 was independent of Tax.

Mapping of the domains of CREB and Tax involved in the interaction. The structures of the various CREB/ATF clones identified by screening of the cDNA library show that the domain interacting with Tax lies within the C-terminal domain of these proteins. To define its position more precisely, vectors expressing either the last 92 or 59 amino acid residues from the C terminus of CREB were constructed (Fig. 3). The former led to strong Tax-dependent activation of TRE-1, and the effect observed was, in fact, more potent than that exerted by the entire CREB protein (Table 5 and Fig. 4). By contrast, the

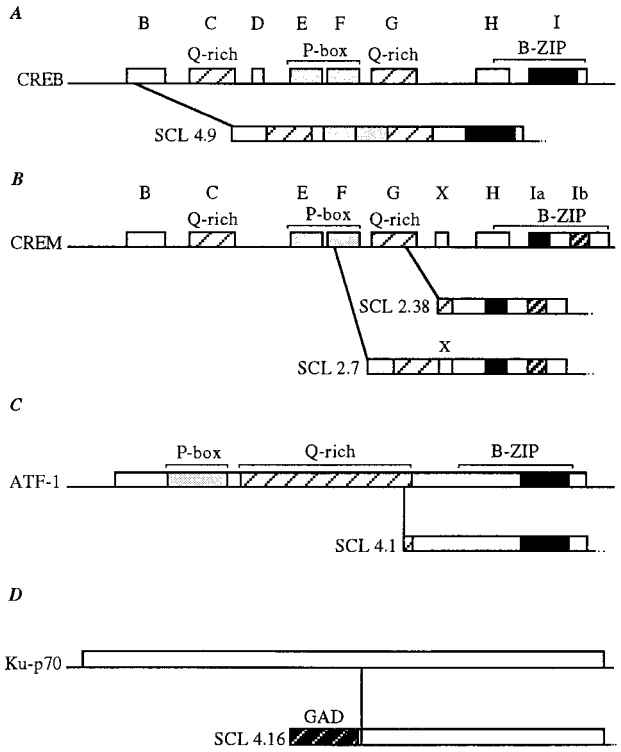

FIG. 2. Schematic representation of the clones identified by screening a library of human cDNAs in strain RH8-ABC3 transformed with plasmid pYU-Tax. The five clones identified in the screening (see Table 3) were sequenced. The structures of the cDNA inserts contained in these clones are shown under the representations of the CREB (A) and CREM (B) genes and of the ATF-1 (C) and Ku p70 (D) cDNAs. CREB and CREM exons are represented as previously reported (58). In clone SCL 4.9, the CREB coding sequence begins at amino acid 8 and contains the 14 amino acid residues of exon D (7, 58). In clones SCL 2.7 and SCL 2.38, the CREM coding sequence begins at amino acid residues 126 and 216, respectively (according to the numbering of Masquilier et al. [41]). SCL 2.7 includes exon X, which is absent in SCL 2.38 (58). In clone SCL 4.1, the ATF-1 coding sequence begins at amino acid 161 (32, 60). Clone SCL 4.16 includes the Ku p70 coding sequence between amino acid residues 325 and 609 (14, 46).

