Isolation of cDNAs for DNA-Binding Proteins Which Specifically Bind to a *tax*-Responsive Enhancer Element in the Long Terminal Repeat of Human T-Cell Leukemia Virus Type I

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Received 10 September 1990/Accepted 12 December 1990

One of the gene products of human T-cell leukemia virus type I (HTLV-I), p40^{tax}, activates its own viral transcription in *trans* through *tax*-responsive enhancers in viral long terminal repeats. Five species of cDNA clones for proteins that bind to the *tax*-responsive enhancer element in HTLV-I were isolated from the Jurkat cell library. The β -galactosidase fusion protein prepared from the lysogen of a clone specifically recognized the cyclic AMP-responsive element in HTLV-I enhancer. The nucleotide sequence of a full-length cDNA clone (TAXREB67) had a coding capacity of 351 amino acids, which contained a basic motif followed by a leucine zipper structure near the carboxy terminus. Its mRNA was detected in human cell lines, including HTLV-I-infected or noninfected hematopoietic cell lines. The mRNA level in Jurkat cells was decreased temporarily by increasing cyclic AMP concentration but increased by increasing Ca²⁺ concentration. Polyclonal antibodies against the fusion protein specifically recognized a 52-kDa protein in Jurkat cells. Analyses of the function of this protein and its interactions with other cellular factors will be useful to help understand the regulatory mechanism through *tax*-responsive enhancers in HTLV-I.

Human T-cell leukemia virus type I (HTLV-I) is an exogenous human retrovirus closely associated with adult T-cell leukemia (17, 46, 65) and a certain type of degenerative neurological disorder (11, 45, 61, 66), although the mechanism of disease development by HTLV-I still remains to be elucidated.

HTLV-I has a unique genomic region termed X in which three species of proteins are translated from a single species of doubly spliced mRNA (39). One of these three proteins, p40^{tax}, works in *trans* as a transcriptional activator for its own viral promoter (9, 58) and also for some promoters of cellular genes such as interleukin-2 (23, 56), interleukin-2 receptor (5, 56), *c-fos* (8, 40), granulocyte-macrophage colony stimulating factor (36), and vimentin (32). These suggest the involvement of this *trans*-activation mechanism by p40^{tax} in some cellular proliferation or differentiation processes during development of adult T-cell leukemia. However, in the promoter regions of cellular genes which can be activated by p40^{tax}, no consensus sequence responsive for p40^{tax} activation has been found. In the promoter region in the long terminal repeat of HTLV-I, the 21-bp elements, which are imperfectly repeated three times, have been identified as *tax*-responsive enhancer elements (2, 49, 55). Among the elements, three parts of oligonucleotide sequences, named domains A, B, and C (Fig. 1) by Fujisawa et al. (10), are known to be completely conserved. Of these three domains, at least B is reported to be essential for *tax*-mediated transcriptional activation through the 21-bp enhancer element (10, 12). However, the importance of the A and C domains is still controversial (10, 12). Also in this 21-bp element, a sequence almost identical to the cyclic AMP (cAMP)-responsive element (CRE) or the activating transcription factor binding site is found in domain B, and a sequence highly homologous to the nuclear factor kappa B

binding site is found in domains B and C (Fig. 1). The transcriptional activation by cAMP and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been reported to occur through the 21-bp element (47, 48, 59). However, accumulating data suggest that the *trans* activation through the 21-bp enhancer element by p40^{tax} utilizes a mechanism different from those involved in the two pathways of signal transduction (10, 25, 47, 48, 59). p40^{tax} has been reported to be unable to bind specifically to DNA strands (1, 24), and requirement of preexisting cellular proteins for its *trans* activation was reported (35, 37). Therefore, multiple factors are considered to be concerned with the transcriptional activation through this 21-bp enhancer element. As one approach to understanding the mechanism of *trans* activation through the 21-bp enhancer element, we isolated cDNA clones whose coding proteins specifically bind to this enhancer element.

MATERIALS AND METHODS

cDNA library. A λ gt11 cDNA library made from poly(A) RNA of Jurkat cells was purchased from Clontech (Palo Alto, Calif.). Another cDNA library constructed with plasmid vector pcDV1 made from poly(A) RNA of simian virus 40-transformed human fibroblast cells was a kind gift from Hiroto Okayama (44).

Cell lines. Jurkat, P30/OHK, TALL-1, Molt4, MT-2, OCH, and C91/PL are CD4⁺ CD8⁻ T-cell lines. BALL-1 is a B-cell line. These cell lines and other cell lines such as U937, HL60, and HeLa are all of human origin. MT-2, OCH, and C91/PL were infected with HTLV-I, and the other cell lines were free from HTLV-I infection. Hematopoietic cell lines were cultured with RPMI 1640 supplemented with 10% fetal bovine serum, and HeLa cells were cultured with Dulbecco's modified minimal essential medium supplemented with 5% fetal bovine serum.

Oligonucleotide probes. Oligonucleotide probes used for this study are listed in Fig. 1. A 26-mer of the oligonucleotide

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FIG. 1. Nucleotide sequences of *tax*-responsive enhancer elements and the probes. (A) Nucleotide sequences of three *tax*-responsive 21-bp enhancer elements in the HTLV-I long terminal repeat. The three enhancers are numbered I, II, and III from distal to proximal. Three domains, A, B, and C, which are conserved in all three enhancers, are shown in the boxes. The consensus sequences of CRE and nuclear factor kappa B are shown below. (B) Nucleotide sequences of the oligonucleotide probes. The wild type (W) has the sequence of the 21-bp enhancer I. R, A(-), B(-), and C(-) have point mutations, as indicated with underlined lowercase letters.

containing the 21-bp sequence at the most distal position from the initiation of transcription was used as a wild-type sequence (W). A randomized sequence (R) and three other oligonucleotides, A(-), B(-), and C(-), with mutations in a few bases in domains A, B, and C, respectively, were also used. For the convenience of catenation, *Hind*III sites were introduced at both ends of each complementary sequence. Oligonucleotides were kinased with [³²P]γ-ATP and T4 polynucleotide kinase, annealed by slowly cooling, and catenized with T4 DNA ligase. These catenized fragments were size fractionated by gel filtration with a Sephacryl S-300 column (diameter, 0.6 cm; length, 20 cm) in 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA–0.1 M NaCl. The fractions containing more than nine tandem repeats of the oligonucleotides were identified by polyacrylamide gel electrophoresis (PAGE), collected, and used as probes.

