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Multiple cDNA clones encoding nuclear proteins that bind to the *tax*-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain

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A *trans*-activator protein, p40^{tax}, of human T cell leukemia virus type 1 (HTLV-1) activates its own promoter and cellular promoters of IL-2, IL-2 receptor α and GM-CSF genes. We isolated three cDNA clones encoding cellular proteins that bind to the p40^{tax}-dependent enhancer of HTLV-1 by screening a λgt11 cDNA library of an HTLV-1 infected cell line. All three proteins, TREB5, TREB7 and TREB36, contained a leucine zipper structure and basic amino acid domain, which are conserved in FOS, JUN and CREB, and also had multiple potential phosphorylation sites. The proteins expressed in *Escherichia coli* bound to the p40^{tax}-dependent enhancer of the 21 bp sequence, but not to an inactive mutant carrying a mutation in the CRE region. In DNase I footprint analysis, all three proteins protected the 21 bp sequences in the LTR; however, the patterns were not identical to each other. TREB7 and TREB36 protected all three repeats of the 21 bp, but TREB5 protected only the second repeat. TREB7 and TREB36 protected the 5′ and middle portions of the 21 bp which are essential for p40^{tax}-mediated *trans*-activation, whereas TREB5 and CREB1 protected a narrower part of the middle region of the second 21 bp repeat containing the CRE consensus sequence. These structural features and DNA binding properties suggest that TREB proteins are members of a CREB protein family and that some of them (i.e., TREB7 and TREB36) may be involved in p40^{tax}-mediated *trans*-activation.

Key words: enhancer binding protein/HTLV-1/leucine zipper/*tax*-responsive element/TREB sequence

Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is an etiologic agent of adult T cell leukemia (ATL) (Poiesz *et al.*, 1980; Yoshida *et al.*, 1982). A regulatory gene, *tax*, of the HTLV-1 genome codes for a nuclear protein of 40 kd (p40^{tax}) that *trans*-activates transcription of the viral genome (Sodroski *et al.*, 1984; Fujisawa *et al.*, 1985). p40^{tax} also activates expression of cellular genes, such as those for interleukin 2 (IL-2), the α subunit of the IL-2 receptor (IL-2Rα) (Inoue *et al.*, 1986; Maruyama *et al.*, 1987; Cross *et al.*, 1987) and GM-CSF (Miyatake *et al.*, 1988). Among these cellular genes, IL-2 and IL-2Rα have been proposed to play critical roles in T cell proliferation. Furthermore, IL-2Rα is generally overexpressed in fresh leukemic cells of ATL patients (Yodoi *et al.*, 1983; Depper *et al.*, 1984). Thus, p40^{tax} is thought to be linked to disordered proliferation of HTLV-1 infected T cells through *trans*-activation of IL-2Rα (Yoshida, 1987).

For *trans*-activation of the long terminal repeat (LTR) of HTLV-1, p40^{tax} requires direct repeats of a 21 bp enhancer that have been identified in the U3 region of the LTR (Fujisawa *et al.*, 1986; Paskalis *et al.*, 1986; Shimotohno *et al.*, 1986), whereas another sequence motif, an NF-κB binding site, is responsible for *trans*-activation of the IL-2Rα gene (Leung and Nabel, 1988; Lowenthal *et al.*, 1988; Ruben *et al.*, 1988). Therefore, p40^{tax} seems to modulate the activities of at least two enhancer motifs. Since there is no evidence that p40^{tax} binds directly to any of these sequences, some cellular protein(s) is suspected to mediate this activation by p40^{tax}. In fact, cellular proteins that bind to the 21 bp enhancer of the LTR or to the NF-κB site of the IL-2Rα have been detected and some of them have been purified (Tan *et al.*, 1989). *Trans*-activation by p40^{tax} was recently found not to require *de novo* synthesis of proteins, suggesting involvement of a pre-existing cellular factor(s) (Jeang *et al.*, 1988).

