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Myb protein binds to human immunodeficiency virus 1 long terminal repeat (LTR) sequences and transactivates LTR-mediated transcription

(transcriptional regulation/DNA–protein interactions/nuclear protooncogenes/human immunodeficiency virus 1 gene regulation)

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ABSTRACT **The protooncogene c-*myb* encodes a nuclear transcription factor that binds to DNA in a sequence-specific manner and transactivates transcription of several viral and cellular genes. The expression of c-*myb* is induced in mitogen-stimulated peripheral blood lymphocytes and is constitutively expressed in several CD4$^+$ T-cell and myeloid cell lines, all of which constitute excellent targets for human immunodeficiency virus (HIV) infection and replication. We looked for the presence of Myb-binding motifs in human retroviral long terminal repeats (LTRs) and tested for Myb binding to HIV-1 LTR sequences by using a highly purified recombinant Myb protein. Our results show that HIV-1 LTR contains one high-affinity Myb-binding site along with two or more low-affinity binding sites. DNase I protection analysis as well as oligonucleotide competition experiments indicate that this binding is sequence specific. Introduction of purified Myb protein directly into HeLa cells harboring HIV-1 LTR chloramphenicol acetyltransferase vectors indicates that Myb protein transactivates HIV-1 LTR-mediated transcription. Thus, Myb protein binding to HIV LTR sequences may constitute one of the signals that regulates HIV-1 transcription.**

Human immunodeficiency virus (HIV) is the etiologic agent of AIDS (1–4). This retrovirus has been shown to infect CD4$^+$ T cells and myeloid cells of monocytic lineage and uses the CD4 molecule as the receptor for viral entry (5–8). Analysis of the frequency of HIV-infected cells in peripheral blood indicates that, although as many as 1 in 100 cells harbor viral DNA, the frequency of cells expressing HIV mRNA is 10- to 100-fold lower (9). The events that trigger the transition from latency to productive viral replication appear to include antigenic stimulation of T cells *in vivo* or mitogenic stimulation *in vitro* (10–12). Thus, signal transduction events in the cell, including activation of DNA-binding transcription factors in stimulated T cells, seem to regulate the production of HIV. Recent studies suggest a role for resting T cells as a reservoir for HIV-1 in the course of AIDS and indicate that an onset of virus production from resting cells may be regulated by the overall level of antigen or mitogen-induced T-cell activation within the infected individual (13). Taken together, these observations suggest that activation of HIV transcription in mitogen-stimulated peripheral lymphocytes requires the induction of cellular factors, which play an important role in the transcription and replication of the HIV genome (reviewed in ref. 14).

Interleukin 2-induced proliferation of T cells after mitogenic or antigenic stimulation is associated with the expression of the cellular protooncogene c-*myb* (15). c-*myb* is also expressed at high levels in immature hematopoietic cells, most notably in CD4$^+$ T cells and myeloid cells, which are the targets of HIV infection (16). This nuclear protein has been demonstrated to bind to a specific nucleotide sequence, YAACKG (17), and act as a transcriptional transactivator of target genes containing this sequence in their promoters (18, 19).

It is interesting to note that induction of c-*myb* in T cells after mitogen stimulation coincides with the activation of transcription of HIV genome in virus-infected T cells. To examine whether Myb protein plays a role in the transcription of HIV, we examined the viral long terminal repeat (LTR) sequences for the presence of Myb binding motifs and tested whether a highly purified recombinant Myb protein binds LTR sequences. Our results show that Myb protein binds and transactivates HIV LTR sequences, suggesting that this protooncogene product could play an important role in the transcriptional regulation of HIV.

MATERIALS AND METHODS

Cells. HeLa cells obtained from the American Type Culture Collection were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and L-glutamine at 37°C with 5% CO_2/95% air. A HeLa cell line stably transfected with HIV LTR chloramphenicol acetyltransferase (CAT) plasmid described earlier (20) was maintained under the same conditions.