Screening of the cDNA library and sequencing. The experiment was performed by following the protocol first reported by Singh et al. (57) and modified by Vinson et al. (63) and Sakura et al. (53). Nucleotide sequence was determined by the dideoxy-chain termination method (54).

Preparation of the β-galactosidase fusion protein from lysogen. *Escherichia coli* Y1089 was used for infection with vector (λgt11) or recombinant phage clones to generate lysogen. Expression of the β-galactosidase fusion protein was induced by temperature treatment and addition of isopropyl-β-D-thiogalactopyranoside (IPTG), as described by Huynh et al. (22). The fusion protein was partially purified by a leach method to isolate insoluble protein (43). Briefly, the bacteria were sonically disrupted in S buffer containing 50 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and fractionated by centrifugation for 20 min in a Sorvall HB-4 rotor at 9,000 rpm. The insoluble fraction was extracted with TD buffer consisting of 1% Triton X-100, 1% deoxycholic acid (Na salt), 150 mM NaCl, 50 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The final insoluble material containing the fusion protein was dissolved in Laemmli's buffer (30) and separated by preparative sodium

dodecyl sulfate (SDS)-PAGE. The fusion protein was eluted from the gel in the electrophoresis buffer containing 0.1% SDS and used for intradermal injection into female Hartley guinea pigs to obtain polyclonal antibody.

Southwestern blot analysis of the β-galactosidase fusion protein. The β-galactosidase fusion protein prepared from the lysogen extract was separated on SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Immobilon, Millipore Co.). The membrane was extensively washed with 50 mM Tris hydrochloride (pH 7.4) before blocking at 4°C for 2 h with the binding buffer used for cDNA screening (63) containing 5% skim milk (Difco). The membrane was then washed with the binding buffer containing 0.25% skim milk for 15 min and incubated at 4°C overnight in the binding buffer [containing 0.25% skim milk, 10 μg of calf thymus DNA per ml, and 4 μg of poly(dI-dC) per ml] and radioactive oligonucleotide probe. Finally the membrane was washed four times for 15 min each with the binding buffer containing 0.25% skim milk and briefly dried for autoradiography.

RNA blot hybridization. Each total cellular RNA was prepared from approximately 5 × 10⁶ cells by using acid guanidinium thiocyanate-phenol-chloroform extraction by the protocol described by Chomczynski and Sacchi (3). For the experiments of addition of chemicals, cells were harvested 0, 2, and 8 h after addition. Ten micrograms of each total cellular RNA was subjected to blot hybridization analysis with nick-translated probes.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under the accession number D90209.

RESULTS

Cloning of cDNAs encoding *tax*-responsive enhancer binding proteins. By using a wild-type 21-bp enhancer sequence as a probe, six species of cDNAs were isolated from approximately 10⁷ clones from a cDNA library of Jurkat cells. Six clones, which had the longest insert in each species of cDNA, were named λ1, λ67, λ107, λ302, λ703, and λ803. Partial nucleotide sequences of the inserts in these six clones were determined. Of these six clones, λ1 was found to have a sequence identical to that of YB-1, which had been reported to bind to the Y box sequence in the promoter region of class II human major histocompatibility complex antigen genes (7). Five other clones did not show any obvious homology with reported DNA sequences (data not shown). A full-length cDNA for the λ67 clone was isolated from a cDNA library of human fibroblasts in pcDV1 by colony hybridization, and its nucleotide sequence was determined. The plasmid clone was named pcD67.

Nucleotide sequence of the insert of pcD67. Figure 2 shows the nucleotide sequence of the insert of pcD67, which had a length of 2,015 nucleotides from its 5' end to the beginning of polyadenylation. The original clone, λ67, which was isolated from the Jurkat cDNA library, contained the fragment corresponding to the region from nucleotide 1,162 to the end of the insert of pcD67 (nucleotide 2,015). Curiously, this insert of λ67 was found to have an extra 48-bp sequence in its 5' end, the nucleotide sequence of which was completely different from that of the corresponding region of pcD67 isolated from the human fibroblast cDNA library (data not shown). It might be due to tissue-specific alternative splicing or might be a cloning artifact. In the sequence of the pcD67 insert, four open reading frames (ORFs) longer than 30