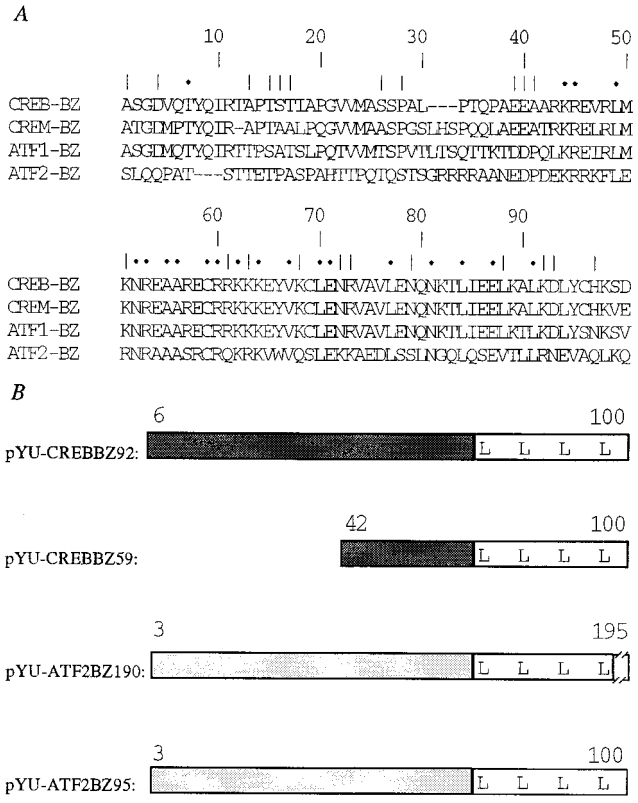

FIG. 3. Schematic representation of the CREB and ATF-2 deletion mutants tested in combination with Tax. (A) Alignment of the B-ZIP (BZ) domains of CREB, CREM, ATF-1, and ATF-2. The sequences of the CREB, CREM, ATF-1, and ATF-2 proteins were aligned by using the CLUSTAL program. The alignment of these proteins is represented for the C-terminal 97 amino acid residues of CREB. The dots above the sequences correspond to identities, and the vertical bars correspond to conservative changes. Numbering starts at amino acid 245 of CREB. (B) Schematic representation of the CREB and ATF-2 deletion mutants tested in combination with Tax. The C-terminal 92 and 59 amino acid residues of CREB were inserted into plasmid pYU, giving plasmids pYU-CREBBZ92 and pYU-CREBBZ59, respectively. Plasmid pYU-ATF2BZ190 includes the C-terminal 190 amino acid residues of ATF-2 (32). Plasmid pYU-ATF2BZ95 expresses the ATF-2 domain located between amino acid residues 316 and 410. The numbers given above the representation of the CREB and ATF-2 sequences indicate the first and last amino acid residues present in the construct, according to the numbering in panel A. L indicates leucine. For all of these constructs, a translational initiation codon precedes the first residue of CREB and ATF-2.

vector expressing the last 59 amino acid residues from the C terminus of CREB was inactive (Table 5). These results show that the CREB domain that binds to TRE-1 in association with Tax lies within the C-terminal 92 amino acid residues, which include the B-ZIP domain.

Smith and Greene have reported a series of point mutations spanning the entire Tax protein (52). These mutant proteins were tested for the ability to induce the HTLV-1 and human immunodeficiency virus type 1 promoters. The intracellular localization of these various mutant proteins was also determined. To delineate the domain of Tax involved in the interaction with CREB, a series of mutations spanning the region between amino acid residues 134 and 320 in Tax was selected. These mutant proteins have been described as nuclear and negative for activation of the HTLV-1 promoter (52). These various mutant proteins were expressed in strain RH8-ABC3 in association with CREB. Mutants M21, M23, M26, M30, and M32 were unable to activate expression of the reporter gene (Fig. 4A). Mutant M35 was weakly active, whereas mutants M41 and M47 gave activity comparable to that of wild-type Tax (Fig. 4A). These mutants were also tested together with the minimal C-terminal domain of CREB. The results were similar to those obtained with the entire CREB protein, except that the activation levels were approximately 10-fold higher (Fig.

TABLE 5. Results of the β-galactosidase filter assay performed with reporter strain RH8-ABC3 and vectors expressing various domains of CREB and ATF-2

Plasmid	Color[a] of strain:	
	RH8-ABC3	RH8-ABC3 (pMD1-Tax)
pYU-CREB	W	BB
pYU-CREBBZ92	W	BBB
pYU-CREBBZ59	W	W
pYU-ATF1	W	BB
pYU-ATF2	W	W
pGAD-ATF2	W	W
pYU-ATF2BZ190	W	W
pYU-ATF2BZ95	W	W

[a] Coloration of colonies was scored as follows: W, white after incubation for 24 h; BB, blue after incubation for 2 h; BBB, blue after incubation for 1 h.

