The Cellular Proto-Oncogene Product Myb Acts as Transcriptional Activator of the Long Terminal Repeat of Human T-Lymphotropic Virus Type I

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The proto-oncogene *c-myb* encodes a nuclear transcription factor that binds to DNA in a sequence-specific manner and activates transcription of several viral and cellular genes. Expression of the *c-myb* gene is induced in mitogen- and/or antigen-stimulated T lymphocytes, which are also the preferential target cells of human T-lymphotropic virus type I (HTLV-I) in vivo and in vitro. We report here that Myb binds to the HTLV-I long terminal repeat (LTR) in four different regions in a sequence-specific manner. Electrophoretic mobility shift assay using labeled LTR fragments as well as labeled double-stranded oligonucleotides show that there are two high-affinity and two low-affinity Myb-binding sites present in the HTLV-I LTR. DNase I footprinting analysis and oligonucleotide competition experiments indicate that this binding is sequence specific. Cotransfection experiments in HeLa cells, using a Myb expression vector and chloramphenicol acetyltransferase reporter gene linked to the HTLV-I LTR, show that Myb activates HTLV-I LTR-mediated transcription by a factor of four- to sixfold. Thus, in HTLV-I-infected T cells, Myb protein binding to the HTLV-I LTR may constitute one of the signals that regulate HTLV-I transcription in vivo.

Human T-lymphotropic virus type I (HTLV-I) is an exogenous human retrovirus that has been shown to be the etiological agent of a type of acute T-cell leukemia (known as adult T-cell leukemia) as well as a neurological disorder known as HTLV-I-associated myelopathy or tropical spastic paraplegia (3, 10, 21, 31, 42). In vivo, HTLV-I has been shown to predominantly infect the helper T cells which are positive for CD4 antigen (11, 30). Efficient expression of the HTLV-I genome requires both virus- and host-specific proteins and is dependent on the DNA sequences in the proviral long terminal repeats (LTR). Two *trans*-acting factors, p40^{tax} (Tax1) and p27^{rex} (Rex1), encoded by the pX region of the HTLV-I genome, have been shown to regulate viral replication at transcriptional and posttranscriptional levels, respectively (14, 15). In addition, several host-specific cellular protein factors have been shown to interact with the HTLV-I LTR and presumably influence the expression of the viral genome. These include HEF-1 (26), a 180-kDa cellular protein (16), multiple members of the ATF/CREB family of proteins (37, 38, 44), HEB-1 and HEB-2 (23), TIF-1 (19), SP-1 (27), and TAXREB67 (40).

Recently, it has been shown that the members of the lymphoid-specific *c-ets* proto-oncogene family, Ets-1 and Ets-2, are activators of HTLV-I LTR-mediated transcription in the absence of Tax1 (3). Like the *c-ets* nuclear proto-oncogene products, the *c-myb* gene product is predominantly expressed in hematopoietic cells and has been shown to be induced by mitogenic or antigenic stimulation of T cells (6, 39), which also has been found to result in enhanced HTLV-I transcription (10). The *c-Myb* protein has been shown to bind DNA in a sequence-specific manner (2) and to mediate transactivation of target genes (8, 9, 17, 24, 25, 41). An examination of the HTLV-I LTR sequences shows the presence of a number of possible Myb-binding sites (8), indicating that this transcription factor might play an impor-

tant role in the transcription of this virus. To determine whether Myb protein plays a role in the transcription of HTLV-I, we have examined whether a highly purified recombinant Myb protein binds to LTR sequences in a sequence-specific manner. Our results indicate that Myb protein indeed binds and transactivates HTLV-I LTR sequences, suggesting that this proto-oncogene product could play an important role in the transcriptional regulation of the HTLV-I LTR in infected T cells.

MATERIALS AND METHODS

Cells. HeLa cells (CCL2) were obtained from the American Type Culture Collection (Rockville, Md.). The cells were grown in Eagle's minimal essential medium with non-essential amino acids and Earle's balanced salt solution, supplemented with 10% (vol/vol) fetal bovine serum and 2 mM L-glutamine. The cells were incubated in a controlled environment at 37°C with 5% CO₂ and 95% air.