	GTTTTCTACTTTGCCCGCCACAGATGTAGTTTCTCTGCGGTGTGC																																	
49	GTTTTCCCTCCTCCCCGCCCTCAGGGTCCACGGCCACCATGGCGTATTAGGGGCAGAGTGCCTGCGGCAGCATTGGCCTTTGACAGCGCGGCAGCAGCACCAGGCTCTGCAGCGGCA																																	
168	ACCCCCAGCGGCTTAAGCCATGGCGTGAGTACCGGGGCGGGTCTGCTCAGCTGTGCTCCTGGGGCGGCGCGGGTTTGGATTGGTGGGGTGGCGCTGGGGCCAGGGCGGTGCCGCCAA																																	
287	GGGGGAAGCATTTAAACGAGCGCCCGGACGCGTGGTCTTTGCTTTGGGTGTCCCGAGACGCTCGCGTGCCTGGGATCGGGAAGCGTAGTCGGGTGCCCGGACTGCTTCCCGAGGAGC																																	
406	CCTACAGCCCTCGGACCCCGAGGCCCGCAAGGTCCCAGGGGTCTTTGGCTGTGCCCCACGAAACGTGCAGGAACCAAGATGGCGGCGGCAGGGCGGCGCGCGGGCGGTGAGTCAAGGGC																																	
525	GGGCGGTGGGCGGGGCGCGGCGCTGGCCGTATTGGACGTGGGGACGGAGCGCTTTCTCTTTGGCGGCGGTGGAAGAATCCCTGGTCTCCGTGAGCGTCCATTTTGTGGAACCTGA																																	
644	GTTGCAAGCAGGGAGGGCAATAACAACCTGCCCTGTTCCCGATTCTCTAGATGGCGCATCTAGAGAAGTCCCGCCTCATAAGTGAAGGATGAAATCTCAGAACAGCTAACCTCTAAT																																	
763	GGGAGTTGGCTTCTGATTCTCATTTCAGGCTTCTCAGGCATTACAGCAGCAGCTTGTGTAACCGACAAAGACACCTTCGAATTAAGCACATTCTCGATTCCAGCAAAAGCACCGCAAC																																	
882	ATG	ACC	GAA	ATG	AGC	TTC	CTG	AGC	AGC	GAG	GTG	TTG	GTG	GGG	GAC	TTG	ATG	TCC	CCC	TTC	GAC	CCG	TCG	GGT	TTG	GGG	GCT	GAA	GAA	AGC				30
	Met	Thr	Glu	Met	Ser	Phe	Leu	Ser	Ser	Glu	Val	Leu	Val	Gly	Asp	Leu	Met	Ser	Pro	Phe	Asp	Pro	Ser	Gly	Leu	Gly	Ala	Glu	Glu	Ser				
972	CTA	GGT	CTC	TTA	GAT	GAT	TAC	CTG	GAG	GTG	GCC	AAG	CAC	TTC	AAA	CCT	CAT	GGG	TTC	TCC	AGC	GAC	AAG	GCT	AAG	GCG	GGC	TCC	TCC	GAA				60
	Leu	Gly	Leu	Leu	Asp	Asp	Tyr	Leu	Glu	Val	Ala	Lys	His	Phe	Lys	Pro	His	Gly	Phe	Ser	Ser	Asp	Lys	Ala	Lys	Ala	Gly	Ser	Ser	Glu				
1062	TGG	CTG	GCT	GTG	GAT	GGG	TTG	GTC	AGT	CCC	TCC	AAC	AAC	AGC	AAG	GAG	GAT	GCC	TTC	TCC	GGG	ACA	GAT	TGG	ATG	TTG	GAG	AAA	ATG	GAT				90
	Trp	Leu	Ala	Val	Asp	Gly	Leu	Val	Ser	Pro	Ser	Asn	Asn	Ser	Lys	Glu	Asp	Ala	Phe	Ser	Gly	Thr	Asp	Trp	Met	Leu	Glu	Lys	Met	Asp				
1152	TTG	AAG	GAG	TTC	GAC	TTG	GAT	GCC	CTG	TTG	GGT	ATA	GAT	GAC	CTG	GAA	ACC	ATG	CCA	GAT	GAC	CTT	CTG	ACC	ACG	TTG	GAT	GAC	ACT	TGT				120
	Leu	Lys	Glu	Phe	Asp	Leu	Asp	Ala	Leu	Leu	Gly	Ile	Asp	Asp	Leu	Glu	Thr	Met	Pro	Asp	Asp	Leu	Leu	Thr	Thr	Leu	Asp	Asp	Thr	Cys				
1242	GAT	CTC	TTT	GCC	CCC	CTA	GTC	CAG	GAG	ACT	AAT	AAG	CAG	CCC	CCC	CAG	ACG	GTG	AAC	CCA	ATT	GGC	CAT	CTC	CCA	GAA	AGT	TTA	ACA	AAA				150
	Asp	Leu	Phe	Ala	Pro	Leu	Val	Gln	Thr	Asn	Lys	Gln	Pro	Pro	Gln	Thr	Val	Asn	Pro	Ile	Gly	His	Leu	Pro	Glu	Ser	Leu	Thr	Lys					
1332	CCC	GAC	CAG	GTT	GCC	CCC	TTC	ACC	TTC	TTA	CAA	CCT	CTT	CCC	CTT	TCC	CCA	GGG	GTC	CTG	TCC	TCC	ACT	CCA	GAT	CAT	TCC	TTT	AGT	TTA				180
	Pro	Asp	Gln	Val	Ala	Pro	Phe	Thr	Phe	Leu	Gln	Pro	Leu	Pro	Leu	Ser	Pro	Gly	Val	Leu	Ser	Ser	Thr	Pro	Asp	His	Ser	Phe	Ser	Leu				
1422	GAG	CTG	GGC	AGT	GAA	GTG	GAT	ATC	ACT	GAA	GGA	GAT	AGG	AAG	CCA	GAC	TAC	ACT	GCT	TAC	GTT	GCC	ATG	ATC	CCT	CAG	TGC	ATA	AAG	GAG				210
	Glu	Leu	Gly	Ser	Glu	Val	Asp	Ile	Thr	Glu	Gly	Asp	Arg	Lys	Pro	Asp	Tyr	Thr	Ala	Tyr	Val	Ala	Met	Ile	Pro	Gln	Cys	Ile	Lys	Glu				
1512	GAA	GAC	ACC	CCT	TCA	GAT	AAT	GAT	AGT	GGC	ATC	TGT	ATG	AGC	CCA	GAG	TCC	TAT	CTG	GGG	TCT	CCT	CAG	CAC	AGC	CCC	TCT	ACC	AGG	GGC				240
	Glu	Asp	Thr	Pro	Ser	Asp	Asn	Asp	Ser	Gly	Ile	Cys	Met	Ser	Pro	Glu	Ser	Tyr	Leu	Gly	Ser	Pro	Gln	His	Ser	Pro	Ser	Thr	Arg	Gly				
1602	TCT	CCA	AAT	AGG	AGC	CTC	CCA	TCT	CCA	GGT	GTT	CTC	TGT	GGG	TCT	GCC	CGT	CCC	AAA	CCT	TAC	GAT	CCT	CCT	GGA	GAG	AAG	ATG	GTA	GCA				270
	Ser	Pro	Asn	Arg	Ser	Leu	Pro	Ser	Pro	Gly	Val	Leu	Cys	Gly	Ser	Ala	Arg	Pro	Lys	Pro	Tyr	Asp	Pro	Pro	Gly	Glu	Lys	Met	Val	Ala				
1692	GCA	AAA	GTA	AAG	GGT	GAG	AAA	CTG	GAT	AAG	AAG	CTG	AAA	AAA	ATG	GAG	CAA	AAC	AAG	ACA	GCA	GCC	ACT	AGG	TAC	CGC	CAG	AAG	AAG	AGG				300
	Ala	Lys	Val	Lys	Gly	Glu	Lys	Leu	Asp	<u>Lys</u>	<u>Lys</u>	Leu	<u>Lys</u>	<u>Lys</u>	Met	Glu	Gln	Asn	<u>Lys</u>	Thr	Ala	Ala	Thr	<u>Arg</u>	Tyr	<u>Arg</u>	Gln	<u>Lys</u>	<u>Lys</u>	<u>Arg</u>				
1782	GCG	GAG	CAG	GAG	GCT	CTT	ACT	GGT	GAG	TGC	AAA	GAG	CTG	GAA	AAG	AAG	AAC	GAG	GCT	CTA	AAA	GAG	AGG	GCG	GAT	TCC	CTG	GCC	AAG	GAG				330
	Ala	Glu	Gln	Glu	Ala	<u>Leu</u>	Thr	Gly	Glu	Cys	Lys	Glu	<u>Leu</u>	Glu	Lys	Lys	Asn	Glu	Ala	<u>Leu</u>	Lys	Glu	Arg	Ala	Asp	Ser	<u>Leu</u>	Ala	Lys	Glu				
1872	ATC	CAG	TAC	CTG	AAA	GAT	TTG	ATA	GAA	GAG	GTC	CGC	AAG	GCA	AGG	GGG	AAG	AAA	AGG	GTC	CCC	TAG	TTG	AGG	ATG	CAG	GAG	CGT	CAAT	GTG	GCT			351
	Ile	Gln	Tyr	<u>Leu</u>	Lys	Asp	Leu	Ile	Glu	Glu	Val	Arg	Lys	Ala	Arg	Lys	Lys	Arg	Val	Pro	***													
1969	TACATAGAGTGCTGTAGCTGTGTGTTCCAATAAATTATTTGTAGGG																																	