By mutagenesis of the 21 bp enhancer of the LTR, a 12 bp sequence in the enhancer has been identified as a *tax*-responsive element (TRE) (Fujisawa *et al.*, 1989). This TRE sequence shares a consensus sequence with the cAMP response element (CRE) (Montminy *et al.*, 1986). However, the CRE alone is not sufficient for activation by p40^{tax}, suggesting that some protein(s) that is distinct from the CRE binding protein (CREB) (Montminy and Bileziikjian, 1987) is involved in the *trans*-activation by p40^{tax} (Fujisawa *et al.*, 1989). To identify the protein(s) that is involved in the transcriptional *trans*-activation by p40^{tax}, we isolated three human cDNA clones that encode a protein(s) which binds to a 21 bp enhancer, and analyzed their structures. All three proteins, predicted from their cDNA sequences, contained a leucine zipper motif juxtaposed to a domain rich in basic amino acids, which are conserved in FOS, JUN, C/EBP and CREB.

Results

Isolation of cDNA clones encoding for TRE binding proteins (TREB)

Purification of nuclear proteins that bind to the 21 bp enhancer revealed the existence of multiple components with different molecular sizes (Tan *et al.*, 1989; Fujisawa,J. and Yoshida,M., unpublished data). On South-western protein blotting analysis, multiple proteins that bind to the 21 bp enhancer were detected in a nuclear extract from HUT 102 cells, an HTLV-1 infected cell line (data not shown). To isolate cDNAs encoding these proteins, a randomly primed

λgt11 cDNA library was prepared from mRNA of HUT 102 cells and screened with a double-stranded oligonucleotide according to the method of Singh *et al.* (1988). The 21 bp enhancer sequence was multimerized five times and used as a probe. Eleven phage clones (λR series) were isolated from about 2×10^6 recombinant phages by several rounds of screening. By hybridization of these clones with each other, eleven clones were classified into three groups: 4 clones in group λR5, 6 clones in group λR7 and 1 clone in group λR36.

The specificity of DNA binding activity of the cDNA encoded protein was confirmed by South-western blot analysis. A bacterial lysate containing a β-galactosidase fusion protein derived from each cDNA was separated by SDS−PAGE and subjected to South-western analysis with a pentamer of the 21 bp enhancer or its mutant. As shown in Figure 1, the 21 bp enhancer probe detected protein bands of 130, 62 and 140 kd in lysates of bacteria infected with λR5, λR7 and λR36, respectively. No significant signal was observed with a mutant probe that was inactive in *trans*-activation. Furthermore, uninfected bacteria did not give any significant band with the wild-type or mutant 21 bp probe. Therefore, we concluded that λR5, λR7 and λR36 carry cDNA encoding TRE binding protein, TREB.

The specific bands detected in cells carrying λR5 or λR36 were larger than β-galactosidase, but, the band detected in cells carrying λR7 was smaller than β-galactosidase. Nucleotide sequence analysis (Figure 2) revealed that the cDNAs in λR5 and λR36 were inserted in frame in the *lacZ* gene, whereas the cDNA in λR7 was inserted out of frame. The λR7 cDNA contained a 207 bp sequence upstream of the first ATG codon; this sequence contained two tandem, in frame termination codons. Thus, independent translation of polycistronic mRNA in *Escherichia coli* can explain the production of non-fused protein.

Structure of TREB proteins

To obtain full-length cDNA clones, we constructed an oligo(dT)-primed λgt11 cDNA library from HUT102 mRNA and screened it with insert cDNA from λR5, λR7 or λR36 as a probe. The nucleotide sequence of the clone carrying the longest insert in each of the respective groups, λT5, λT7 and λT36, was analyzed (Figure 2). Of these, λT7 has already been reported as CRE-BP1 (Maekawa *et al.*, 1989).

