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Involvement of Transcription Factor YB-1 in Human T-Cell Lymphotropic Virus Type I Basal Gene Expression

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Sequences which control basal human T-cell lymphotropic virus type I (HTLV-I) transcription likely play an important role in initiation and maintenance of virus replication. We previously identified and analyzed a 45-nucleotide sequence (downstream regulatory element 1 [DRE 1]), +195 to +240, at the boundary of the R/U5 region of the long terminal repeat which is required for HTLV-I basal transcription. We identified a protein, p37, which specifically bound to DRE 1. An affinity column fraction, containing p37, stimulated HTLV-I transcription approximately 12-fold in vitro. We now report the identification of a cDNA clone (15B-7), from a Jurkat expression library, that binds specifically to the DRE 1 regulatory sequence. Binding of the cDNA fusion protein, similarly to the results obtained with purified Jurkat protein, was decreased by introduction of site-specific mutations in the DRE 1 regulatory sequence. In vitro transcription and translation of 15B-7 cDNA produced a fusion protein which bound specifically to the HTLV-I +195 to +240 oligonucleotide. The partial cDNA encodes a protein which is homologous to the C-terminal 196 amino acids of the 36-kDa transcription factor, YB-1. Cotransfection of a YB-1 expression plasmid increases HTLV-I basal transcription approximately 14-fold in Jurkat T lymphocytes. On the basis of the molecular weight, DNA-binding characteristics, and in vivo transactivation activity, we suggest that the previously identified DRE 1-binding protein, p37, is YB-1.

The human T-cell lymphotropic virus type I (HTLV-I) is associated with a clinically aggressive form of adult T-cell leukemia and the degenerative neuromuscular disease tropical spastic paraparesis/HTLV-I-associated myelopathy. HTLV-I gene expression is regulated at the transcriptional and post-transcriptional levels by viral gene products Tax_1 ($p40^{tax}$) and Rex_1 ($p27^{rex}$) (1, 10, 15, 35). The U3 region of the HTLV-I long terminal repeat (LTR) contains several important elements needed for Tax_1 transactivation. The 21-bp repeat elements (Tax_1-responsive element 1 [TRE-1]) confer Tax_1 responsiveness to the HTLV-I and heterologous promoters, function in either orientation, and interact with Tax_1 indirectly (6, 11, 24, 31, 34). In addition, Tax_1-responsive element 2 (TRE-2) contains binding sites for Ets1, Myb, Sp1, and TIF-1 (4, 5, 12, 23, 28).

Sequences downstream of the RNA initiation site, encompassing the R region and the 5′ portion of the U5 region, have been shown to be important for basal gene expression (17, 22, 27, 33). Nakamura et al. (27) reported that sequences from +104 to +240 were important for HTLV-I basal gene expression. In agreement with this report, Seiki et al. (33) reported that sequences between +32 and +266 were important for HTLV-I basal mRNA synthesis. The downstream element was orientation independent but was effective only when it was located between the transcription initiation site and the translation initiation site. The downstream element was not Tax_1 responsive. A distinct regulatory element (33), which may function as an RNA element, is located between +266 and +347. In addition, we have previously shown that sequences from +195 to +240 (downstream regulatory element 1 [DRE 1]) downstream of the start site at the R/U5 region are important for basal transcription. A cellular protein of 37 kDa was responsible for specific binding to this region and transactivating the promoter (17). We now report the cloning and identification of a transcription factor which binds and transactivates the HTLV-I LTR through the DRE 1 regulatory sequence.