Plasmids. Construction of HIV 1 LTR CAT plasmids has been described (21). A bacterial expression vector expressing the Myb protein was constructed by using a cDNA clone of mouse c-*myb* (22). This cDNA was truncated at the 3′ end such that it contained 396 N-terminal amino acids that encoded the DNA-binding as well as the transactivating domains but lacked the negative regulatory domain. The expression plasmid contained λpL promoter and the gene product was expressed as a fusion protein with 16 amino acids of λcII protein at the N terminus (23). This recombinant protein is referred to as t-Myb. The construction of this plasmid has been described elsewhere (24) and is shown in Fig. 1*A*.

Purification of Bacterially Synthesized Myb Protein. The bacteria carrying the Myb expression plasmid were thermally induced by shifting the temperature from 32°C to 42°C and further processed as described (25) to purify the Myb protein. Purified recombinant Myb protein preparations were analyzed by SDS/PAGE (26) and used only when they were found to be at least 90–95% pure.

Mobility-Shift and DNase I Footprinting Assays. Mobility-shift assays were performed as described by Chodosh (27)

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Abbreviations: LTR, long terminal repeat; HIV, human immunodeficiency virus; CAT, chloramphenicol acetyltransferase; HTLV, human T-cell lymphotropic virus.

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and the DNase footprint analysis was performed as described by Brenowitz *et al.* (28).

Protein Scrape Loading and CAT Assays. The purified Myb protein was introduced into cells by the scrape-loading technique as described (29). CAT assays were performed as described (30). TLC plates were autoradiographed as well as quantitated by Laserimage 1000 (Ambis Microbiology Systems, San Diego, CA).

RESULTS

A Survey of Human Retroviral LTR Sequences for the Presence of Myb-Binding Motif. Recently it has been shown that proteins of the Myb family bind a sequence motif YAACG/TG (Y, pyrimidine) and function as transcriptional transactivators (17–19). We first examined human T-cell-tropic retroviral LTR sequences for the presence of this motif. This motif was seen in all the four retroviral LTRs examined, which included HTLV-1, HTLV-2, HIV-1, and HIV-2 (HTLV, human T-cell lymphotropic virus). In HIV-1, we observed one such motif in the U3 region, which had a perfect match with the consensus sequence (Table 1). However, when one mismatch was allowed, there were 14 other possible sites that could constitute a binding site for the Myb family of proteins. These motifs are present along the entire length of the HIV-1 LTR, which included the U3, U5, and R regions (listed in Table 1).

To carry out DNA-binding studies, we expressed a truncated version of the c-*myb* gene in bacteria by using an expression vector that has been described (23, 25). The recombinant protein contained the DNA-binding and transactivation domains and was shown by us to bind DNA efficiently and to transactivate reporter genes linked to promoters containing the Myb-responsive element (unpublished data). The recombinant protein (designated t-Myb) used in these studies was purified and was found to be at least 90–95% pure as determined by SDS/PAGE analysis (Fig. 1*B*).

To determine whether Myb binds to HIV-1 LTR sequences, we generated four overlapping DNA fragments of the LTR by polymerase chain reaction, as shown in Fig. 2*A*. The labeled DNA fragments were then systematically examined by mobility-shift assays for their ability to bind to the recombinant Myb protein. These studies revealed that fragment 2 (which contained the perfect Myb-binding motif) showed very strong Myb-binding activity, while fragments 3 and 4 showed weak binding and fragment 1 exhibited no binding (Fig. 2*B*). These results suggest that the sequence motif present in fragment 2, which spans the sequence from position 151 to 156, constitutes a high-affinity binding site for Myb protein, while some of the other motifs present in fragments 3 and 4 represent low-affinity binding sites.