The inserts in the λT5, λT7 and λT36 clones were ~1.8, 4.0 and 2.4 kb long, respectively, and were very similar in size to those of the mRNAs (Figure 4). Therefore, each cDNA clone seems to be an almost full-length copy of the respective mRNA. Each clone has a long open reading frame (ORF) starting with ATG, which is preceded by an in frame stop codon. The ORFs in λT5, λT7 and λT36, respectively, contain 783, 1515 and 813 nucleotides, which could code for 261, 505 and 271 amino acids. These proteins are calculated to have molecular weights of 28.7, 54.5 and 29.2 kd, respectively. We named these proteins TREB5, TREB7 (identical to CRE-BP1 previously reported by Maekawa *et al.*, 1989) and TREB36, respectively.

Interestingly, the deduced amino acid sequences of all three proteins contained a motif of the leucine zipper structure that has been found in several enhancer binding proteins such as C/EBP, JUN and FOS (Landschulz *et al.*, 1988) (Figure 3). Leucine residues are repeated 6 times in TREB5, 5 times in TREB7 and 4 times in TREB36 at every seventh amino acid. A stretch of basic amino acid residues adjacent to the leucine zipper structure was also found in these TREB proteins, although similarity among the TREB proteins was not striking (Figure 3). These conserved motifs were located at the C-terminal regions of TREB7 and TREB36 as observed in JUN and CREB, but in the middle of the TREB5 proteins, as for those in FOS (Figure 2).

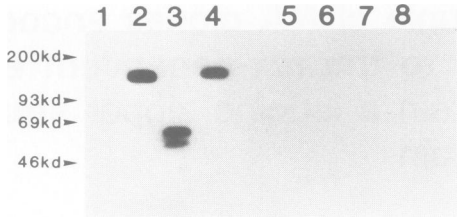

Fig. 1. Binding activities of TREB proteins, produced in bacteria, to the 21 bp enhancer of HTLV-1. Y1089 lysogens carrying no phage (**lanes 1 and 5**), λR5 (**lanes 2 and 6**), λR7 (**lanes 3 and 7**) and λR36 (**lanes 4 and 8**) were induced for production of β-galactosidase fusion proteins and the extracts were analyzed by South-western blotting with the wild-type 21 bp sequence, CTAGGCCCTGACGTGTCCCCCTG, (**lanes 1−4**) or the mutant sequence, CTAGGCCCACACGTGT-CCCCCTG, (**lanes 5−8**).

The amino acid sequence of TREB5 has no significant homology to any known sequence except several motifs such as the leucine zipper structure and basic domain. However, TREB36 shows striking homology to the CREB protein: the homologies of the total nucleotide and amino acid sequences of TREB36 and rat CREB (Gonzalez *et al.*, 1989) are 68% and 65%, respectively. Using λT36 cDNA as a probe, we were able to isolate human CREB cDNAs as weakly hybridizing clones under non-stringent conditions. These cDNA clones of human CREB had at least two types of coding sequence (Figure 2C). One of them, CREB1, encoded a human counterpart of the rat CREB reported by Gonzalez *et al.* (1989), whereas the other, CREB2, lacked the amino acid sequence SSCKDLKRLFSGTQ and was similar to the human CREB cDNA reported by Hoeffler *et al.* (1988). The CREB1 and CREB2 cDNAs were identical except for the presence or absence of the 42 bp region for the extra stretch of amino acids. Therefore, these two types of mRNA were very probably formed by alternative splicing.

Binding properties of TREB proteins to HTLV-1 LTR

Twelve nucleotides in the 5′ region of the 21 bp enhancer sequence in the LTR are essential for *tax*-dependent enhancer activity (Fujisawa *et al.*, 1989). We therefore suspected that the TREB protein, which is responsible for *trans*-activation by $p40^{tax}$, would recognize this 12 bp sequence. Accordingly, we carried out a DNase I footprinting assay to characterize the recognition sequences of the proteins encoded by these cDNAs.

To avoid any effects of the large mass of β-galactosidase in the fusion protein, we used the φ10 promoter for T7 RNA polymerase (Rosenberg *et al.*, 1987). This system adds only three amino acids at the N-terminal of the protein encoded by cDNA. Plasmids constructed for TREB5, TREB7, TREB36 and CREB1 expressed proteins of ~38, 61, 34 and 45 kd, respectively (data not shown), all of which were larger than those predicted from the respective sequence, as previously observed for rat CREB (Gonzalez *et al.*, 1989).