Generation of a full-length LTR by PCR from an HTLV-I proviral molecular clone. The restriction map of an HTLV-I proviral DNA clone (p73RM) has been described elsewhere (29). This is an 8-kbp *Sst*I genomic DNA fragment which has been subcloned into the SP64 vector (Promega, Madison, Wis.). A full-length proviral LTR was derived from this clone by polymerase chain reaction (PCR) amplification of the 5' and 3' halves of the LTR and subsequent ligation at the *Sst*I site. The outer primers were designed such that they contained *Xho*I and *Hind*III sites, which were used to subclone the LTR into the Bluescript KS vector (Stratagene, La Jolla, Calif.). The sequence of the reconstituted LTR in the Bluescript vector (pHTLV-I LTR) was verified by nucleotide sequence analysis (20, 33).

Plasmids. pHTLV-I LTR-chloramphenicol acetyltransferase (CAT) was constructed as follows: Bluescript KS vector-derived pHTLV-I LTR was digested with *Hind*III and *Xho*I, and the 766-bp *Xho*I-*Hind*III LTR fragment (which contains the entire 755-bp proviral LTR sequence and an additional

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FIG. 1. Schematic representation of the HTLV-I LTR and locations of PCR-amplified LTR fragments 1 (nt 1 to 213), 2 (nt 205 to 447), 3 (nt 426 to 647), and 4 (nt 545 to 755).

11-bp non-LTR sequence derived from the PCR primers) was purified by agarose gel electrophoresis. pU3RI-CAT (36) was also digested with *Hind*III and *Xho*I, and the resultant larger DNA fragment (which contains the CAT gene) was similarly purified. These two purified DNA fragments were ligated, and the resultant HTLV-I LTR-CAT plasmid was verified by dideoxy sequencing, using appropriate primers from the LTR region.

The *c-myb* expression vector pCMVtMyb was generated as follows. A cDNA fragment containing the 396 N-terminal amino acids was generated by PCR amplification from a full-length mouse *c-myb* clone. This PCR fragment was ligated with the expression vector pBC12/CMV/IL-2 (7) from which the interleukin-2 sequences had been removed by *Hind*III and *Bam*HI digestions. The construction of pRSVLacZ plasmid has been described elsewhere (1).

Generation of overlapping HTLV-I LTR fragments by PCR. Four overlapping HTLV-I LTR fragments (hereafter designated fragments 1 to 4) were generated by using pHTLV-I LTR as the template and the following four pairs of primers (Fig. 1): for fragment 1, OL-50 (5'-GGAATTCTGACAATGACCATGAGCCCCAAA-3') and OL-51 (5'-GGAATTCGGTGGCTTCCCCGAGGTCTGAG-3'); for fragment 2, OL-52 (5'-GGAATTCACCGGAACCACTTCTCTCC-3') and OL-53 (5'-GGAATTCAGGCACCACAGCGGGAGGCGG C-3'); for fragment 3, OL-54 (5'-GGAATTCGCTCCCGCTGTGGTGCCTCC-3') and OL-55 (5'-GGAATCGAATGAAAGGGGAGGGGTGAA-3'); and for fragment 4, OL-56 (5'-GGAATTCACGCTTGCCTGACCCTGCTTG-3') and OL-57 (5'-GGAATTCTGTGTACTAAGTTTCTCTCTGG-3'). Each of these primers (OL-50 to OL-57) contained 6 or 7 additional bases at their 5' ends in order to create *Eco*RI restriction sites for subsequent cloning of the PCR-amplified products. All four of these HTLV-I LTR fragments were subcloned into the Bluescript KS vector at the *Eco*RI site, and their sequences were verified by nucleotide sequence analysis (20, 33).

Recombinant Myb protein. The recombinant Myb expression vector has been described elsewhere (8). The *c-myb* cDNA was truncated at the 3' end such that it encoded 396 N-terminal amino acids which encompassed the DNA-binding and transactivation domains but lacked the negative regulatory domain (32). This recombinant Myb protein is referred to as tMyb.