A lambda gt11 Jurkat cDNA expression library was screened to isolate proteins which specifically interacted with a ^{32}P-labeled +195 to +240 oligonucleotide probe. The cDNA library was plated at 10^6 PFU on *Escherichia coli* Y1090 and induced with 10 mM IPTG (isopropylthiogalactoside)-soaked nitrocellulose membrane. Nitrocellulose filters were denatured and renatured with guanidinium hydrochloride as described previously (39). For screening, membranes were blocked with 5% nonfat dry milk in TNE 200 (50 mM Tris [pH 8.0], 200 mM NaCl, 1 mM EDTA) for 2 h at 4°C and subsequently incubated with approximately 5×10^6 cpm of ^{32}P-radiolabeled +195 to +240 oligonucleotide probe per ml (10^6 cpm/pmol) and poly(dI-dC) (Pharmacia) for 2 h at 4°C. Filters were washed three times in TNE 200 at room temperature. After four rounds of screening, we isolated a recombinant lambda phage which expressed a fusion protein capable of specifically binding to the HTLV-I downstream regulatory sequences. The wild-type +195 to +240 oligonucleotide, but not a five-base-substitution mutant (17), specifically bound to recombinant clone 15B-7 (data not shown). The five-base-substitution mutant of oligonucleotide +195 to +240 was previously shown to decrease binding of the Jurkat DRE 1-binding protein, p37 (17).

Recombinant phage clone 15B-7 was subsequently sequenced, by using multiple overlapping primers (Fig. 1). Computer analysis of the 15B-7 amino acid sequence demonstrated greater than 99% homology, over a 196-amino-acid stretch, to the C-terminal domain of a previously identified transcription factor, YB-1 (Fig. 1). This 36-kDa protein binds

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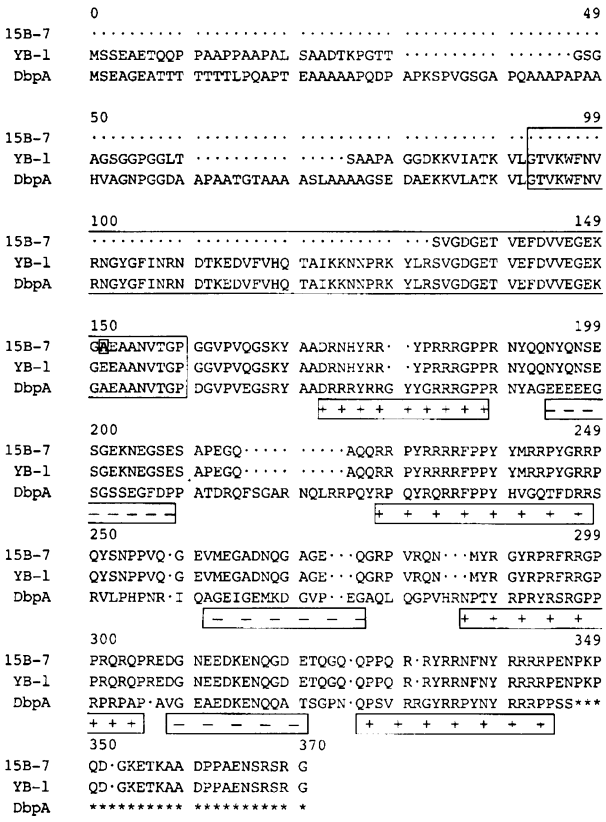

FIG. 1. Sequence analysis of lambda gt11 cDNA clone 15B-7 and comparison with human YB-1 and human DNA-binding protein A (38). A single positive plaque was aspirated into a tube containing lambda gt11 primers 1218 and 1222 (New England Biolabs) and PCR amplified, and the cDNA was subcloned into the pCRII plasmid (Invitrogen) for both DNA sequencing and DNA expression. The 15B-7 amino acid sequence was homologous to the C-terminal 196 amino acids of YB-1, except for glutamic acid at position 151, which was replaced with alanine. Interesting features including a conserved C-terminal box (□), a protamine-like domain (+++), and an acidic amino acid domain (---) are indicated.