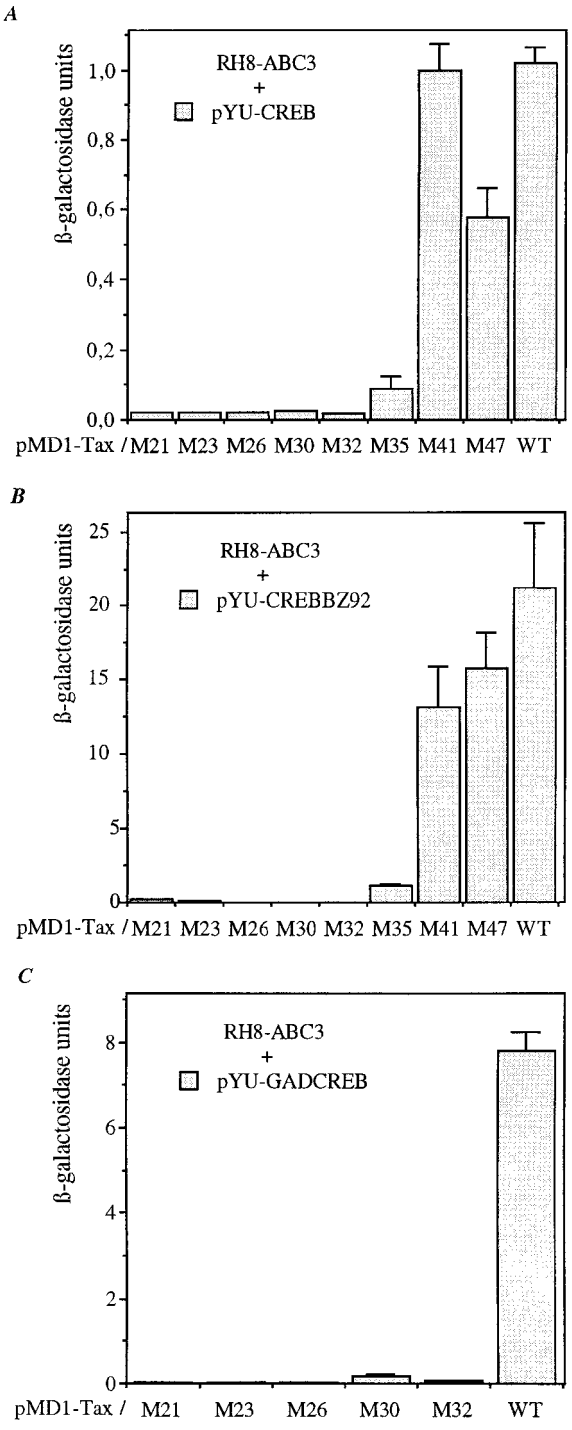

FIG. 4. Determination of the activities of various Tax mutants in combination with CREB, CREBBZ 92, or GAD-CREB. Strain RH8-ABC3 was transformed with either pYU-CREB (A) or pYU-CREBBZ92 (B) plus wild-type pMD1-Tax and mutants M21, M23, M26, M30, M32, M35, M41, and M47. (C) Strain RH8-ABC3 was transformed with pYU-GAD-CREB plus wild-type pMD1-Tax and mutants M21, M23, M26, M30, and M32. β-Galactosidase activity was quantified and is represented as described in the legend to Fig. 1. The various Tax mutants were expressed to identical levels as tested by Western blotting (immunoblotting) (data not shown).

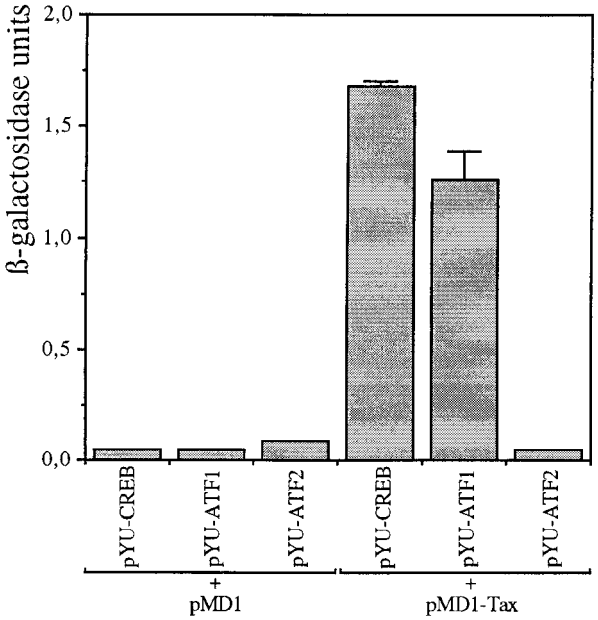

FIG. 5. Determination of the abilities of various members of the CREB/ATF family to transactivate TRE-1 in the presence of Tax. Strain RH8-ABC3 was transformed with either pYU-CREB, pYU-ATF1, or pYU-ATF2. These plasmids were transformed with either pMD1 or pMD1-Tax as indicated. β-Galactosidase activity is represented as described in the legend to Fig. 1B.

4B). Mutants M21, M23, M26, M30, and M32 could be affected in the ability to interact either with CREB or with the transcriptional machinery. Addition of a transcriptional activation domain to CREB did not reverse the lack of activity of these five mutants (Fig. 4C). Thus, these mutations affect the interaction with CREB. These results show that the Tax domain between amino acid residues 134 and 197 plays an important role in the formation of the TRE-1–CREB–Tax ternary complex.