EMSA. HTLV-I LTR fragments (PCR fragments 1 to 4) were labeled at either end by end-filling reaction using the Klenow fragment of DNA polymerase I and an appropriate α -³²P-labeled deoxynucleoside triphosphate (dNTP) following restriction enzyme cleavage by either *Hind*III or *Bam*HI (present in the Bluescript polylinker region) and then release of the labeled DNA fragment by digestion with a second enzyme (*Bam*HI or *Hind*III). The labeled LTR fragments were gel purified prior to use in the electrophoretic mobility

shift assay (EMSA). EMSA was performed as described by Chodosh (5). The reaction mixture contained 12 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9), 4 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10% (vol/vol) glycerol, 0.5 to 1.0 μ g of poly(dI-dC), 10,000 to 20,000 cpm of labeled LTR fragment, and different amounts of purified tMyb protein in a total volume of 20 μ l. In control experiments, tMyb protein was substituted by acetylated bovine serum albumin. A 4% polyacrylamide gel was run in 0.5 $\times$ Tris-borate-EDTA (TBE) buffer in the cold at 4°C. The gel was dried in a gel dryer and then subjected to autoradiography.

DNase I footprinting. The general method of Brenowitz et al. (4) was followed. Binding reactions for DNase I footprinting were carried out by using 2 to 4 μ g purified tMyb protein as described for EMSA. The binding reaction was performed at 30°C for 15 min, which was immediately followed by DNase I (0.5 U of RNase-free DNase I; Pharmacia LKB, Piscataway, N.J.) treatment at 20°C for 30 s. The reactions were terminated by adding 50 μ l of chloroform-isoamyl alcohol-phenol mixture (24:1:25). After vortexing and centrifugation, the aqueous phase was separated and the labeled DNA was precipitated with 2.5 volumes of alcohol (-20°C) in the presence of 10 μ g of carrier yeast tRNA. The DNA pellet was washed twice with 80% (vol/vol) alcohol, dissolved into 10 μ l of gel loading buffer, and electrophoresed on a 6 or 8% sequencing gel in 1 $\times$ TBE buffer (20).

Oligonucleotides for EMSA and competition experiments. On the basis of the results of DNase I footprinting analysis using HTLV-I LTR fragments 2, 3, and 4, the following four double-stranded oligonucleotides were made from the LTR sequence: for LTR fragment 2, oligonucleotide 1 (ON-1) (5'-TATAAAGCGCGGAGACAGTTCAGGAGGGG-3'/3'-TTCGCGCTCTGTCAAGTCTCCCTCCCGAGC-5') and ON-2 (5'-GCCATCCACGCGGTTGAGTCGCTTCTGCG-3'/3'-GGTGC GGCCA ACTCAGCGCAAGACGGCGGA-5'); for fragments 3 and 4, ON-3 (5'-TCTGCGTCTTTGTTTCG TTTTCTGTTCTGC-3'/3'-CAGAAACAAAGCAAAAGAC AAGACGCGGCG-5'); for fragment 4, ON-4 (5'-AAGTACC GGCAGTCCGTTGGCTCGGAGCC-3'/3'-GGCCGCTGA GGCAACCGAGCCTCGGTCTCGCT-5'); and a nonspecific oligonucleotide for competition experiments (ON-5; 5'-ACT TCTGTGCCTCTTAAAAAGCAAGCCCTGACT-3'/3'-AC ACACGGAGAATTTTCGTTCCGGGACTGAAGT-5').

All of these oligonucleotides were blunt-ended with the Klenow fragment of DNA polymerase I and purified by extensive dialysis before using in competition experiments. When these oligonucleotides were used in EMSA, an appropriate α -³²P-labeled dNTP was included in the Klenow reaction mixture and then purified by Sephadex G-50 chromatography.

DNA transfection. For DNA transfection experiments, 5 $\times$ 10⁵ to 7 $\times$ 10⁵ HeLa cells were spread onto 60-mm plates and incubated overnight (24 h) at 37°C. A typical transfection experiment contained 6 μ g of total plasmid DNA and 30 μ l of Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, Md.) for each 60-mm plate. The manufacturer's suggested protocol was followed for all transfection experiments. Opti-Mem reduced-serum medium (3 ml) was used during transfection, which was carried out for 5 h and then blocked by addition of 3 ml of complete medium containing 20% (vol/vol) fetal bovine serum. At 24 h after transfection, the medium was changed (6 ml of fresh complete medium containing 10% [vol/vol] fetal bovine serum was added), incubation was continued for 24 h, and then the cells were harvested by trypsinization. The cells were washed twice

with 1× HBS (140 mM NaCl, 20 mM HEPES, pH 7.4) and lysed by freeze-thawing (three cycles) and sonication (1 min at 4°C) in 150 µl of lysis buffer containing 50 mM HEPES (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. After sonication, cell lysates were centrifuged at 12,000 × *g* for 10 min at 4°C, and the clear supernatant was used for β-galactosidase and CAT assays.