Fig. 2. Nucleotide sequence and predicted amino acid sequence of cDNA clones of TREB5 (**A**), TREB36 (**B**) and human CREB1 (**C**). The nucleotide sequences were determined by the dideoxy method (Sanger *et al.*, 1977) and the sequences are numbered from the initiation codon ATG. Open boxes indicate leucine residues in the leucine zipper structure and the thick line indicates the basic amino acid domain. An arrow with λR-5 or -36 is the site for the fusion with the *lacZ* gene in λR5 or λR36; thin underlining with (a), (c), and (ck) indicates potential sites for phosphorylation by protein kinase A, kinase C and casein kinase II, respectively. The wavy line in (**C**) indicates the sequence that is missing in CREB2.

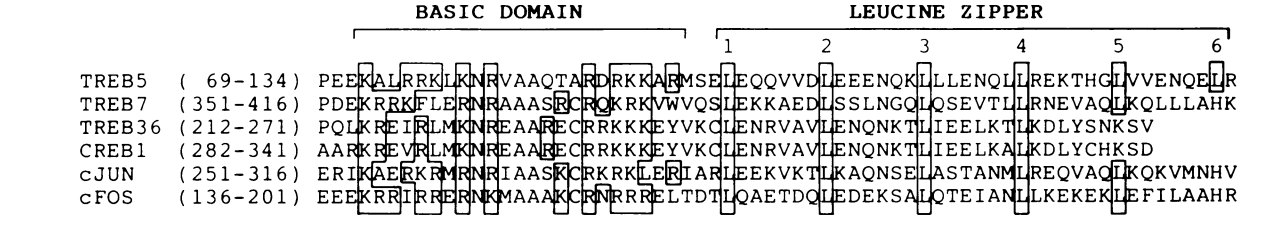

Fig. 3. Sequence homology between proteins containing leucine zippers. The name of the protein and the numbers of the first and last amino acids are shown on the left of the sequences. In the basic domain, Lys (K) and Arg (R) are boxed. In the leucine zipper, Leu (L) is boxed.