Specific activity of the cyclic AMP-responsive members of the CREB/ATF family. Interestingly, a particular subgroup of the CREB/ATF factors was identified by screening the cDNA library. ATF-1, CREM, and CREB are indeed closely related. These three factors are substrates of protein kinase A, and since they are active in the phosphorylated form, they participate in cyclic AMP signaling. That other members of the CREB/ATF family were not obtained in the screening could be due either to chance or to their inability to bind to TRE-1 in association with Tax. To investigate this point, vectors expressing ATF-2 (32), alone or in a fusion with GAD, were constructed and used to transform the RH8-ABC3 reporter strain. In the absence or presence of Tax, both constructs were unable to activate TRE-1 (Table 5 and Fig. 5). To determine whether the absence of response of ATF-2 to Tax could be related to the inhibiting effect of a domain outside the B-ZIP region, two additional vectors expressing either the 192 amino acid residues from the C terminus or the 95-amino-acid domain similar to the C terminus of CREB were also tested (Fig. 3). These two vectors were found to be negative, in the presence or the absence of Tax (Table 5). In these experiments, it was verified that the entire ATF-1 protein has an activity similar to that of CREB (Table 5 and Fig. 5). This shows that CREB, CREM, and ATF-1 are probably the only members of the CREB/ATF family to allow the association of Tax with TRE-1.

DISCUSSION

Establishment of the Tax-CREB complex on TRE-1. Expression of Tax confers a strong enhancer activity on TRE-1 of the HTLV-1 promoter (9, 26, 44). DNA affinity precipitation experiments have shown that Tax binds to TRE-1 in a sequence-specific manner via cellular factors (6, 54). Studies performed by electrophoretic mobility shift assay led to divergent results; a complex including Tax was observed by some groups but not others (2, 21, 57, 61). This could be related to the poor stability of such a complex during electrophoretic separation. That transactivation of TRE-1 results from the indirect binding of Tax has been established uniquely on the basis of in vitro experiments. Since Tax is active on TRE-1 in a wide variety of mammalian cell lines (16), this has not been supported by clear genetic evidence. This report shows that such genetic studies can be conveniently performed with *S. cerevisiae*. Both Tax and the cellular factor CREB are, by themselves, inactive on TRE-1 in this microorganism, but in combination they lead to its activation. Experiments with CREB fused to GAD showed that CREB does not bind by itself to wild-type TRE-1 or that mutated in domains A and C. This is in agreement with in vitro data which indicate that a palindromic cyclic AMP response element (CRE) is a better binding site for CREB than TRE-1 (21, 59). Binding of CREB to TRE-1 occurred in the presence of Tax. This cooperative effect between Tax and CREB was clearly observed with a minimal CREB domain corresponding to the C-terminal 92 amino acid residues. This part of CREB lacks the transcriptional activation domains located in the N-terminal region. This indicates that activation of transcription of the reporter gene is exerted by Tax, which is present in the complex. Although various constructs were tested, a direct protein-protein interaction between Tax and CREB was not observed with the two-hybrid system. However, binding of Tax to CREB in the absence of TRE-1 has been observed with bacterially produced proteins and recently with a two-hybrid assay performed with mammalian cells (1, 2, 59). This suggests that in *S. cerevisiae*, the intracellular concentrations of CREB and Tax are too low for this interaction to occur. Since association of the two factors intervenes on TRE-1, this DNA sequence plays an active role in the formation of the ternary complex. This notion is also supported by the results obtained with TRE-1 mutated in both domains A and C. Compared with that of the wild type, this altered sequence caused weaker activation in response to the Tax-CREB combination. These data clearly show that in vivo transactivation results from formation of the TRE-1–CREB–Tax ternary complex.

This assay with *S. cerevisiae* was used to delineate the domains of CREB and Tax that mediate formation of the complex on TRE-1 in vivo. Deletion of the N terminus of CREB up to amino acid 282, just upstream of the first stretch of basic amino acid residues, impaired the cooperative effect with Tax. The N-terminal limit of the domain permitting binding of Tax on TRE-1 therefore lies between amino acid residues 250 and 282. This domain is therefore slightly larger than the B-ZIP region. Several Tax point mutants were also evaluated by this assay. Mutant M35 (^{206}Met-Ile→Ala-Ser) appears to mark a limit, as mutants with mutations on the N-terminal side are inactive and those with mutations on the C-terminal side have activity similar to that of the wild type. These data are in good agreement with a recent study which shows that the N terminus of Tax is required for the interaction with CREB (1). It is noteworthy that mutations M41 and M47, which abrogate the activity of Tax when tested in mammalian cells on the HTLV-1 promoter (52) and as a fusion protein with GB (13), did not have any effect in *S. cerevisiae*. This suggests that Tax acts on the transcriptional machinery through different mechanisms in *S. cerevisiae* and mammalian cells.