Binding of Myb to fragment 2 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 protein (Fig. 2*C*). The sequence specificity of this binding was tested by using homologous and heterologous competitor DNA fragments (Fig. 2*D*). These results show that unlabeled homologous fragment 2 could readily compete with the observed binding and, at a concentration of 0.5 μg per reaction mixture, complete inhibition of the binding activity was observed. On the other hand, a heterologous DNA fragment (such as fragment 1) that did not bind Myb (Fig. 2*B*) could not inhibit the binding even at a concentration of 2 μg per reaction mixture. These results suggest that the observed Myb–DNA interaction is sequence specific and could be mediated via the Myb-binding motif present in this fragment.

DNase I Footprint Analysis. To determine the precise nature of the DNA-binding site in fragment 2, we performed

Table 1. Myb-binding motifs (YAACKG) in the LTR of human retroviruses

LTR region	HIV-1		HIV-2		HTLV-1		HTLV-2	
	Position	Sequence	Position	Sequence	Position	Sequence	Position	Sequence
U3	21	CAACGA	17	tcactc	51	tcactg	6	CAATGG
	110	tatctg	70	TAATTG	139	CACCGG	91	TAAAGG
	117	CCACTG	151	ctactg	255	caacgc	122	GAACTG
	124	caaagg	188	TCACTG	343	gaactg	131	CCACGG
	151	caactg	264	caaggg			195	taaggg
	160	tatctg	278	taacta			305	gaactg
	182	TAAAGG	376	taaatg				
	323	GAACTG	380	taacta				
	347	CAAGGG	396	tagctg				
	358	cagcgg	422	TAACTA				
	433	CAGCTG	426	TAACAG				
	433	cagctg	435	CAGCTG				
			435	cagctg				
			474	CAAGGG				
			535	CCGCTA				
R	511	cagtgg	608	ctactg	389	TACCTG	344	TCACGG
			614	tacctg	411	ccaccg	387	caaccg
			653	caagtg	454	GAACTG	402	ccacgg
			660	caccgg	503	caaagg	435	GAACTG
			719	taactg	518	caaggg	483	taaggg
					576	CAACTC		
U5	568	caacag	730	taactt	611	taacgg	598	tgacgg
	585	TAACTA	734	TAAGTG	652	CGACTG	601	TCACTG
			783	CACCTG	656	TGACTG	619	CAACGC
			806	taacag	672	CCACGG	660	ccacgg
					683	TACCGG	673	CAAGGG
					694	caacgg		

The sequences were obtained from the Human Retroviruses and AIDS Databank, Los Alamos National Laboratory, 1989. The first base in the U3 region was given number 1. A computer search was carried out with both strands of the LTR sequence. Capital letters indicate the sequence in the positive strand and lowercase letters indicate the sequence in the negative strand.

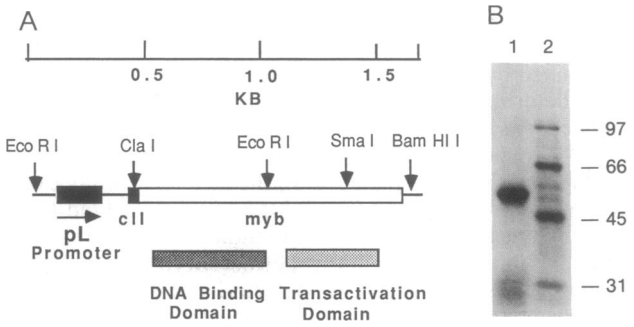

FIG. 1. (*A*) Restriction map of the bacterial expression vector used for expression of a truncated form of Myb protein in bacteria. The position of the λpL promoter sequence is indicated as a solid box. The cDNA of c-*myb*, which was fused to the first 16 codons of λ*c*II gene, is shown as an open box. The *Cla* I site occurs within the coding region of the λ*c*II gene. The *Eco*RI and *Sma* I sites occur within the c-*myb* cDNA. The position of the DNA binding and transactivation domains of Myb are indicated below the restriction map. (*B*) SDS/PAGE analysis of purified recombinant Myb protein. The recombinant Myb protein was purified, analyzed on an acrylamide gel (26), and stained with Coomassie blue. Lane 1, purified Myb protein; lane 2, molecular weight markers. Molecular weights ($\times 10^{-3}$) of the marker proteins are indicated on the right.