FIG. 2. Nucleotide sequence of the insert of pcD67. Numbers of the nucleotides begin at the first nucleotide of the insert next to the G tail. The amino acid sequence corresponding to the longest ORF is also shown. Basic amino acids of the basic motif are underlined. Five leucines which can make a leucine zipper structure are underlined twice.

nucleotides, which relatively matched Kozak's consensus (29), could be found. The original clone, λ 67, contained only one ORF, which corresponded to the longest among these four. The amino acid sequence of this longest ORF, 351 amino acids long, is shown in Fig. 2. It had a leucine zipper structure (31) near its carboxy terminus (from amino acids 306 to 334). A stretch of basic amino acid residues, which is suggested to be necessary for sequence-specific DNA binding (6, 28, 42), was found just next to the amino terminus of the leucine zipper. This polypeptide was named TAXREB67.

Expression of the β -galactosidase fusion protein encoded by λ 67. To analyze the characteristics of the phage clone λ 67, a lysogen of this clone was established by using *E. coli* Y1089. The expected molecular mass of the β -galactosidase fusion protein produced by this lysogen was approximately 142 kDa, while intact β -galactosidase produced by a lysogen with λ gt11 vector alone has a molecular mass of 116 kDa (22, 26). Intact β -galactosidase was shown to be abundantly expressed in the lysogen made with λ gt11 after 2 h of IPTG induction (Fig. 3A). In contrast, when the extract of lysogen with λ 67 was used, several protein bands larger than 116 kDa were specifically observed after 2 h of IPTG induction. Among those bands, the largest one corresponded to the expected size of the fusion protein encoded by λ 67. These protein bands were confirmed by the immunostaining assay shown in Fig. 3B by using antibody against β -galactosidase.

FIG. 3. Expression of TAXREB67 and the β -galactosidase fusion protein. (A) Coomassie staining after SDS-PAGE of lysogen extract. Extracts of λ gt11 lysogen collected at 0 h (lane 1) and at 2 h after IPTG induction (lane 3) and extracts of λ 67 lysogen collected at 0 h (lane 2) and 2 h after IPTG induction (lane 4) were analyzed. (B) Immunostaining after SDS-PAGE of lysogen extract. An immunoblot obtained after SDS-PAGE was treated with anti- β -galactosidase antibody. Four lanes are aligned in exactly the same order as those described for panel A. Arrowheads indicate β -galactosidase (lane 3) and the fusion protein (lane 4).

FIG. 4. Southwestern blot analysis. Southwestern blot analysis of lysogen extract. Extract of the lysogen with λ gt11 was probed with W (lane 1), R (lane 3), A(-) (lane 5), B(-) (lane 7), or C(-) (lane 9). Extract of the lysogen with λ 67 was probed with W (lane 2), R (lane 4), A(-) (lane 6), B(-) (lane 8), and C(-) (lane 10).