CAT assay. Tissue extracts containing identical levels of β-galactosidase activities were used for the assay of CAT activity at 37°C followed by thin-layer chromatography (12). Autoradiograms were developed 48 h after exposure (at -70°C), and the thin-layer chromatography plate was quantitated by using a Laserimage 1000 (Ambis Microbiology Systems, San Diego, Calif.).

β-Galactosidase assay. The β-galactosidase assay was performed according to a protocol (assay instruction no. 0890) provided by Boehringer Mannheim (Indianapolis, Ind.). Briefly, to 1 ml of 3 mM chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim catalog no. 884308) solution in buffer A (2.83 g of HEPES, 0.9 g of NaCl, 65 mg of magnesium hemi-L-aspartate, 1 g of protease-free bovine serum albumin, and 50 µl Tween 20 in 100 ml of water, pH adjusted to 7.25), 10 to 20 µl of tissue extract was added, and the mixture was incubated at room temperature for 1 h. The reaction was stopped by addition of 2 µl of 1 M zinc acetate,

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      U3
1   tgacaatgaccatgagcccaaatatccccgggggcttagagcctccca
51  gtgaaaaacatttccgcgaacagaagtctgaaaaggctcagggccagac
101 taaggctctgacgtctccctccggaggagcagctcagcaccggctcaggc
151 caggccctgacgtgtccccctgaagacaaatcataagctcagacctccgg
201 gaagccaccggaaccaccatttctctcccatgtttgtcaaggccctca
251 ggcgttgacgacaacccctcacctcaaaaaacttttcattgacgcatat
      Poly A          TATA-Box          High affinity
301 ggctaaataaactaacaggagtctataaaagcgcgcgagacagttcaggag
      |
      R
351 ggggctcgcattctctcttcacgcgcgcgcgcctacctgaggccgcca
      Low affinity
401 tccacgcgcgttgagtcgcgtttctgcgcctccgcctgtggtgcctct
      Sst I
451 gaactcgcgcgcgtctaggttaagtttagagctcaggtcgagaccgggc
501 ctttgctcggcgctcccttgaggcctacctagactcagccggctctccac
      U5          Low affinity
551 gctttgcctgaccctgctgttcaactctgcgtctttgtttcgtttctg
      High affinity
601 ttctgcgcgcctacagatcgaaagttccaccctttcccttcattcagc
651 actgactgccggcttgccccacggccaagtaccgagcactccgttggctc
701 ggagccagcgacagcccatcctatagactctccaggagagaaacttagt
751 acaca

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FIG. 2. Complete nucleotide sequence of the HTLV-I LTR in the proviral clone p73RM (29). The nucleotides are numbered from 1 to 755. Vertical lines denote the boundaries of the U3, R, and U5 regions. Bases protected by tMyb in DNase I footprinting experiments are underlined. High and low affinities above the footprint regions indicate high- and low-affinity Myb-binding sites.

FIG. 3. Binding of tMyb with HTLV-I LTR fragments. Binding reactions were performed as described in Materials and Methods. Lanes 1, 3, 5, and 7 contain labeled DNA fragments only; lanes 2, 4, 6, and 8 contain labeled DNA fragments and 50 ng of tMyb protein.

and the color of the reaction was read at 570 nm against a reagent blank.

PCR. PCR was carried out by using reagents from the GeneAmp kit (Perkin Elmer-Cetus, Norwalk, Conn.). A general protocol suggested by the manufacturer was followed. A 100-µl reaction mixture contained 50 to 100 ng of template DNA, 1 µg each of 5' and 3' primers, 2.5 U of *Taq* DNA polymerase, buffer, and dNTP. After the PCR (25 cycles), the product was purified by passage through Tip-5 column (Qiagen, Inc., Studio City, Calif.) as recommended by the manufacturer.

Nucleotide sequence accession number. The GenBank accession number for the HTLV-I LTR sequence reported here is M81248.

RESULTS

Sequence of the HTLV-I LTR in the proviral molecular clone p73RM. The sequence of the 755-bp LTR used in this study is shown in Fig. 2. The bases are numbered from 1 to 755, starting at the first base of the U3 sequence. A comparison of this LTR sequence with that of clone λATK-1 (34) revealed 98% identity. The majority of the base substitutions were found to be transitional (e.g., C→T or A↔G), and only two substitutions were found to be transversional (e.g., A↔C) (data not shown).