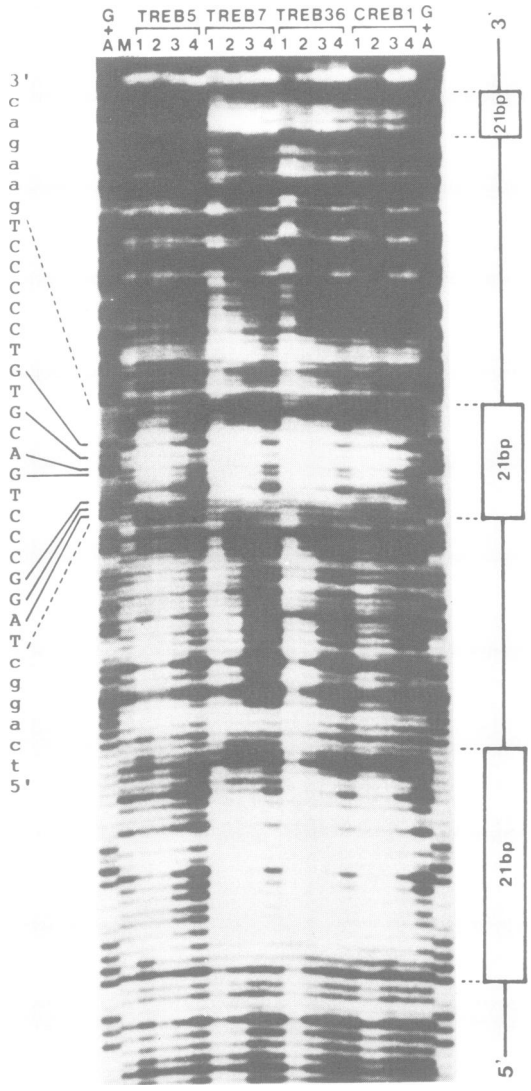

Fig. 4. DNase I footprinting activity of partially purified TREB proteins. TREB proteins produced in bacteria were partially purified on heparin–Sepharose and subjected to footprinting analysis. The probe was the LTR fragment labeled at the 5′ end of the sense strand (see Materials and methods). **G+A**, sequence ladders for G and A residues; **M**, mock; **TREB5**: lanes 1, 2, 3 and 4 contain ~90, 45, 23 and 11 μg of protein, respectively. 'Mock' was an extract of bacteria with pET3a vector alone. The position of the 21 bp enhancer and the flanking sequences are shown diagrammatically, and their sequences are indicated on the left in upper- and lower-cases, respectively.

These proteins were partially purified on a heparin–Sepharose column and used for DNase I footprinting assay (Figure 4). When the LTR sequence was used as a probe, all four proteins preferentially protected the 21 bp sequence, although the protection patterns were different between these proteins: no TREB and CREB proteins efficiently protected the 3′ region of the 21 bp sequences and TREB7 and TREB36 protected the 5′ and middle regions of the 21 bp sequence, while TREB5 and CREB1 preferentially protected a narrower portion of the middle of the second 21 bp sequence. These results with TREB7 and TREB36 are consistent with the requirement for protein involved in the *trans*-activation, since the 5′ and the middle portions of the 21 bp repeat are essential for *trans*-activation by $p40^{tax}$ and the 3′ portion is dispensable (Fujisawa *et al.*, 1989). We also

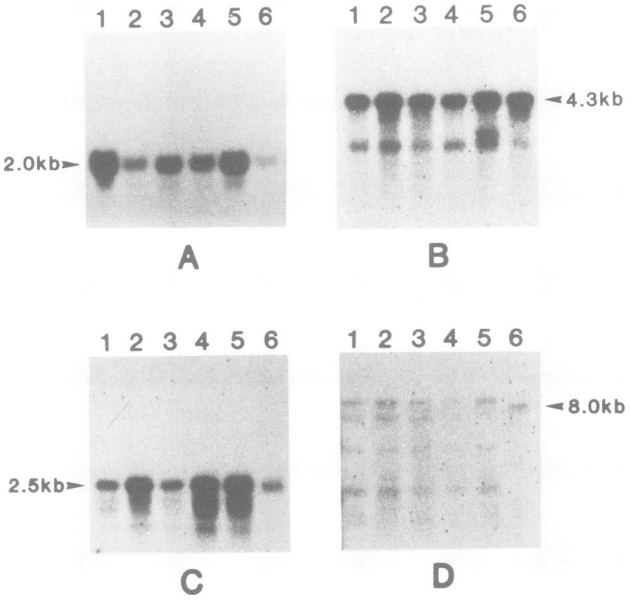

Fig. 5. Expression of TREB mRNAs in various cell lines. Poly(A)$^+$ RNA from different cell lines was analyzed by RNA blotting using each cDNA as a probe. **Panel A**, T5 cDNA; **panel B**, T7; **panel C**, T36; **panel D**, CREB. **Lane 1**, HUT102 (HTLV-1 infected human T cell line); **lane 2**, Jurkat (human T cell line); **lane 3**, Namalwa (human Burkitt cell line); **lane 4**, HeLa (human carcinoma cell line); **lane 5**, FL (human epithelial cell line); **lane 6**, PC 12 (rat pheochromocytoma cell line).

noticed other variations in the protection: a given protein showed different affinities for each of the three 21 bp units. For example, the 21 bp sequence closest to the 3′ end was not protected by TREB5, whereas it was protected by the other proteins (see Figure 4); furthermore, the second repeat of the 21 bp sequence was protected differently from other repeats with CREB1. These observations indicate that the flanking sequence and/or small variations in the 21 bp sequence affect the binding affinities of TREB and CREB proteins. In addition, the TREB5, TREB7 and TREB36 proteins protected sequences outside the 21 bp repeats and induced some DNase I sensitive sites (Figure 4).