Unlike most of the cellular proteins, the fusion protein was found to be resistant to the solubilizers, such as Triton X-100, deoxycholic acid, and a high salt concentration, and to be enriched in the insoluble pellet after centrifugation (data not shown). The sample enriched in the fusion protein was then subjected to preparative SDS-PAGE to obtain partially purified protein for immunization.

Southwestern blot analysis. To examine the DNA-binding specificity of the fusion protein, Southwestern blot analysis was performed with a series of probes (Fig. 1). The results are shown in Fig. 4. In the case of the extract of the λ 67 lysogen, a single specific band was observed at the expected size of the fusion protein in the lanes probed with the W, A(-), and C(-) probes. No band was detected in the lanes probed with either the R or the B(-) sequence. As for the lysogen extract made with λ gt11, none of the probes bound to any protein.

Expression of TAXREB67 mRNA in human cell lines. The expression of TAXREB67 mRNA was analyzed with various human cell lines by RNA blot hybridization by using the insert of λ 67 as a probe. As a result, TAXREB67 mRNA was expressed as a single species of approximately 2 kb in all the cell lines examined, including four HTLV-I-negative T-cell lines (P30/OHK, TALL-1, Molt4, and Jurkat), three HTLV-I-producing cell lines (MT-2, OCH, and C91/PL), a B-cell line (BALL-1), a monocytic cell line (U937), an undifferentiated myelocytic leukemia cell line (HL60), and a HeLa cell line (data not shown).

Effect of cAMP and calcium concentrations on the expression of TAXREB67 mRNA. Effects of intracellular cAMP and Ca^{2+} on the expression of TAXREB67 mRNA were analyzed with Jurkat cells by RNA blot hybridization. The concentration of intracellular cAMP was increased by addition of 300 ng of *Vibrio cholerae* toxin (Sigma) per ml, 20 μM forskolin (Sigma), or 8 mM dibutyryl cAMP (Sigma) to the culture medium of Jurkat cells. Changes in levels of TAXREB67 mRNA in the presence of each of these reagents were similar to one another. The mRNA level decreased significantly after incubating cells with each reagent for 2 h (Fig. 5A). After the incubation for 8 h, however, the level increased and was almost comparable to that of the initial period. On the other hand, a significant increase in the level of mRNA of TAXREB67 was observed when the cells were treated with 10 ng of TPA per ml or 200 nM calcium ionophore (A23187 [Sigma]) for 2 h (Fig. 5B). No synergistic

FIG. 5. Effect of intracellular cAMP and calcium levels on the expression of TAXREB67 mRNA in Jurkat cells. (A) Effect of cAMP level. Total cellular RNAs from Jurkat cells before stimulation (lane 1), after 2 h of incubation with *V. cholerae* toxin (lane 2), forskolin (lane 3), or dibutyryl cAMP (lane 4), and after 8 h of incubation with *V. cholerae* toxin (lane 5), forskolin (lane 6), or dibutyryl cAMP (lane 7) were probed with the insert of λ 67. (B) Effect of calcium level. Total cellular RNAs from Jurkat cells before stimulation (lane 1) and after 2 h of stimulation with TPA (lane 2), A23187 (lane 3), or TPA and A23187 (lane 4) were probed with the insert of λ 67.

effect on the mRNA level of TAXREB67 after 2 h of incubation was observed with A23187 plus TPA (Fig. 5B).

Identification of authentic TAXREB67 protein. To raise polyclonal antibody against TAXREB67, guinea pigs were immunized with highly purified β -galactosidase fusion protein prepared from the lysogen made with λ 67. After immunization at 2-week intervals for 8 weeks, antisera were collected from the animals. The resultant polyclonal antibody specifically reacted with the fusion protein used for immunization, with *E. coli* β -galactosidase (Sigma), and with full-length recombinant TAXREB67 protein produced in *E. coli* that had no β -galactosidase fusion (data not shown). By using this antibody, the authentic TAXREB67 in Jurkat cells was detected by Western immunoblot analysis (Fig. 6). The protein specifically recognized by the antibody had an apparent molecular mass of 52 kDa, which was slightly larger than the calculated molecular mass of TAXREB67 encoded by the full-length cDNA.

DISCUSSION

By using the 21-bp enhancer sequence in the long terminal repeat of HTLV-I as a probe, six species of cDNAs that code for DNA-binding proteins were isolated from the cDNA library derived from Jurkat cells. One of the six, YB-1, was reported by Didier et al. (7) to be a DNA-binding protein which specifically binds to the Y box sequence TAACC in the promoter region of class II human major histocompatibility complex antigen genes. However, this protein also bound to the 21-bp enhancer sequence in HTLV-I and to the randomized 21-bp probe, R (data not shown). Therefore YB-1 seems to have a broader binding specificity than had been previously thought. Didier et al. (7) reported a nucleotide sequence of 1,512 bp, while our clone contained a longer sequence and its ORF can be continued further in the 5' direction (data not shown).

Two of the other five clones showed sequence specificity

FIG. 6. Western blot analysis of authentic TAXREB67. Jurkat cells washed with phosphate-buffered saline were resuspended in Laemmli's buffer and heated for 5 min at 100°C to provide crude cell extract for Western blot analysis. The blot was incubated with control serum obtained from an uninjected animal (lane 1) or with antiserum to the fusion protein containing the carboxy-terminal region of TAXREB67 (lane 2). Molecular mass markers were purchased from Sigma (SDS-6H and SDS-7): myosin, 205 kDa; β -galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa.

for binding to domain B, and the other three showed sequence specificity for binding to domain C (unpublished results). No clone with a sequence specificity for binding to domain A was isolated. This result seems to be consistent with the fact that there has been no report of identification of any protein which specifically binds to domain A. However, mutations introduced in this domain are reported to significantly reduce the enhancer function of transcriptional activation caused by p40^{tax} (10). Therefore, this sequence of domain A might be necessary not for protein binding but for some other function. Alternatively, regulatory protein binding to domain A might be expressed on a very specific occasion.