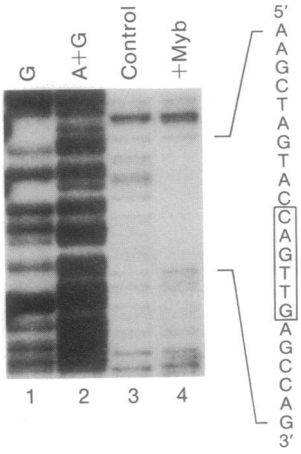

FIG. 3. DNase I footprint analysis of fragment 2 with recombinant Myb. DNA fragment 2 labeled at one end was used for footprint analysis as described (28). Lane 1, G ladder (31); lane 2, A+G ladder (31); lane 3, probe treated with DNase I in the presence of bovine serum albumin; lane 4, probe treated with recombinant Myb protein. The sequence of the region protected is given. The boxed portion of the sequence represents the consensus Myb-binding site.

DNase I footprint analysis with purified Myb. Both strands of DNA were used for this analysis and the results showed that the same region was protected in each of the strands. Fig. 3 shows the protected region of the positive strand. The protected region consists of 22 base pairs (bp) and contains the expected Myb-binding motif (boxed) in the region, which had a perfect match to the sequence described by Biedenkapp *et al.* (17).

Oligonucleotide Competition Experiments. To further establish the nature of the binding sequence in fragment 2, we synthesized a double-stranded oligonucleotide (35 bp) encompassing the sequence that is protected in DNase I footprint analysis, and we tested this oligonucleotide for Myb binding. This oligonucleotide was found to bind Myb readily and could be detected as a retarded band in mobility-shift assays (Fig. 4). We could also demonstrate sequence-specific binding of Myb, since unlabeled homologous oligonucleotide readily competed for this binding site, while a random oligonucleotide, which did not contain the Myb-binding motif, failed to compete for binding (Fig. 4). The homologous

