Expression of the *c-myc* Proto-oncogene Is Essential for HIV-1 Infection in Activated T Cells

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Summary

We previously found that activation of primary CD4^{+} T cells via both the T cell antigen receptor (TCR) and CD28 is required for HIV-1 DNA to be translocated from the cytoplasm to the nucleus. Here we report that expression of c-Myc protein in CD4^{+} T cells is induced only after such costimulation. In addition, cyclosporin A not only inhibits nuclear import of HIV-1 DNA but also inhibits expression of c-Myc protein. Because of these correlations, we tested whether c-Myc is necessary for nuclear import of HIV-1 DNA. Specific *c-myc* antisense, but not sense or non-sense, phosphorothioate oligodeoxynucleotides selectively induced the accumulation of two NH$_2$-terminally truncated c-Myc proteins and abolished HIV-1 genome entry into host nuclei. Consequently, both virus replication and HIV-1–induced apoptotic cell death were inhibited. Synthesis of viral full-length DNA was not affected. Specific *c-myc* antisense oligonucleotide inhibited HIV-1 infection under conditions that did not affect cell cycle entry or proliferation. Thus, c-Myc appears to regulate HIV-1 DNA nuclear import via a mechanism distinct from those controlling entry into the cell cycle.

Key words: c-Myc • HIV-1 DNA • T cell • nuclear import • apoptosis

The life cycle of human immunodeficiency virus 1 (HIV-1) in infected cells can be divided into pre- and postintegrated stages. After HIV-1 binds to the surface of T cells through interaction of the envelope protein gp120 with CD4 and a seven-span transmembrane chemokine receptor, the virus fuses with the host cell, enters the cytoplasm, and disassociates from the cell membrane (1–4). Viral reverse transcription is initiated, then linear double-stranded viral DNA is synthesized, followed by formation of virus preintegration complexes (PIC)[1] (5). Double-stranded viral DNA within these complexes migrates to the host nucleus and integrates into the host cell genome, or forms circular molecules without the capacity to integrate (6, 7). After a latency period, proviruses can be induced by a variety of stimuli to replicate and this in turn can lead to depletion of CD4^{+} T cells by a process of programmed cell death (8, 9).

Full-length HIV-1 DNA synthesis and translocation to the nucleus are dependent upon activation of T cells (10–16). Two T cell activation signals are required for the synthesis and nuclear translocation of simian immunodeficiency virus (SIV) or HIV-1 DNA (14, 17): one signal through the TCR, which normally regulates the G0 to G1 transition, induces full-length viral DNA synthesis; the second signal, through CD28 or the IL-2 receptor complex (IL-2R), which regulates the G1 to S transition, controls viral DNA entry into the nucleus. Furthermore, cyclosporin A (CSA), a T cell activation inhibitor, and mimosine, a late G1 phase inhibitor, abrogate nuclear import of SIV or HIV-1 genomes (14, 17, 18). However, the cellular factors involved in regulation of this process are not well understood.

One candidate molecule regulated by lymphocyte activation is c-Myc, a transcription factor that has been implicated in regulation of cell activation, differentiation, cell cycle progress, transformation, and apoptosis (19–25). The *c-myc* proto-oncogene is an immediate-early gene rapidly induced during the G0 to G1 transition in activated T cells (20–22). An IL-2R–dependent signaling pathway is required for induction of *c-myc* expression (26–30) and CSA suppresses c-myc gene transcription (31). These observations suggested that c-Myc might play a key role in the regulation of HIV-1 DNA nuclear import.

Here we present evidence that expression of c-Myc occurs as a consequence of T cell costimulation. In addition, blocking c-Myc by CSA correlates with this drug's inhibitory effect on translocation of HIV-1 genome to the nucleus. Furthermore, specific *c-myc* antisense, but not corresponding sense, non-sense, or scrambled phosphorothioate oligodeoxynucleotides (PS-ODNs), selectively abolished HIV-1 DNA entry into host nuclei and induced 46- and 50-kD truncated c-Myc proteins whose NH$_2$-terminal

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[1]*Abbreviations used in this paper:* c-MycS, c-Myc short; CSA, cyclosporin A; PS-ODN, phosphorothioate oligodeoxynucleotide; PIC, pre-integration complexes; TUNEL, TdT-mediated dUTP nick-end labeling.

transactivation domains are deleted. As a result, both replication and the cytopathic effects of HIV-1 were inhibited. Specific *c-myc* antisense PS-ODNs inhibited HIV-1 infection without affecting cell cycle entry or proliferation, suggesting that c-Myc regulates HIV-1 DNA nuclear import via a mechanism distinct from those controlling entry into the cell cycle.