TAXREB67 has a leucine zipper structure just carboxy terminal to a stretch of basic amino acids. To date, more than 10 DNA-binding proteins have been reported to have similar structures, and they constitute a gene family. It includes the AP-1 family of *c-fos*, *c-jun*, and related proteins (4, 18, 50, 51, 52, 62, 68), yeasts YAP-1 (38) and GCN4 (16), CRE binding proteins (13, 19, 34, 67), and certain CRE binding protein-like proteins derived from plants (27). Some amino acids in the basic motifs of various proteins in this family are known to be well conserved, and TAXREB67 shares most of them. This result shows that TAXREB67 is a member of this family. Yoshimura et al. (67) also reported the isolation of three cDNA clones which code for 21-bp enhancer binding proteins of similar structure. However, the nucleotide sequences of those three are all different from those of TAXREB67 and the other five cDNA clones we isolated. Therefore, at least eight proteins capable of binding to the 21-bp element seem to be expressed in various cells. Proteins in this family are known to form homo- and/or heterodimers to bind to DNA strands (15, 21, 28, 41, 64, 68).

However, it is difficult to predict only from the amino acid sequence whether TAXREB67 can make a dimeric structure. Further experiments using intact protein will be necessary. However, as mentioned above, as many as eight different proteins, all having leucine zipper structures adjacent to the carboxy terminus of their basic domains, have been identified as the 21-bp element-binding proteins. Therefore, it is likely that all or some of them would form homo- or heterodimers with each other to regulate the expression of the genes downstream of the element.

The transcription factors with basic motifs next to the leucine zipper usually have an acidic region which is considered to be important for its transcriptional activator function (20, 33). In the amino acid sequence of TAXREB67, there are two relatively acidic domains; one is from amino acid residues 83 to 121 (14 of 39 amino acid residues [36%] are acidic), and the other is from amino acids 181 to 218 (11 of 38 amino acids [29%] are acidic). Further functional studies will be necessary to determine the significance of these acidic regions.

A significant amount of TAXREB67 mRNA was expressed in all the cell lines examined. Since there was no clear difference in the levels of mRNA between HTLV-I-producing and -nonproducing cell lines, the expression of TAXREB67 mRNA does not seem to be affected by the presence of Tax. The increase in the level of TAXREB67 mRNA was amplified by addition of TPA or a calcium ionophore. The same condition is known to induce proliferation of normal resting T cells (60). Therefore, change of the expression level of TAXREB67 may have a relation to T-cell proliferation. Since adult T-cell leukemia caused by HTLV-I is a disease of abnormal proliferation of T cells, it seems to be important to analyze the relationship between the function of TAXREB67 and the process of leukemogenesis caused by HTLV-I. To approach the problem, it is necessary to characterize the intact TAXREB67 protein without β -galactosidase fusion. Furthermore, possible interaction between TAXREB67 and other regulatory factors of transcription should be analyzed to understand the mechanism of transcriptional activation through the 21-bp enhancer sequence of HTLV-I.

During preparation of the manuscript, we learned that Hai et al. reported a part of the amino acid sequence of ATF4 (14) which was identical to the TAXREB67 sequence.

ACKNOWLEDGMENTS

We thank Takashi Sugimura of the National Cancer Center for continuous encouragement for the work and Shunsuke Ishii for *E. coli* Y1089.

This work was supported by a Grant-in-Aid for Comprehensive 10-Year Strategy of Cancer Control from the Ministry of Health and Welfare, Japan. A.T. is the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

REFERENCES

1. Beimling, P., and K. Moelling. 1989. Isolation and characterization of the tax protein of HTLV-I. *Oncogene* 4:511-516.
2. Brady, J., K. T. Jeang, J. Duvall, and G. Khoury. 1987. Identification of p40^{tax}-responsive regulatory sequences within the human T-cell leukemia virus type I long terminal repeat. *J. Virol.* 61:2175-2181.
3. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
4. Cohen, D. R., and T. Curran. 1988. *fra-1*: a serum-inducible, cellular immediate-early gene that encodes a Fos-related antigen. *Mol. Cell. Biol.* 8:2063-2069.