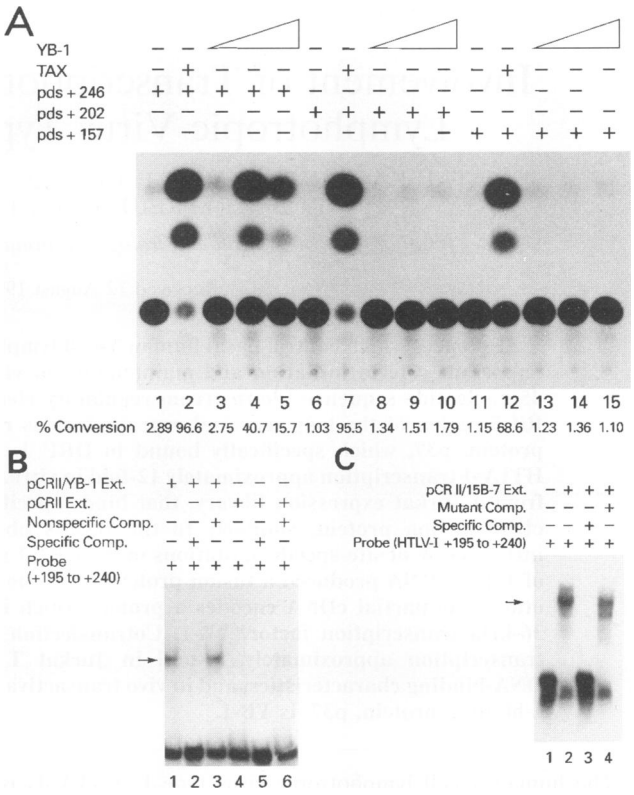

FIG. 2. CAT assay and gel shift pattern of YB-1 in the presence of the HTLV-I +195 to +240 element. (A) Jurkat cells were transfected by electroporation with a constant amount of reporter HTLV-I promoter plasmid (5 μg of pds+157, pds+202, or pds+246) and various amounts of the YB-1 expression plasmid (1, 5, or 10 μg of DNA). (B) Gel mobility band shift assay of in vitro-translated YB-1 on the HTLV-I DRE 1 sequence +195 to +240 oligonucleotide probe. The rabbit reticulocyte TNT system (Promega) was used to translate the recombinant 15B-7 cDNA clone isolated from the lambda gt11 library. Lane 1, 1 μl of a 1:5 dilution of TNT extract (Ext.) containing the 15B-7 clone and 6 ng of the DRE 1 +195 to +240 ^{32}P-end-labeled probe. The gel shift complex (arrow) is inhibited by 100-fold excess of the same unlabeled oligonucleotide (lane 2) but not by a nonspecific oligonucleotide spanning −38 to −8 of the HIV promoter (lane 3). The bands are not seen with a TNT extract without the input 15B-7 plasmid (lanes 4 to 6). Comp., competitor. (C) Gel shift competition analysis using a 100-fold excess of unlabeled wild-type DRE 1 +195 to +240 oligonucleotide (lane 3) or a DRE 1 base substitution mutant oligonucleotide (lane 4) (17).

specifically and transactivates the Y-box sequence containing an inverted CCAAT box (8).

To determine whether, in fact, YB-1 was able to regulate transcription of the HTLV-I LTR, we obtained a eukaryotic expression vector containing YB-1 (a generous gift of B. D. Schwartz). The YB-1 expression plasmid was transfected along with HTLV-I wild-type or mutant reporter chloramphenicol acetyltransferase (CAT) plasmids into Jurkat cells as described previously (17). Extracts were prepared 18 h after transfection for CAT assays. Results of such an experiment are shown in Fig. 2A. Cotransfection of the +246 HTLV-I promoter CAT plasmid with Tax_1 led to a 32-fold increase in CAT gene expression (Fig. 2A, lanes 1 and 2). Similarly, cotransfection of the YB-1 expression plasmid, in increasing amounts, led to a maximal stimulation of approximately 14-fold (Fig. 2A, lanes 3 to 5). An apparent squelching effect was observed at higher concentrations of input YB-1. The YB-1 transactivation is not seen with plasmids that do not contain downstream sequences beyond +202 (Fig. 2A, lanes 8 to 15).