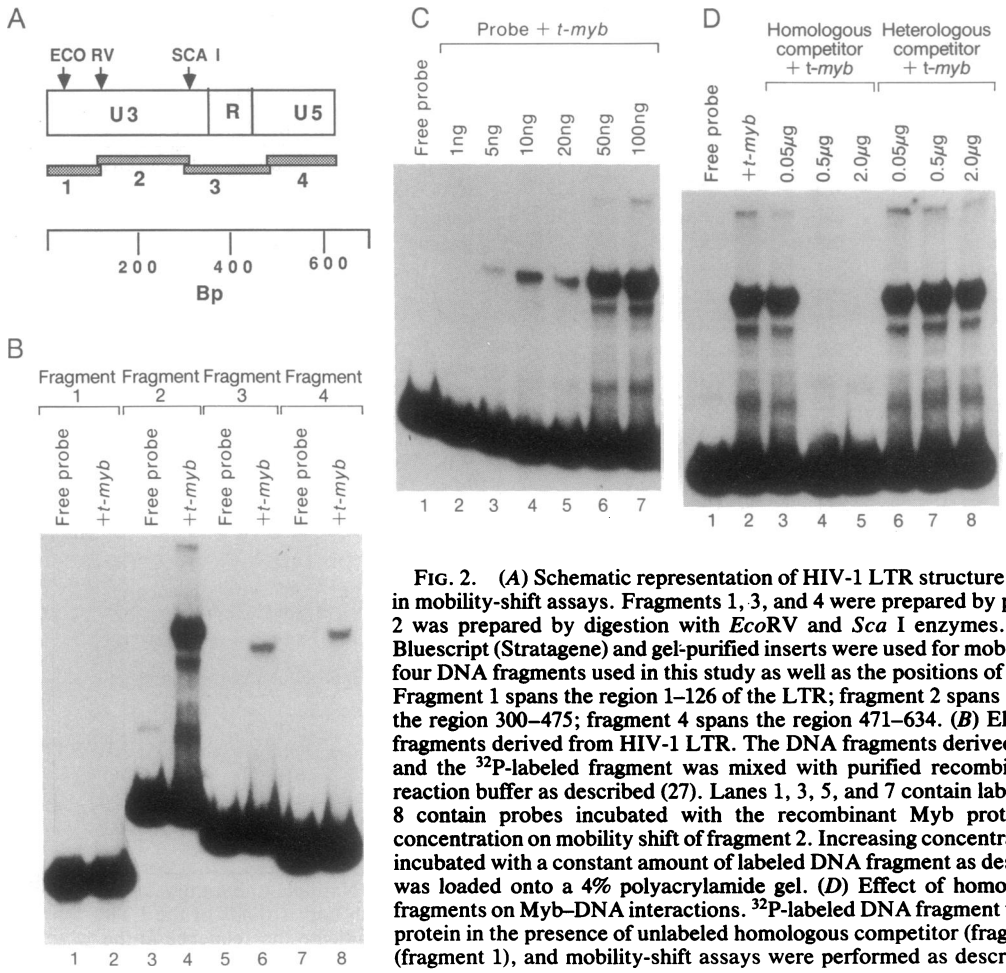

FIG. 2. (*A*) Schematic representation of HIV-1 LTR structure and location of DNA fragments used in mobility-shift assays. Fragments 1, 3, and 4 were prepared by polymerase chain reaction. Fragment 2 was prepared by digestion with *Eco*RV and *Sca* I enzymes. All fragments were subcloned into Bluescript (Stratagene) and gel-purified inserts were used for mobility-shift assays. The positions of the four DNA fragments used in this study as well as the positions of U3, R, and U5 regions are indicated. Fragment 1 spans the region 1–126 of the LTR; fragment 2 spans the region 115–315; fragment 3 spans the region 300–475; fragment 4 spans the region 471–634. (*B*) Electrophoretic mobility-shift assay of fragments derived from HIV-1 LTR. The DNA fragments derived from the LTR (*A*) were end-labeled and the ^{32}P-labeled fragment was mixed with purified recombinant Myb protein and incubated in reaction buffer as described (27). Lanes 1, 3, 5, and 7 contain labeled free probe and lanes 2, 4, 6, and 8 contain probes incubated with the recombinant Myb protein. (*C*) Effect of increasing Myb concentration on mobility shift of fragment 2. Increasing concentrations of Myb protein (1–100 ng) were incubated with a constant amount of labeled DNA fragment as described (27), and the reaction mixture was loaded onto a 4% polyacrylamide gel. (*D*) Effect of homologous and heterologous competitor fragments on Myb–DNA interactions. ^{32}P-labeled DNA fragment was incubated with recombinant Myb protein in the presence of unlabeled homologous competitor (fragment 2) or a heterologous competitor (fragment 1), and mobility-shift assays were performed as described above.

oligonucleotide, when used in competition experiments in conjunction with labeled fragment 2, was also found to readily compete for the binding site (data not shown).

Transactivation of HIV LTR by Myb. To examine whether the Myb-binding sequences in HIV LTR can confer Myb-dependent transcriptional regulation, we used a HeLa cell line that was stably transfected with the HIV LTR CAT plasmid. This cell line expresses low levels of CAT activity and can be readily induced by the HIV-1 TAT peptide upon scrape loading (20, 29). Proteins such as bovine serum albumin had no effect on the levels of CAT activity (data not shown). This cell line was used for scrape loading with various amounts of purified recombinant Myb protein and the transactivation of HIV LTR was assayed by determining the levels of CAT activity produced at various time points after scrape loading. The results of this experiment, shown in Fig. 5, demonstrate that scrape loading of Myb protein into HeLa cells results in 10- to 20-fold transactivation of HIV LTR in a time-dependent manner, with a maximum transactivation occurring at a time point of 6 hr. The activity gradually decreased after ≈12 hr, which coincides with the half-life of the Myb protein. These results suggest that the Myb-binding sites present in the HIV-1 LTR act to positively regulate transcription in the presence of Myb protein.