5. Cross, S. L., M. B. Feinberg, J. B. Wolf, N. J. Holbrook, F. Wong-Staal, and W. J. Leonard. 1987. Regulation of the human interleukin-2 receptor α chain promoter; activation of a non-functional promoter by the transactivator gene of HTLV-I. *Cell* 49:47-56.
6. Dang, C. V., M. McGuire, M. Buckmire, and W. M. F. Lee. 1989. Involvement of the 'leucine zipper' region in the oligomerization and transforming activity of human c-myc protein. *Nature (London)* 337:664-666.
7. Didier, D. K., J. Schiftenbauer, S. L. Woulfe, M. Zacheis, and B. D. Schwartz. 1988. Characterization of the cDNA encoding a protein binding to the major histocompatibility complex class II Y box. *Proc. Natl. Acad. Sci. USA* 85:7322-7326.
8. Fujii, M., P. Sassone-Corsi, and I. M. Verma. 1988. c-fos promoter trans-activation by the tax₁ protein of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. USA* 85:8526-8530.
9. Fujisawa, J., M. Seiki, T. Kiyokawa, and M. Yoshida. 1985. Functional activation of the long terminal repeat of human T-cell leukemia virus type I by a trans-acting factor. *Proc. Natl. Acad. Sci. USA* 82:2277-2281.
10. Fujisawa, J.-I., M. Toita, and M. Yoshida. 1989. A unique enhancer element for the trans activator (p40^{tax}) of human T-cell leukemia virus type I that is distinct from cyclic AMP- and 12-O-tetradecanoylphorbol-13-acetate-responsive elements. *J. Virol.* 63:3234-3239.
11. Gessain, A., F. Barin, J. C. Vernant, O. Gout, L. Maurs, A. Calender, and G. De-The. 1985. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* ii:407-409.
12. Giam, C. Z., and Y.-L. Xu. 1989. HTLV-I Tax gene product activates transcription via pre-existing cellular factors and cAMP responsive element. *J. Biol. Chem.* 264:15236-15241.
13. Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. Biggs III, W. W. Vale, and M. R. Montminy. 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature (London)* 337:749-752.
14. Hai, T., F. Liu, W. J. Coukos, and M. R. Green. 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* 3:2083-2090.
15. Halazonetis, T. D., K. Georgopoulos, M. E. Greenberg, and P. Leder. 1988. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* 55:917-924.
16. Hinnebusch, A. G. 1984. Evidence for transcriptional regulation of the activator of general amino acid control in yeast. *Proc. Natl. Acad. Sci. USA* 81:6442-6446.
17. Hinuma, Y., K. Nagata, M. Hanaoka, M. Nakai, T. Matsumoto, K. Kinoshita, S. Shirakawa, and I. Miyoshi. 1981. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. USA* 78:6476-6480.
18. Hirai, S.-I., R.-P. Ryseck, F. Mechta, R. Bravo, and M. Yaniv. 1989. Characterization of junD: a new member of the jun proto-oncogene family. *EMBO J.* 8:1433-1439.
19. Hoeffler, J. P., T. E. Meyer, Y. Yun, J. L. Jameson, and J. F. Habener. 1988. Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. *Science* 242:1430-1433.
20. Hope, I. A., and K. Struhl. 1986. Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* 46:885-894.
21. Hope, I. A., and K. Struhl. 1987. GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. *EMBO J.* 6:2781-2784.
22. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Constructing and screening cDNA libraries in λ gt10 and λ gt11, p. 49-78. In D. Goller (ed.), *DNA cloning: a practical approach*, vol. I. IRL Press, Oxford.
23. Inoue, J., M. Seiki, T. Taniguchi, S. Tsuru, and M. Yoshida. 1986. Induction of interleukin 2 receptor gene expression by p40^{tax} encoded by human T-cell leukemia virus type I. *EMBO J.* 5:2883-2888.
24. Jeang, K. T., I. Boros, J. Brady, M. Radonovich, and G. Khoury. 1988. Characterization of cellular factors that interact with the human T-cell leukemia virus type I p40^{tax}-responsive 21-base-pair sequence. *J. Virol.* 62:4499-4509.
25. Kadison, P., H. T. Poteat, K. M. Klein, and D. V. Faller. 1990. Role of protein kinase A in tax transactivation of the human T-cell leukemia virus type I long terminal repeat. *J. Virol.* 64:2141-2148.
26. Kalnins, A., K. Otto, U. Ruther, and B. Muller-Hill. 1983. Sequence of the lacZ gene of *Escherichia coli*. *EMBO J.* 2:593-597.
27. Katagiri, F., E. Lam, and N.-H. Chua. 1989. Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. *Nature (London)* 340:727-730.
28. Kouzarides, T., and E. Ziff. 1988. The role of the leucine zipper in the fos-jun interaction. *Nature (London)* 336:646-651.
29. Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 15:8125-8132.
30. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
31. Landschulz, W. H., P. F. Johnson, and S. L. McKnight. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240:1759-1764.
32. Lilienbaum, A., M. Duc Dodon, C. Alexandre, L. Gazzolo, and D. Paulin. 1990. Effect of human T-cell leukemia virus type I Tax protein on activation of the human vimentin gene. *J. Virol.* 64:256-263.
33. Ma, J., and M. Ptashne. 1987. Deletion analysis of GAL4 defines two transcriptional activating segments. *Cell* 48:847-853.
34. Maekawa, T., H. Sakura, C. Kanei-Ishii, T. Sudo, T. Yoshimura, J. Fujisawa, M. Yoshida, and S. Ishii. 1989. Leucine zipper structure of the protein CRE-BP-1 binding to the cyclic AMP response element in brain. *EMBO J.* 8:2023-2028.
35. Marriott, S. J., I. Boros, J. F. Duvall, and J. N. Brady. 1989. Indirect binding of human T-cell leukemia virus type I tax¹ to a responsive element in the viral long terminal repeat. *Mol. Cell. Biol.* 9:4152-4160.
36. Miyatake, S., M. Seiki, M. Yoshida, and K. Arai. 1988. T-cell activation signals and human T-cell leukemia virus type I-encoded p40^{tax} protein activate the mouse granulocyte-macrophage colony-stimulating factor gene through a common DNA element. *Mol. Cell. Biol.* 8:5581-5587.
37. Montagne, J., C. Beraud, I. Crenon, G. Lombard-Platet, L. Gazzolo, A. Sergeant, and P. Jalinot. 1990. Tax1 induction of the HTLV-I 21bp enhancer requires cooperation between two cellular DNA-binding proteins. *EMBO J.* 9:957-964.
38. Moye-Rowley, W. S., K. D. Harshman, and C. S. Parker. 1989. Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. *Genes Dev.* 3:283-292.
39. Nagashima, K., M. Yoshida, and M. Seiki. 1986. A single species of pX mRNA of human T-cell leukemia virus type I encodes trans-activator p40^{tax} and two other phosphoproteins. *J. Virol.* 60:394-399.
40. Nagata, K., K. Ohtani, M. Nakamura, and K. Sugamura. 1989. Activation of endogenous c-fos proto-oncogene expression by human T-cell leukemia virus type I-encoded p40^{tax} protein in the human T-cell line, Jurkat. *J. Virol.* 63:3220-3226.
41. Nakabeppu, Y., and D. Nathans. 1989. The basic region of Fos mediates specific DNA binding. *EMBO J.* 8:3833-3841.
42. Neuberg, M., M. Schuermann, J. B. Hunter, and R. Muller. 1989. Two functionally different regions in Fos are required for the sequence-specific DNA interaction of the Fos/Jun protein complex. *Nature (London)* 338:589-590.
43. Nyunoya, H., T. Ogura, M. Kikuchi, H. Iwamoto, K. Yamashita, M. Maekawa, Y. Takebe, K. Miyamura, S. Yamazaki, and K. Shimotohno. 1990. Expression of HTLV-I envelope protein fused to hydrophobic amino-terminal peptide of baculovirus polyhedrin in insect cells and its application for serological assays. *AIDS Res. Hum. Retroviruses* 6:1311-1321.