We next analyzed the binding of YB-1 to the sequence +195 to +240 in a band shift assay. Figure 2B shows the result of such an experiment; specific binding is seen with an extract that contained in vitro-transcribed and -translated 15B-7 (Fig. 2B, lanes 1 to 3) but not the in vitro-translated pCRII control extract (Fig. 2B, lanes 4 to 6). The consensus Y-box sequence is 5′-CTGATTGGCCAA-3′ (38). The HTLV-I +195 to +240 sequence contains the sequence 5′-CTGCTTGCTCAA-3′ (+210 to +222; the underlined bases differ from the consensus YB-1 sequence). The results presented in Fig. 2B and C demonstrate that the wild-type HTLV-I sequence, but not a nonspecific (Fig. 2B, lane 3) or mutant HTLV-I +195 to +240 oligonucleotide containing the sequence 5′-ATGATTACTC AA-3′ (Fig. 2C, lane 4), was capable of competing for 15B-7 binding. As pointed out above, the base substitution mutant of oligonucleotide +195 to +240 was previously shown to decrease binding of the Jurkat DRE 1-binding protein, p37 (17).

It was of interest to determine whether cells expressing HTLV-I regulatory proteins resulted in the altered expression

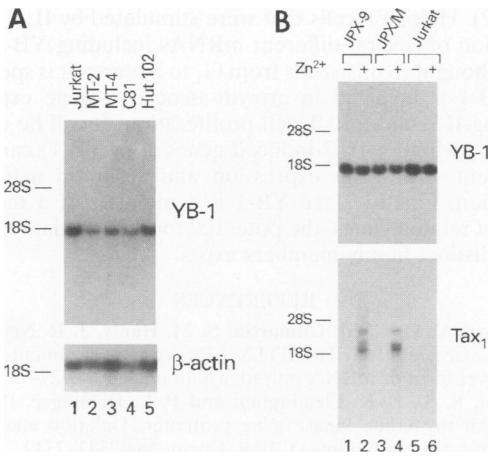

FIG. 3. YB-1 RNA analysis of HTLV-I-transformed cell lines and HTLV-I Tax_1-expressing cells. (A) Poly(A)$^+$ RNAs were prepared from the HTLV-I-transformed cell lines MT-2, MT-4, C81, and Hut 102 and from uninfected Jurkat cells. Fourteen micrograms of poly(A)$^+$ RNA was separated on a 1.2% denaturing agarose gel, blotted and hybridized under conditions described elsewhere (9). YB-1 cDNA was labeled by [^{32}P]dCTP to a specific activity of 6×10^8 cpm/μg of DNA by using the primer extension reaction. The blot was exposed to Kodak XAR X-ray film for 2 h by using an intensifying screen. Rehybridization with a β-actin-specific probe was carried out as described elsewhere (9). (B) JPX-9 and JPX/M cells, permanently transformed with a metallothionein-inducible gene encoding native or frameshift mutant Tax_1, respectively (26), and Jurkat cells were incubated for 22 h in the absence or presence of 150 μM $ZnCl_2$, and poly(A)$^+$ RNA was isolated. Ten micrograms of RNA was used for Northern blot analysis as described previously (9). For hybridization with Tax_1-specific sequences, a full-length Tax_1 cDNA probe was used.

of YB-1. Therefore, we compared YB-1 mRNA levels in the HTLV-I-transformed cell lines MT-2, MT-4, C81, and Hut 102 and control uninfected Jurkat cells. A Northern (RNA) blot was performed with 14 μg of poly(A)$^+$ RNA isolated from each of the cell lines. The blot was probed with a ^{32}P-labeled YB-1-specific probe as described previously (9). The data, presented in Fig. 3A, show similar levels of the 1.5-kb YB-1 mRNA in all five cell lines. It is possible that mRNA synthesis in cell lines stably expressing HTLV-I viral regulatory proteins would not show continuous elevated expression of inducible mRNAs.