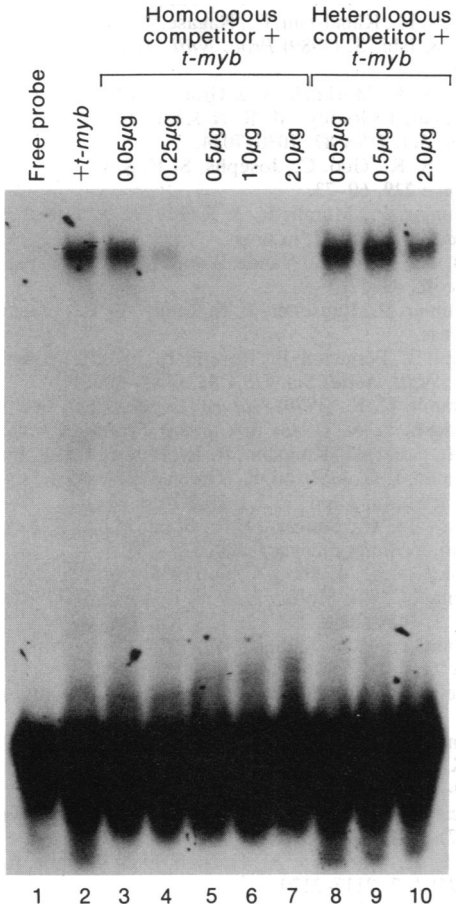

FIG. 4. Electrophoretic mobility-shift assay of a synthetic oligonucleotide probe derived from the DNase I protected region of fragment 2 (see Fig. 3). The ^{32}P-labeled probe was incubated with recombinant Myb protein either in the absence of competitor DNA (lane 2) or in the presence of various concentrations of either unlabeled homologous oligonucleotide probe (lanes 3–7) or unlabeled heterologous oligonucleotide probe (lanes 8–10). The sequence of the double-stranded homologous oligonucleotide probe used for labeling was 5′-CTACAAGCTAGTACCAGTTGAGCCAGATAAGATAG-3′. The sequence of the double-stranded heterologous oligonucleotide was 5′-TTCTGTGCCTCTTAAAAAGCAAGCCCTG-3′.

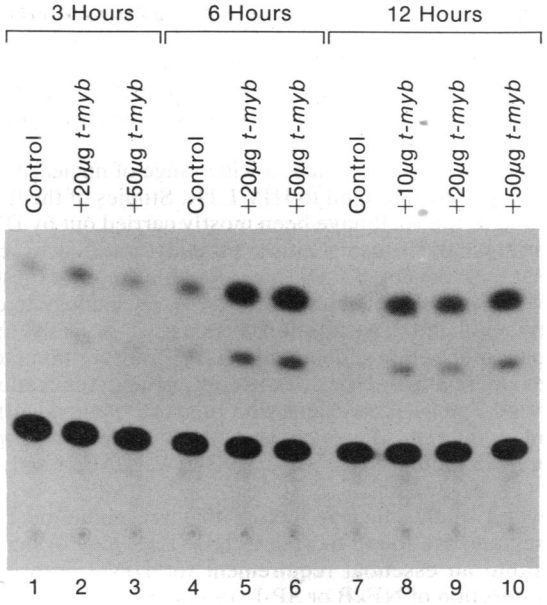

FIG. 5. Transactivation of HIV-1 LTR CAT activity in HeLa cells by Myb protein. HeLa cells stably transfected with a HIV LTR CAT plasmid were scrape loaded either with bovine serum albumin (lanes 1, 4, and 7) or with various concentrations of recombinant Myb protein (lanes 2, 3, 5, 6, and 8–10). CAT activity in scrape-loaded cells was measured after incubation for 3, 6, or 12 hr.