Binding of Tax to TRE-1 is permitted by the cyclic AMP-responsive members of the CREB/ATF family. Screening of a library of human cDNAs for proteins binding to TRE-1 in the presence of Tax led to the isolation of several factors belonging to the CREB/ATF family. Interestingly, these three factors, CREB, CREM, and ATF-1, are activated via phosphorylation by protein kinase A (17, 29). It has been proposed that they form a particular subgroup within the CREB/ATF family (34). Disruption of the CREB gene in mice is compensated for by an increase in the level of expression of CREM (34). The CREB and CREM genes express multiple isoforms (7, 20, 24, 37, 58). For CREM, depending on the presence or the absence of the Q1 and Q2 domains, these isoforms are either activators or repressors of CREs. As shown by the structure of the CREM clones obtained and by the results of the experiments with truncated CREB proteins, the binding of Tax depends on a C-terminal domain. Hence, Tax probably binds to both activator and repressor forms of CREM. Thus, it is likely that Tax binds to TRE-1, whatever predominant isoform is expressed in the cell. That ATF-1 can also cooperate with Tax on TRE-1 is in disagreement with previous in vitro studies (2, 61). In our assay, it was clear that ATF-1 was as active as CREB. It is noteworthy that the Tax-binding domain defined in CREB (the 92 C-terminal amino acid residues) is highly conserved in ATF-1 (71.7% homology), as well as in CREM (82.6% homology). This domain is less conserved in other members of the CREB/ATF family (23.9% homology between CREB and ATF-2 and 26.1% homology between CREB and ATF-3). In agreement with these levels of similarity, ATF-2 was unable to allow binding of Tax to TRE-1. On the basis of in vitro experiments, it has been reported that Tax increases the dimerization and DNA binding of ATF-2 (21, 57). In agreement with a recent in vitro study (10), ATF-2 is probably less sensitive to the effect of Tax than ATF-1, CREB, and CREM. At intracellular concentrations, this poorer reactivity would result in the absence of binding to TRE-1. Thus, although these different CREB/ATF factors should be similar in terms of the overall geometry of the B-ZIP domain, this region of ATF-1, CREB, and CREM certainly has particular features which result in strong binding to Tax. It is intriguing that the B-ZIP domains of these three factors that are activated by the protein kinase A pathway possess a common specificity. It will be interesting to identify the structural aspect of this region which confers reactivity on the Tax protein.

The p70 subunit of the Ku autoantigen interacts with TRE-1. Unexpectedly, a clone coding for the C-terminal moiety of the p70 subunit of the autoantigen Ku was also isolated by screening for cellular factors binding to TRE-1. Ku has been identified as a nuclear protein against which patients suffering from systemic lupus erythematosus develop autoantibodies (14, 46). Ku has been involved in DNA repair, homologous recombination, and transcriptional regulation (27, 42, 47, 51). Ku corresponds to a heterodimer of two subunits, p70 and p86. In association with a third catalytic subunit, it forms a complex exhibiting a DNA-dependent kinase activity (DNA-PK) (12, 53). Our data show that p70 of Ku binds to TRE-1 in a cellular context. Future studies should clarify how Ku intervenes in the transactivation of TRE-1 by Tax. It will be interesting to study whether binding of the p70 subunit of Ku to DNA causes activation of DNA-PK at TRE-1. This event might cause modification via phosphorylation of several components of the transcriptional initiation complex.

In conclusion, the data obtained with the experimental system that we have developed with *S. cerevisiae* confirm and

extend our understanding of the mechanism of transactivation of HTLV-1 TRE-1 by Tax. The cellular factors which respond to an increase in the level of cyclic AMP mediate the specific binding of Tax to TRE-1. This property depends on a C-terminal domain which includes the B-ZIP region. This model illustrates how a viral transactivator subverts specific cellular transcription factors to the benefit of a pathogenic virus.

ACKNOWLEDGMENTS

We thank S. Elledge for providing us with the cDNA library in pACT; R. Bernards, M. R. Green, and P. Sassone-Corsi for the generous gift of plasmids; and J. Broach for the yeast S150-2B strain. Special thanks to E. Gilson for introducing us to yeast manipulation and to C. B. Bluink for critical reading of the manuscript.

This work was supported by the Agence Nationale de Recherches sur le SIDA, the Association pour la Recherche contre le Cancer, and the Ligue Nationale contre le Cancer (F.B.).

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