Binding of Myb with the HTLV-I LTR. To carry out DNA-binding studies, we expressed a truncated version of the *c-myc* gene in bacteria, using an expression vector that has been previously described (35). The recombinant Myb protein contained the DNA-binding and transactivation domains (32) and was found to bind DNA efficiently and to transactivate reporter genes linked to promoters containing the Myb-responsive element (unpublished data). The recombinant protein, designated tMyb, used in these studies was purified and was found to be at least 90 to 95% pure, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (8).

To determine whether tMyb binds to HTLV-I LTR se-

FIG. 4. DNase I footprinting analysis of HTLV-I LTR fragments with tMyb. (A and B) DNase I footprints of fragment 2; (C) DNase I footprint of fragment 3; (D) DNase I footprint of fragment 4. The fragments were labeled on the positive strand of the LTR. Lanes: 1, G ladder; 2, A+G ladder; 3, labeled LTR fragment treated with DNase I in the presence of bovine serum albumin; 4 and 5, labeled LTR fragment treated with DNase I in the presence of 2 (lane 4) or 4 (lane 5) μ g of tMyb protein. The boxed portion of the protected sequence represents the putative Myb-binding site (2).

quences, we generated four overlapping LTR fragments by PCR amplification (Fig. 1). The end-labeled LTR fragments were then systematically examined by EMSA, using the recombinant tMyb protein. As shown in Fig. 3, LTR fragments 2 and 4 both showed very strong Myb-binding activity, while fragment 3 showed weak binding and fragment 1 showed very little or no binding activity. The presence of two bands with fragments 2 and 4 suggests the possibility that these fragments contained two Myb-binding sites. Binding of tMyb protein with fragments 2, 3, and 4 was found to be concentration dependent, and this binding could be readily detected in the presence of as little as 5 ng of the recombinant tMyb protein (data not shown).

Mapping of Myb-binding sites in the HTLV-I LTR. To further map the Myb-binding sites in the HTLV-I LTR, we used fragments 2, 3, and 4 to perform DNase I footprinting analysis. Both strands of the three fragments were analyzed, and the results showed that the same regions were protected in both strands. Further, in all DNase I footprinting experiments, the same DNA regions were found to be occupied at about the same points when titrated with two different

concentrations of tMyb protein (2 and 4 μ g). Figure 4 (see also Fig. 2) shows the protected regions of the LTR fragments corresponding to the positive strand of the proviral LTR sequence. LTR fragment 2 was found to be protected by tMyb protein in two distinct regions which encompassed nucleotides (nts) 329 to 349 and nt 400 to 420. The putative Myb-binding sequences in these two regions are CAGTTC and CCGTTG, respectively (Fig. 4A and B). LTR fragment 3 was protected by the recombinant tMyb protein in one distinct region encompassing nt 583 to 601. The protected region contained the putative Myb-binding motif TCGTTT in this fragment (Fig. 4C). LTR fragment 4 was found to be protected in two distinct regions. One of these two regions was common to fragment 3, and the second protected region in fragment 4 was located between nt 683 and 702. This particular protected region in LTR fragment 4 has been found to contain a perfectly matching Myb binding motif, CCGTTG (Fig. 4D). The results of DNase I protection experiments are in agreement with those of EMSA studies. Thus, both fragments 2 and 4 were found to be protected in two distinct regions of the DNA, whereas fragment 3 binds with tMyb only in one region.

Oligonucleotide binding and competition studies. To confirm the results of the DNase I footprinting analysis, double-stranded oligonucleotides were synthesized from the sequences corresponding to the four footprinted regions in LTR fragments 2, 3, and 4 (see Materials and Methods for the sequences of these oligonucleotides). The labeled oligonucleotides were tested by EMSA using tMyb (Fig. 5). ON-1 (corresponding to fragment 2, LTR nt 324 to 358) and ON-4 (corresponding to fragment 4, LTR nt 677 to 711) both were found to bind strongly with as little as 2.5 ng of tMyb protein (lanes 8 and 11, respectively). ON-2 (corresponding to fragment 2, LTR nt 397 to 431) showed less avid binding and

FIG. 5. EMSA of synthetic double-stranded oligonucleotides derived from the DNase I-protected regions of LTR fragments 2, 3, and 4 (see Fig. 4). End-labeled oligonucleotides were treated with the indicated amounts of tMyb protein. ON-1 and ON-2 were derived from LTR fragment 2; ON-3 and ON-4 were derived from LTR fragments 3 and 4, respectively.