DISCUSSION

Results presented in this communication suggest that human T-cell-tropic retroviral LTR sequences contain several Myb-binding motifs. Within HIV-1 LTR, we could identify one site within the U3 sequences that had a perfect match with the consensus sequence YAACG/TG. However, when one mismatch was allowed, 14 other possible sites could be identified. Our results show that the site that had a perfect match with the consensus sequence in the U3 region, at position 151–156, constitutes a high-affinity binding site. Some of the other sites that had an imperfect match also seem to participate in Myb binding, although with low affinity. However, not all these sites identified in Table 1 seem to participate in Myb binding under the conditions used in this study. For example, fragment 1 was found to contain at least two possible sites but neither of them seem to participate in Myb binding. Similarly, in fragment 2 there are at least five possible sites, of which only one (with perfect match to the consensus sequence) seems to bind to the Myb protein. In fragments 3 and 4 there appears to be at least one low-affinity site in each that binds to Myb. Recently, Ness *et al.* (19) identified a Myb-regulated gene termed *mim-1*, which also contains three Myb-binding sites in its promoter/enhancer element. One of these three sites seems to be a high-affinity binding site for Myb, while the other two seem to constitute low-affinity binding sites (unpublished results). Such clustering of Myb-regulatory elements may be a common pattern for target genes regulated by Myb.

DNase I footprint analysis as well as oligonucleotide competition experiments clearly demonstrate that the Myb protein binds to HIV LTR in a sequence-specific manner. Scrape-loading experiments also demonstrate that addition of Myb to HeLa cells stably transfected with a HIV-1 LTR CAT plasmid results in 10- to 20-fold transactivation of LTR-mediated transcription, as measured by the increase in CAT activity. Taken together, these results suggest that Myb binds to HIV LTR and transactivates LTR-mediated transcription. This is particularly significant since mitogenic stimulation of T cells, a prerequisite for HIV transcription and replication,

is known to induce Myb production in these cells (15). It is also significant to note that $CD4^+$ T cell lines, such as CCRF-CEM and SupT1, as well as myelomonocytic cell lines, such as U937, which support productive infection of HIV, are known to constitutively express high levels of Myb (16).

During the past few years, a wide range of nuclear factors have been shown to bind to HIV LTR. Studies of the factors that bind to the LTR have been mostly carried out by DNase footprint analysis using either partially purified or crude nuclear extracts (14, 32–34). Most notable amongst these are the NF-κB and Sp-1 factors (14, 32–35). By analogy to other highly regulated transcription systems, it is likely that a complex interaction of many individual transcription factors serves to regulate HIV-1 transcription and replication in activated T cells. Consistent with this view is the identification of binding sites for several other eukaryotic transcription factors, including SP-1, AP-1, NFAT-1, USF, NF-1, and LBP (14, 32–36).

Several of the nuclear factors that are ubiquitously expressed and are found to bind to HIV LTR may or may not constitute an essential requirement for HIV transcription. Thus, deletion of NFκB or SP-1 sites does not seem to affect transcription of the HIV-1 genome, as the mutant proviruses lacking these sites seem to replicate as efficiently as wild-type viruses (33). In this context, it is important to note that Myb is one of the tissue-specific nuclear transcription factors that is expressed predominantly in immature hematopoietic cells that constitute targets for HIV infection and thus could play an essential role in the transcription of HIV.

The region surrounding the NF-κB binding site has often been referred to as the enhancer region of the viral LTR, based on deletional analysis of the LTR sequences (37). Similarly, the 5′ end of the LTR has been designated as the negative regulatory element, since deletion of these sequences has been found to enhance LTR-mediated transcription (37). It is interesting to note that HIV-1 LTR has multiple Myb-binding sites, as has been observed by gel-shift assays, and the high-affinity binding site occurs in the negative regulatory domain. It is possible that binding of Myb to this sequence overcomes the negative regulatory effect of this sequence, and this in combination with its binding to other sites could result in transcriptional transactivation. Further mutagenic analysis of these sites should provide a clearer picture of the exact role of various Myb-binding sites present in HIV LTR in viral transcription and replication.

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