Expression of TREB mRNAs in various cells

The $p40^{tax}$ protein *trans*-activates the HTLV-1 enhancer in many types of cell lines including mouse and chicken cell line (Sodroski *et al.*, 1984; Fujisawa *et al.*, 1985), whereas *trans*-activation of other promoters, such as those of the genes for IL-2Rα (Inoue *et al.*, 1986; Maruyama *et al.*, 1987; Cross *et al.*, 1987) or GM-CSF (Miyatake *et al.*, 1988) has been observed only in some cell types. These facts prompted us to examine the expression of the TREB5, TREB7, TREB36 and CREB in mRNAs in various cell lines (Figure 5). The expression of the mRNAs of all these proteins, including CREB, were detected by Northern blot analysis in all of the cell lines tested. The ubiquitous expression of the three genes may partly explain the specificity of the *trans*-activation by $p40^{tax}$ in a wide variety of cell lines.

The cDNA probes of TREB5, TREB7 and TREB36 detected main bands of ~2.0, 4.3 and 2.5 kb, respectively, and some other minor bands. We detected multiple bands of CREB mRNA of almost equal intensities in human cells, but a single major band in rat cells (Figure 5), as reported

by Gonzalez *et al.* (1989). Preliminary analysis suggested that the multiple RNAs reflect both alternative splicing at several sites and alternative polyadenylation (data not shown).

Discussion

In this study, we isolated three different human cDNA clones encoding proteins that bind specifically to the 21 bp enhancer of HTLV-1. These proteins are novel species and are strong candidates for factors involved in transcriptional *trans*-activation by $p40^{tax}$, because they have the properties of *trans*-activators predicted from previous studies; namely, that they have the ability to interact with the 12 bp sequence in the 21 bp sequence and that they differ from the CREB and from AP-1 and NF-kB (proteins responsive to TPA stimulation) (Fujisawa *et al.*, 1989).

Sequence analysis of these clones showed that all the proteins have a leucine zipper structure and an adjacent basic amino acid domain (Figure 3). These motifs are conserved in other DNA binding proteins such as FOS (Straaten *et al.*, 1983), JUN (Bohmann *et al.*, 1987) and CREB (Gonzalez *et al.*, 1989) and can form a hetero- or homodimer and bind to the respective DNA element (Rausher *et al.*, 1988; Halazonetis *et al.*, 1988; Yamamoto *et al.*, 1988). Therefore, the presence of the leucine zipper motif and basic domain in these TREBs suggests that the functional unit may be a homo- or heterodimer. Heterodimers could be formed with different TREBs, or CREB, or even with proteins outside this family such as FOS and JUN, expanding the complexity of gene regulation in the signal transduction cascade.

In accordance with this idea, TREBs contain multiple putative activation motifs for transcription that are rich in acidic amino acids, proline or glutamine (Mitchell and Tjian, 1989) (see Figure 2). The locations and contents of these amino acids are as follows: acidic amino acids: 145–154 (6/15 = 40%) in TREB5 and 37–46 (50%) in TREB36; proline: 8–56 (18%) in TREB5 and glutamine: 174–253 (18%) in TREB5 and 102–180 (19%) in TREB36. In addition, all TREBs have several potential sites for phosphorylation by protein kinase A (Kemp *et al.*, 1977) and/or protein kinase C (Kishimoto *et al.*, 1985), and casein kinase II (Marin *et al.*, 1986; Kuenzel *et al.*, 1987) (see Figure 2). Phosphorylation of these sites could affect the activity of these proteins.