Materials and Methods

Reagents. PS-ODNs used in this study were synthesized by Oligo Etc. Sequences used were as previously described (23): *c-myc* antisense, AACGTTGAGGGGCAT, located in exon 2 of initiation site of translation; sense *c-myc*, ATGCCCCTCAACGTT; non-sense, AGTGGCGGAGACTCT; and scrambled, AAGCATACGGGGTGT containing a GGGG motif (32). The oligonucleotides were dissolved in 30 mM Hepes (pH 7.0). Purified mAbs to human CD8 (G10-1, IgG2a), CD16 (FC-2, IgG2b), CD20 (1F5, IgG2a), and HLA-DR (HB10a, IgG2a) were produced in our lab and used to purify human primary CD4+ T cells as previously described (14). Goat anti–mouse IgG conjugated to magnetic microbeads was purchased from Miltenyi Biotec. mAbs to human CD3 (64.1, IgG2a) and CD28 (9.3, IgG2a) were used to activate CD4+ T cells as previously described (14). Phospho–c-Myc (Thr58/Ser62) polyclonal antibody was purchased from New England Biolabs. Anti–human c-Myc mAb (9E10, IgG1), rabbit polyclonal antibody specific to NH$_2$-terminal region 1–262 amino acids of c-Myc (N-262), and rabbit polyclonal anti-ERK1 (c-16) antiserum were obtained from Santa Cruz Biotechnology. PE-conjugated anti–HIV-1 p24 protein mAb was purchased from Coulter Corp. TUNEL (TdT-mediated dUTP nick-end labeling) detection kits were obtained from Boehringer Mannheim.

CD4+ T Cell Isolation. Enriched preparations of human CD4+ T cells were isolated from peripheral blood samples from healthy, HIV-seronegative donors as follows: PBLs were obtained by centrifugation over Ficoll-Hypaque, and then E-rosette–positive (Er+) cells were isolated as previously described (33). CD4+ T cells were obtained by negative selection of Er+ cells depleting CD8+, CD16+, CD20+, and HLA-DR+ cells with mAb-coated beads. The purity of isolated CD4+ cells was >97% as monitored by flow cytometry. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 U/ml penicillin, 10 mg/ml streptomycin, 1 mM pyruvate, and nonessential amino acids.

HIV-1 Infection. HIV-1 strain Lai was prepared as previously described (14). Cells were infected with HIV-1 at a multiplicity of infection of 0.01 per cell.

PCR to Monitor Initiation and Elongation of HIV-1 DNA Synthesis and Viral DNA Nuclear Import. DNA was extracted from HIV-1– and heat-inactivated HIV-1–infected cells as previously described (14). PCR was performed as described (14) with some modifications including: 50 ng of DNA/sample for amplification of β-globin, 100 ng for LTR/LTR products, 250 ng for LTR/gag products, and 750 ng for LTR/circle products. PCR mixtures contained 1 μM of each primer, 200 μM each of the four deoxynucleoside triphosphates, 1.5 mM MgCl$_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.2 U Taq DNA polymerase (GIBCO BRL). The final volume was 50 μl. The reaction was subjected to 32 cycles (30 cycles for β-globin) of denaturation for 45 s at 94°C, annealing for 1 min at 60°C, and elongation for 2 min at 70°C. PCR products were subjected to 2% agarose gel

containing 0.01 μg/ml ethidium bromide and were visualized by UV light. Primers used in this study have been described (14).