FIG. 6. Oligonucleotide competition experiments with labeled LTR fragments. (A) Competition experiments using labeled fragment 2. Since fragment 2 contains two DNase I footprint regions, oligonucleotides derived from both of these regions were used independently for competition experiments. Lanes: 1 and 8, free probe (labeled fragment 2); 2 and 9, probe plus tMyb (50 ng); 3 to 6, probe plus tMyb (50 ng) in the presence of 0.2, 0.4, 0.8, and 1 μ g, respectively, of unlabeled homologous competitor ON-1; 7 and 14, probe plus tMyb (50 ng) in the presence of unlabeled heterologous competitor ON-5; 10 to 13, probe plus tMyb (50 ng) in the presence of 0.2, 0.4, 0.8, and 1 μ g, respectively, of unlabeled homologous competitor ON-2. (B) Competition experiments using labeled fragment 3. Lanes: 1, free probe (labeled fragment 3); 2, probe plus tMyb (50 ng); 3 to 6, probe plus tMyb (50 ng) in the presence of 0.2, 0.4, 0.8, and 1 μ g, respectively, of unlabeled homologous competitor ON-3; 7, probe plus tMyb (50 ng) in the presence of unlabeled heterologous competitor ON-5. (C) Competition experiments using labeled fragment 4. Since fragment 4 contains two DNase I footprint regions, oligonucleotides derived from both of these regions were used independently for competition experiments. Lanes: 1 and 8, free probe (labeled fragment 4); 2 and 9, probe plus tMyb (50 ng); 3 to 6, probe plus tMyb (50 ng) in the presence of 0.2, 0.4, 0.8, and 1 μ g, respectively, of unlabeled homologous competitor ON-3; 7 and 14, probe plus tMyb (50 ng) in the presence of unlabeled heterologous competitor ON-5; 10 to 13, probe plus tMyb (50 ng) in the presence of 0.2, 0.4, 0.8, and 1 μ g, respectively, of unlabeled homologous competitor ON-4.

required 5 ng of tMyb protein to exhibit significant binding (lane 6). On the other hand, ON-3 (corresponding to both fragments 3 and 4, LTR nt 577 to 611) showed weak binding even when 5 ng of tMyb protein was used (lane 3).

To test whether the oligonucleotides derived from the footprinted regions could also compete with their corresponding LTR fragments for Myb-binding activity, different amounts of unlabeled oligonucleotides were allowed to compete for Myb binding in the presence of labeled LTR fragments (Fig. 6). With increasing oligonucleotide concentration, a gradual decrease in labeled protein-DNA complex formation was observed for all four oligonucleotides tested. On the other hand, this complex formation was not inhibited by a noncompetitive unlabeled oligonucleotide (ON-5) even when this oligonucleotide was used at 1 μ g per reaction (Fig. 6).

Transactivation of HTLV-I LTR-CAT by Myb. To examine whether Myb was able to increase the basal promoter activity of HTLV-I LTR *in vivo*, an expression plasmid encoding tMyb protein (pCMVtMyb) was cotransfected into

HeLa cells with a reporter plasmid containing the CAT gene under the control of HTLV-I LTR (pHTLV-I LTR-CAT). As negative controls, the reporter plasmid was either transfected alone or cotransfected with the same expression vector lacking the tMyb DNA insert. A recombinant plasmid containing the bacterial *lacZ* gene under the control of the Rous sarcoma virus LTR (pRSVLacZ) was also included in all transfection experiments to correct for the variations in transfection efficiencies. The results are expressed as *n*-fold stimulations of CAT activity compared with values for the reporter plasmid alone. Using this procedure, we reproducibly observed four- to sixfold stimulation of HTLV-I LTR-mediated transcription of the reporter gene when the tMyb expression vector was cotransfected with the reporter plasmid (Fig. 7, lane 3). On the other hand, cotransfection of the reporter plasmid along with the same expression vector lacking the tMyb insert failed to enhance the CAT activity (lane 4). These results suggest that HTLV-I LTR-mediated expression can be transactivated by coexpression of Myb protein.