Therefore, we next analyzed the effect of Tax_1 on YB-1 expression in the Jurkat-derived JPX-9 cell line which is stably transformed with a metallothionein-inducible Tax_1 gene (26). Of the HTLV-I proteins that are encoded by the viral genome, Tax_1 has been found to play an important role in cellular transformation and viral gene expression. In addition, Tax_1 has been shown to transactivate a number of cellular genes (20). Upon incubation with 150 μM $ZnCl_2$ for 22 h, Tax_1 was expressed at high levels (Fig. 4B). In contrast, YB-1 mRNA levels were the same in the presence and absence of $ZnCl_2$. Since the Tax_1 produced in JPX-9 cells was functional, as determined by transient transfection using the Tax_1-responsive HTLV-I LTR-CAT plasmid pU3R-CAT (data not shown), these results suggest that Tax_1 does not regulate YB-1 expression.

Interestingly, we have also found a YB-1 control element in the human immunodeficiency virus (HIV) LTR. This sequence, 5′-CTGATTGGCAGA-3′, is present approximately 370 bases upstream of the transcription start site and has been previously footprinted and named site A (7, 29). Site A is present at the very 5′ end of the negative regulatory element (NRE), and its function is unknown. All HIV promoters sequenced to date, except isolates HIVHXB2 (G to A, 364 bases upstream of the start site) and HIVRF (T to C, 366 bases upstream of start site), have the above wild-type sequence (25). We examined the effect of YB-1 on two HIV promoter constructs (Fig. 4A) (3). The first construct, CD12, contains the entire HIV promoter. The second construct, CD7, contains 273 bases upstream of the RNA start site, deleting the putative YB-1 binding site. Upon transfection of YB-1 into Jurkat cells along with the HIV LTR-CAT plasmids, an increase in basal transcription with the wild type (Fig. 4A, lanes 3 to 5), but not the deletion mutant construct (Fig. 4A, lanes 8 to 10), was observed. The ability of YB-1 to transactivate HIV transcription has been reproduced in several independent experiments. As with the results presented above, an apparent squelching effect was observed at higher concentrations of YB-1 (Fig. 4A, lane 5).

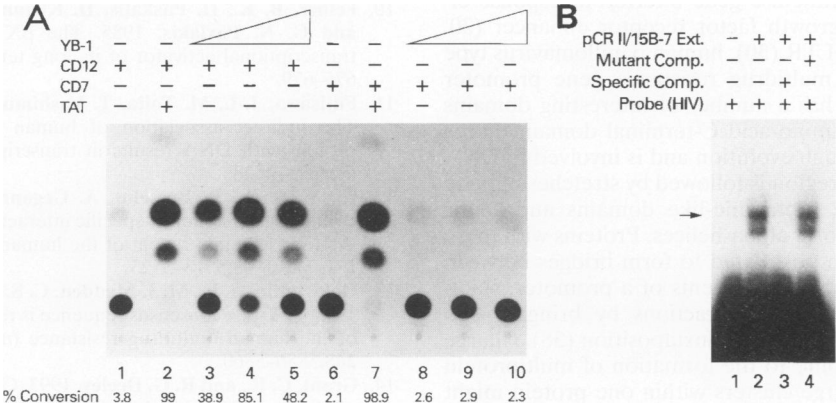

FIG. 4. CAT assay and gel shift pattern of YB-1 on an upstream HIV promoter element. (A) Jurkat cells were transfected with reporter plasmids CD12 (wild-type LTR; 5 μg) or deletion mutant CD7 (−273; 5 μg) (3) and various concentrations (1, 5, or 10 μg) of the YB-1 expression plasmid. (B) Gel shift assay using in vitro-transcribed and -translated 15B-7 from a TNT extract (Ext.) and an HIV YB-1-binding-site 5′-CCCTGATTGGCAGAA-3′ oligonucleotide. One microliter of a 1:5 dilution of the TNT extract was used in the gel shift reactions. The specific gel shift complex (arrow; lane 2) was inhibited by a 200-fold excess of unlabeled wild-type oligonucleotide (lane 3) but not by a five-base mutant oligonucleotide 5′-CCCTGGCCCACAGAA-3′ (lane 4).