Western Blot Analysis. After various treatments, 5×10^6 primary CD4+ T cells were lysed in 500 μl lysis buffer (2% NP-40, 0.5% sodium deoxycholate, 0.2% SDS, 25 mM Tris-HCl, 50 mM NaCl, 1 mM PMSF, 1 mM Na$_3$VO$_4$, 10 μM E-64 [trans-epoxy-succinylt-1-leucylamido (4-guanidino)-butane], 1 μg/ml pepstatin, 10 μg/ml leupeptin, and 0.1% aprotinin). After incubation on ice for 30 min the cells were sonicated. The cell lysates (equivalent to 10^6 primary CD4+ T cells) were mixed with $2\times$ SDS loading buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 83 mM dithiothreitol, and 0.02% bromophenol blue), incubated at 100°C for 5 min, electrophoresed by 8% SDS-polyacrylamide gel, and then transferred to nitrocellulose membranes (Schreicher & Schuell). The membranes were blocked with 5% nonfat milk-TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) at 4°C overnight, followed by incubation with primary antibodies (in 5% BSA-TBST) at 4°C overnight. After washing, membranes were incubated with horseradish peroxidase–conjugated second antibodies at room temperature for 1 h. Bands on the blotted membranes were detected by incubation with enhanced chemiluminescence reagent (ECL) (Amersham) for 1 min and exposure to Kodak X-Omat film (Eastman-Kodak Co.).

Flow Cytometry. Apoptotic cell death followed by HIV-1 infection was detected by TUNEL according to the manufacturer's protocol (Boehringer Mannheim). After TUNEL staining, cells were resuspended in 100 μl PBS containing 1% BSA; PE-conjugated anti–HIV-1 p24 mAb was added and incubated at 4°C for 20 min. The cells were washed with cold PBS, suspended in 1% paraformaldehyde (Sigma Chemical Co.), and kept at 4°C in the dark until flow cytometry analysis by means of FACScan® (Becton Dickinson). Cell cycle stages were determined by measuring DNA content with propidium iodide as previously described (34).

Cell Proliferation. Cell proliferation was estimated by [³H]thymidine incorporation: 10^5 primary CD4+ T cells in the presence or absence of oligodeoxynucleotides were stimulated with CD3 (10 μg/ml) and CD28 (20 μg/ml) mAbs in triplicate in 96-well plates. The cells were incubated at 37°C in a 5% CO$_2$ incubator for 3 d. Each well was pulsed for 16 h with 0.5 μCi [³H]thymidine, and then the incorporation of [³H]thymidine radioactivity was monitored by a beta counter.