FIG. 7. Transactivation of HTLV-I LTR CAT activity in HeLa cells by a tMyb expression vector. Lanes: 1, HeLa cells transfected with the tMyb expression vector (4 μ g); 2, HeLa cells transfected with HTLV-I LTR-CAT plasmid (1 μ g); 3, HeLa cells cotransfected with HTLV-I LTR-CAT (1 μ g) and tMyb (4 μ g) expression plasmids; 4, HeLa cells transfected with HTLV-I LTR-CAT (1 μ g) and empty tMyb (4 μ g) expression plasmids. As an internal control, 1 μ g of pRSVLacZ (1) was included in all transfection experiments; tissue extracts containing identical amounts (0.10 optical density equivalent) of β -galactosidase activities were used in all CAT assays. Also, lanes 1 and 2 contain Bluescript KS vector (1 and 4 μ g, respectively) in order to maintain equal amounts (6 μ g) of DNA in all transfections. Transactivation of CAT activity is fourfold greater in lane 3 than in lane 2.

DISCUSSION

The results presented in this report show that Myb protein binds to HTLV-I LTR sequences at four distinct regions: two high-affinity and two low-affinity binding sites. The two high-affinity binding sites occur in the U3 and U5 regions of the LTR, spanning nt 329 to 349 and nt 683 to 702, respectively (Fig. 2). The low-affinity binding sites map to locations in the R and U5 regions, spanning nt 400 to 420 and nt 583 to 601, respectively. DNase I footprinting analysis and oligonucleotide competition experiments show that binding of Myb to these four sites in the LTR is sequence specific. Co transfection of the HTLV-I LTR-CAT construct along with a *myb* expression vector into HeLa cells resulted in a four- to sixfold increase in the transcriptional activation of the LTR. Taken together, these results suggest that Myb protein binds to HTLV-I LTR and activates LTR-mediated transcription. This is particularly significant since mitogenic stimulation of T cells, a prerequisite for HTLV-I transcription and replication, is also known to induce Myb production in these cells.

Transcription of genes in eukaryotic cells is a complex process involving multiple steps and a variety of transcriptional factors (22). To date, a number of cellular protein factors have been described which interact either with the HTLV-I LTR 21-bp repeat elements or with distinct responsive regions in the LTR to confer transcriptional activation of viral gene expression (reviewed in reference 18; 19, 23, 26, 37, 38, 40, 43, 44). Many of these cellular protein factors are ubiquitously expressed and may or may not actively participate in transcriptional regulation of HTLV-I in the target cells. HTLV-I is predominantly T lymphotropic, and the virus infects the helper/inducer subset of T cells defined by the surface antigen CD4 (11, 13, 30). Therefore, it is reasonable to believe that lymphoid-specific putative transcription factors may play a crucial role in the expression of viral RNA. Recently, the HTLV-I LTR has been shown to contain a sequence motif which is recognized by the *ets-1* and *ets-2* proto-oncogene products (3). This sequence, which also contributes to Tax1 responsiveness of the HTLV-I LTR, is characterized by the presence of four repeats of a pentanucleotide sequence, CC(T/A)CC, and binding of the

ets-1 or *ets-2* product to this region seems to result in three- to fourfold transcriptional activation of the HTLV-I LTR (3). Our results show that Myb protein binds to sequences in the HTLV-I LTR and activates transcription of a reporter gene linked to the LTR. While a number of nuclear factors have been shown to bind to the HTLV-I LTR, Myb and Ets proteins constitute a special case in that they activate HTLV-I LTR transcription in the absence of Tax1 protein. Further, both Myb and Ets proteins are preferentially expressed at high levels in lymphoid tissues, particularly in the CD4⁺ T cells which are also natural targets for HTLV-I infection. Therefore, the simultaneous presence of both Myb and Ets proteins in activated T cells may play a critical role in HTLV-I gene expression and propagation of the virus in T cells in vivo. Recently, it has been shown that the *v-myb* and *v-ets* genes of E26 virus cooperate in mitogenic stimulation of fibroblasts (28). The observation that Myb and Ets activate HTLV-I LTR transcription in the absence of Tax1 suggests that these transcription factors play a crucial role in early viral transcription and thus the synthesis of mRNA coding for Tax1 and Rex1. A better understanding of the function of Myb and Ets proteins in HTLV-I transcription could provide valuable insights into the regulation of viral gene expression in vivo.

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