44. Okayama, H., and P. Berg. 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell. Biol.* 3:280-289.
45. Osame, M., K. Usuku, S. Izumo, N. Ijichi, H. Amatani, A. Igata, M. Matsumoto, and M. Tara. 1986. HTLV-I associated myelopathy, a new clinical entity. *Lancet* i:1031-1032.
46. Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo. 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA* 77:7415-7419.
47. Poteat, H. T., P. Kadison, K. McGuire, L. Park, R. E. Park, J. G. Sodroski, and W. A. Haseltine. 1989. Response of the human T-cell leukemia virus type I long terminal repeat to cyclic AMP. *J. Virol.* 63:1604-1611.
48. Radonovich, M., and K.-T. Jeang. 1989. Activation of the human T-cell leukemia virus type I long terminal repeat by 12-*O*-tetradecanoylphorbol-13-acetate and by Tax(p40x) occurs through similar but functionally distinct target sequences. *J. Virol.* 63:2987-2994.
49. Rosen, C. A., R. Park, J. G. Sodroski, and W. A. Haseltine. 1987. Multiple sequence elements are required for regulation of human T-cell leukemia virus gene expression. *Proc. Natl. Acad. Sci. USA* 84:4919-4923.
50. Ryder, K., A. Lanahan, E. Perez-Albuern, and D. Nathans. 1989. Jun-D: a third member of the Jun gene family. *Proc. Natl. Acad. Sci. USA* 86:1500-1503.
51. Ryder, K., L. F. Lau, and D. Nathans. 1988. A gene activated by growth factors is related to the oncogene v-jun. *Proc. Natl. Acad. Sci. USA* 85:1487-1491.
52. Ryder, K., and D. Nathans. 1988. Induction of protooncogene c-jun by serum growth factors. *Proc. Natl. Acad. Sci. USA* 85:8464-8467.
53. Sakura, H., T. Maekawa, F. Imamoto, K. Yasuda, and S. Ishii. 1988. Two human genes isolated by a novel method encode DNA-binding proteins containing a common region of homology. *Gene* 73:499-507.
54. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
55. Shimotohno, K., M. Takano, T. Teruuchi, and M. Miwa. 1986. Requirement of multiple copies of a 21-nucleotide sequence in the U3 regions of human T-cell leukemia virus type I and type II long terminal repeats for trans-activation of transcription. *Proc. Natl. Acad. Sci. USA* 83:8112-8116.
56. Siekevitz, M., M. B. Feinberg, N. Holbrook, F. Wong-Staal, and W. C. Greene. 1987. Activation of interleukin 2 and interleukin 2 receptor (Tac) promoter expression by the trans-activator (tat) gene product of human T-cell leukemia virus, type I. *Proc. Natl. Acad. Sci. USA* 84:5389-5393.
57. Singh, H., J. H. LeBowitz, A. S. Baldwin, and P. A. Sharp. 1988. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. *Cell* 52:415-423.
58. Sodroski, J. G., C. A. Rosen, and W. A. Haseltine. 1984. *trans*-Acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. *Science* 225:381-385.
59. Tan, T.-H., R. Jia, and R. G. Roeder. 1989. Utilization of signal transduction pathway by the human T-cell leukemia virus type I transcriptional activator *tax*. *J. Virol.* 63:3761-3768.
60. Truneh, A., A. F. Golstein, and A.-M. Schmitt-Verhulst. 1985. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol esters. *Nature (London)* 313:318-320.
61. Tsujimoto, A., T. Teruuchi, J. Imamura, K. Shimotohno, I. Miyoshi, and M. Miwa. 1988. Nucleotide sequence analysis of a provirus derived from HTLV-I-associated myelopathy (HAM). *Mol. Biol. Med.* 5:29-42.
62. Van Beveren, C., F. van Straaten, T. Curran, R. Muller, and I. M. Verma. 1983. Analysis of FBJ-MuSV provirus and *c-fos* (mouse) gene reveals that viral and cellular *fos* gene products have different carboxy termini. *Cell* 32:1241-1255.
63. Vinson, C. R., K. L. LaMarco, P. F. Johnson, W. H. Landschulz, and S. L. McKnight. 1988. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes Dev.* 2:801-806.
64. Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs III, and M. R. Montminy. 1988. Phosphorylation induced binding and transcriptional efficacy of nuclear factor CREB. *Nature (London)* 334:494-498.
65. Yoshida, M., I. Miyoshi, and Y. Hinuma. 1982. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. USA* 79:2031-2035.
66. Yoshida, M., M. Osame, K. Usuku, M. Matsumoto, and A. Igata. 1987. Viruses detected in HTLV-I-associated myelopathy and adult T-cell leukemia are identical on DNA blotting. *Lancet* i:1085-1086.
67. Yoshimura, T., J. Fujisawa, and M. Yoshida. 1990. 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. *EMBO J.* 9:2537-2542.
68. Zerial, M., L. Toschi, R.-P. Ryseck, M. Schuermann, R. Muller, and R. Bravo. 1989. The product of a novel growth factor activated gene, *fos B*, interacts with JUN proteins enhancing their DNA binding activity. *EMBO J.* 8:805-813.