Results and Discussion

In our previous study we found that HIV-1 nuclear import required a CSA-sensitive pathway, and that both TCR and CD28 ligation are essential for this process (14). Similarly, the expression of c-Myc in primary CD4+ T cells required costimulation with CD3 and CD28 mAbs (Fig. 1 A); neither CD3 nor CD28 ligation alone induced c-Myc expression. Time course experiments showed that c-Myc expression increased by 4 h, peaked at 24 h after costimulation, and was sustained for 48 h. Moreover, CSA inhibited c-Myc expression (Fig. 1 A, bottom). Because of this correlation, we tested whether c-Myc might be a key regulator of HIV-1 DNA nuclear import in primary T cells. Since no c-Myc–specific inhibitor is yet available, we used a *c-myc* antisense PS-ODN to inhibit c-Myc function. By competitively inhibiting HIV-1 reverse transcriptase binding to the virus genome–cellular primer complex, PS-
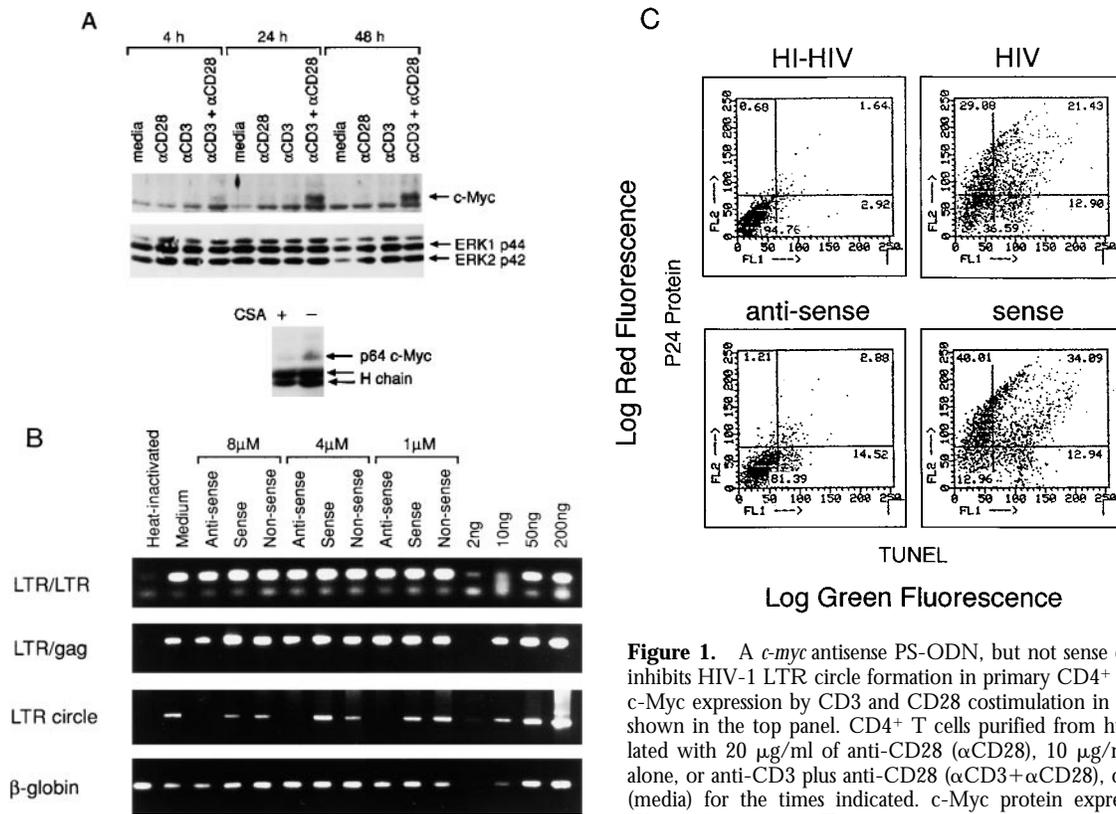
Figure 1. A *c-myc* antisense PS-ODN, but not sense or non-sense PS-ODNs, inhibits HIV-1 LTR circle formation in primary CD4$^+$ T cells. (A) Induction of c-Myc expression by CD3 and CD28 costimulation in primary CD4$^+$ T cells is shown in the top panel. CD4$^+$ T cells purified from human PBLs were stimulated with 20 μg/ml of anti-CD28 (αCD28), 10 μg/ml of anti-CD3 (αCD3) alone, or anti-CD3 plus anti-CD28 (αCD3+αCD28), or were left unstimulated (media) for the times indicated. c-Myc protein expression was evaluated by Western blot analysis. The same blot was stripped and reprobed with ERK1 antisera as a protein loading control. (Bottom panel) CSA inhibited c-Myc expression. Primary CD4$^+$ T cells were stimulated with CD3 and CD28 mAbs in the presence or absence of CSA (1 μg/ml) for 24 h. Cell lysates were performed by Western blot analysis to detect c-Myc protein expression. Similar results were obtained in three additional experiments. (B) *c-myc* antisense, but not sense or non-sense, PS-ODN inhibits HIV-1 LTR circle formation in primary CD4$^+$ T cells. Human peripheral blood CD4$^+$ T cells were stimulated with anti-CD3 mAb plus anti-CD28 mAbs and infected with HIV-1 at the same time. After 24 h, cells were treated with graded doses of *c-myc* antisense, sense, or non-sense PS-ODNs or were left untreated. DNA was extracted at 3 d after infection, and levels of initiation of reverse transcription (LTR/LTR), full-length viral DNA synthesis (LTR/gag), and formation of LTR circles were monitored by PCR. DNA from heat-inactivated HIV-1 (HI–HIV-1)–treated cells was run in parallel. Different amounts of DNA extracted from HIV-1–infected C8166 T cells were run as a positive control. One of three similar experiments is shown. (C) *c-myc* antisense PS-ODN inhibits HIV-1 replication and apoptosis of host T cells. Human CD4$^+$ T cells were infected with HIV-1, and PS-ODNs (1 μM) were added to the cultures 24 h later and refreshed every 2 d. At day 6, intracellular double-staining with PE-conjugated anti-HIV p24 protein mAb and FITC-conjugated TUNEL were performed to detect HIV-1 replication and induction of apoptosis. One of two representative experiments is shown.

ODNs have an inhibitory effect on the initiation of HIV-1 reverse transcription in a sequence-independent manner (35, 36). However, sequence-independent PS-ODNs do not exhibit any anti–HIV-1 activity once initiation of virus reverse transcription has begun (35–38). To avoid nonspecific anti-HIV activity of sequence-independent PS-ODNs, we first infected activated CD4$^+$ T cells with HIV-1 for 24 h and then administrated graded doses of *c-myc* antisense, sense, or non-sense PS-ODNs to the infected cells. As shown in Fig. 1 B, initiation of reverse transcription (LTR/LTR product) and full-length viral DNA synthesis (LTR/gag product) were not affected by the *c-myc* antisense PS-ODN.