Since the CRE consensus sequence is essential for TREB binding, TREB might be classified into the CREB family. In fact, one of the three TREBs, TREB36, shows sequence homology to the CREB protein. However, each TREB protein differs slightly in DNA binding properties: in DNase I footprinting, all four proteins protected the 21 bp sequences; however, TREB7 and TREB36 protected the 5′ and middle regions of the 21 bp, which are essential for $p40^{tax}$ mediated *trans*-activation (see Figure 4), while TREB5 and CREB1 protected a narrower region of the 21 bp repeat containing the CRE, although this was apparent only on the middle repeat of the 21 bp sequence. Therefore, TREB7 and TREB36 are the most likely proteins to be involved in *trans*-activation by $p40^{tax}$, although we do not have any direct evidence that TREB is involved in *trans*-activation. As another variation, TREB proteins also protected the flanking sequences of the 21 bp sequence, but differed in their bindings to the flanking sequences. Another interesting finding was that the protection patterns of each unit of three 21 bp repeats in the LTR were not identical even with a given protein, for example, TREB5 preferentially protected only the middle unit of the 21 bp, and CREB1 protected the middle unit of 21 bp differently from the other two units. These observations indicate that the binding of each protein differs depending on the flanking sequences and/or a few base substitutions in each 21 bp sequence.

$p40^{tax}$ also enhances the transcription of the IL-2α gene, which depends on the binding activity of the NF-κB-like factor (Leung and Nabel, 1988). The NF-κB-like factor is certainly different from the TREBs described here, so $p40^{tax}$ can modulate the activities of at least two transcriptional factors, although there is still a possibility that an increase of NF-κB-like activity is mediated through transcriptional induction of its gene by $p40^{tax}$. Further studies on protein–protein interaction between $p40^{tax}$ and cellular transcriptional factors should be very useful for understanding the molecular mechanisms of transcriptional activation.

During the preparation of this manuscript, Hai *et al.* (1989) reported the isolation of cDNA clones encoding transcription factor ATF from HeLa and MG63 λgt11 libraries. Comparing our data with the amino acid sequences deduced from their partial cDNA clones, we found that TREB7 and TREB36 correspond to their factors ATF-2 and ATF-1, respectively.

Materials and methods

Preparation and screening of cDNA libraries

RNA was prepared from HUT 102, an HTLV-1 infected human T cell line, by the ribonucleoside–vanadyl complex method (Berger, 1987), and RNA containing poly(A) was selected on oligo(dT)–cellulose. cDNAs were prepared by the method of Gubbler and Hoffman (1983) using either random hexanucleotides (for the R series) or oligo$(dT)_{12-18}$ (for the T series) as primers. Each cDNA preparation was cloned into *Eco*RI digested λgt11 vector according to the method of Huynh *et al.* (1985).

Recombinant λ phages of the R-series were infected into *E.coli* Y1090 and induced to produce β-galactosidase fusion proteins as described by Huynh *et al.* (1985). Protein replica filters were prepared by transferring colonies to nitrocellulose filters and screened with the five repeats of the double stranded wild-type 21 bp sequence (CTAGGCCCTGACGTGTCCCCCTG) as described by Singh *et al.* (1988). A cDNA library of the T series was screened using cDNA of λR5, λR7 or λR36 as a probe by the standard hybridization method.

Preparation of proteins produced in E.coli

E.coli Y1089 lysogens harboring λR5, λR7 or λR36 were induced to express their respective products and extracts were prepared (Huynh *et al.*, 1985). The extracts were subjected to electrophoresis in 7% SDS–PAGE followed by South-western analysis using a double-stranded oligonucleotide.

For expression of TREB proteins in *E.coli*, the respective coding sequence was inserted between the *Nhe*I and *Bam*HI sites of the pET3a vector (Rosenberg *et al.*, 1987) using *Xba*I or *Nhe*I, and *Bam*HI or *Bgl*II linkers. The expression plasmids were then transfected into BL21(DE3)pLysS, an *E.coli* strain, and induced to express their cDNA product. The lysate was partially purified on heparin–Sepharose as described previously (Maekawa *et al.*, 1989), and used for a DNase I footprinting assay.

DNase I footprint analysis

The probe DNA used for the DNase I footprint was the *Hind*III–*Xho*I fragment of the LTR isolated from pLTR-CAT (Fujisawa *et al.*, 1985). The sense strand was labeled at the 5′ terminus. Protein that had been partially purified on heparin–Sepharose was mixed with the DNA probe and subjected to a DNase I footprinting assay as described previously (Maekawa *et al.*, 1989).

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