We next analyzed a 15-base oligonucleotide containing the HIV YB-1-box homology, 5′-CCCTGATTGGCAGAA-3′, in a gel shift assay with in vitro-translated 15B-7. Figure 4B shows the results of a representative gel shift experiment. A specific gel shift complex was observed when the HIV YB-1 probe was incubated with in vitro-translated 15B-7 (Fig. 4B, lane 2). The gel shift complex was inhibited with a 100-fold excess of unlabeled specific oligonucleotide but not with a 100-fold excess of a five-base mutant (Fig. 4B, lanes 3 and 4; mutant oligonucleotide 5′-CCCTGGCCCACAGAA-3′).

We have previously reported that a 37-kDa protein bound specifically to the HTLV-I DRE 1 regulatory sequence (17). Using a radiolabeled DRE 1 probe, we have identified a partial cDNA clone (15B-7) from a Jurkat lambda gt11 cDNA library which binds specifically to DRE 1. The DNA-binding properties of the 15B-7 protein were similar to those of p37. Interestingly, the amino acid sequence of Jurkat cDNA clone 15B-7 was identical to the C-terminal 196 amino acids of the 36-kDa transcription factor YB-1. Moreover, cotransfection of YB-1 with the HTLV-I LTR resulted in a sequence-specific transactivation. On the basis of the similar molecular weight, DNA-binding properties, and in vivo transactivation, we speculate that the previously identified DRE 1 binding protein, p37, was YB-1.

Several distinct families of proteins which bind to the Y-box sequence have been identified. YB-1 family members have been found to be markedly conserved in sequence throughout evolution from bacteria to vertebrates (19, 40). Several functions have been associated with YB-1. In the chicken, YB-1 expression in early liver development is positively associated with DNA synthesis or cell proliferation (14). Interestingly, YB-1 family members in *Xenopus* frogs, FRG Y1 and FRG Y2, have apparent functional differences at various stages of development (38). Expression of YB-1 appears to have limited tissue specificity. High levels of YB-1 mRNA were present in the heart, muscle, liver, lung, adrenal gland, and brain tissues in a 24-week-old human fetus (36). In contrast, small amounts of YB-1 mRNA were found in the thymus, kidney, bone marrow, and spleen tissues. In the pancreas, bladder, stomach, and testis tissues, YB-1 mRNA could not be detected by Northern hybridization. Sequences similar to the Y-box element have been reported to bind transcription factors and effect expression of several promoters such as hsp70 (38), thymidine kinase (2, 18, 21), HLA-DR beta-chain (8), mouse (2′–5′)oligoadenylate synthetase gene interferon response element (41), epidermal growth factor receptor enhancer (30, 37), Rous sarcoma virus LTR (30), human papillomavirus type 18 enhancer (36), and multidrug resistance gene promoter (13). The YB-1 protein has a number of interesting domains (Fig. 1). There is a 68-amino-acid C-terminal domain that is perfectly conserved through evolution and is involved in DNA binding. This conserved region is followed by stretches of basic amino acids resembling protamine-like domains and acidic amino acids which may form alpha-helices. Proteins with these characteristics have been postulated to form bridges between distal and proximal regulatory elements of a promoter, stabilizing specific protein-protein interactions by bringing two regions of the same DNA helix into juxtaposition (38). Charge clusters may also contribute to the formation of multiprotein complexes. Multiple charge clusters within one protein might contribute to cooperative protein-protein and protein-nucleic acid interactions (16). The acidic and basic domains also contain six putative nuclear localization signals (8).

The role of YB-1 in the HTLV-I or HIV viral replication cycle remains to be established. Interestingly, there seems to be increased expression of YB-1 in interleukin 2 (IL-2)-induced T cells (32). Helper T cells that were stimulated by IL-2 showed expression of several different mRNAs including YB-1. Since IL-2 is thought to push cells from G_1 to S phase, it is speculated that YB-1 is involved in growth-associated gene expression including IL-2-induced T-cell proliferation. It will be of interest to see whether IL-2-induced genes (i.e., YB-1) can stimulate latent viral gene expression and result in active viral replication. Finally, since YB-1 is a member of a multigene family of related genes, the potential for differential regulation by the distinct family members exists.

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