However, nuclear import of HIV-1 DNA (LTR circles) was blocked by *c-myc* antisense PS-ODN even at doses as low as 1 μM. Doses below to 0.2 μM were less efficient at inhibiting LTR circle formation (data not shown). Neither *c-myc* sense nor non-sense PS-ODN had any effect on viral DNA nucleus translocation up to 8 μM (Fig. 1 B). Consequently, HIV-1–infected cells treated with *c-myc* antisense PS-ODN did not produce p24 gag protein or undergo

apoptosis (Fig. 1 C). Under conditions in which HIV-1 had already entered the nucleus (e.g., at 48 h), *c-myc* antisense PS-ODN did block viral p24 expression (data not shown). Lack of an effect by *c-myc* antisense PS-ODN on full-length viral DNA synthesis was not simply because the oligonucleotides were added too late to the cultures (after 24 h infection), as full-length viral DNA was not detectable until at least 40 h after HIV infection in activated CD4$^+$ T cells (reference 14 and data not shown). Thus, *c-myc* antisense PS-ODN apparently selectively acts on the stage of HIV-1 DNA nuclear import.

We next studied whether *c-myc* antisense PS-ODN specifically inhibited full-length c-Myc protein expression. Using mAb 9E10 specific to the COOH-terminal end of c-Myc (39), we consistently observed that in the presence of *c-myc* antisense, sense, or non-sense PS-ODNs, the two major forms of c-Myc proteins, p64 and p67, remained relatively unchanged (Fig. 2). However, *c-myc* antisense PS-ODN selectively induced the accumulation of 46- and 50-kD proteins, whose expression levels were higher than
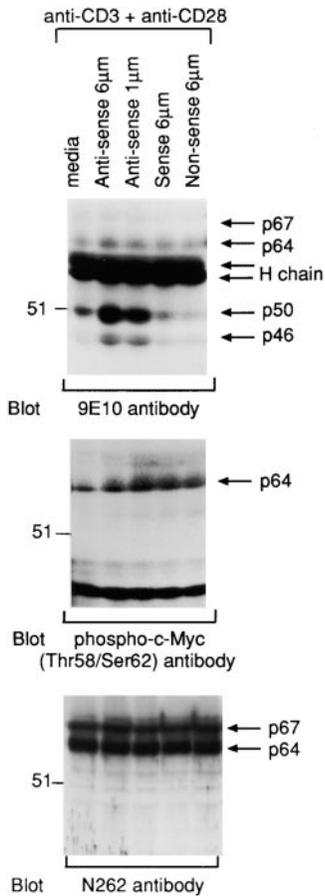
Figure 2. *c-myc* antisense PS-ODN induces accumulation of 46- and 50-kD NH₂-terminally truncated c-Myc proteins in anti-CD3 and anti-CD28 activated CD4⁺ T cells. Human PBL CD4⁺ T cells were stimulated with CD3 and CD28 mAbs for 24 h followed by incubation with PS-ODNs for another 24 h at indicated concentrations. c-Myc proteins were detected by Western blotting with 9E10 mAb (top), or with an antibody specific to phosphorylated Thr58/Ser62 of c-Myc (middle), or with an antibody specific to the NH₂-terminal region of c-Myc, respectively (bottom). The migration of mol wt markers is indicated on the left. Similar results were obtained in three additional experiments.

duce NH₂-terminally truncated c-Myc proteins that are known to act as dominant negative inhibitors by competitively suppressing full-length c-Myc functions (45–48). Blockage of HIV-1 DNA nuclear import by *c-myc* antisense

Figure 3. *c-myc* antisense PS-ODN has no effect on cell cycle progression or proliferation of T cells under conditions of inhibition of HIV-1 infection. Human PBL CD4⁺ T cells were stimulated with CD3 plus CD28 mAbs for 24 h and then incubated with PS-ODNs or the cell cycle inhibitor mimosine as indicated. (A) At day 4, cells were stained with propidium iodide and DNA content was detected by flow cytometry. The percentages of cells in each cell cycle phase were determined with the use of MCycle plus software (Phoenix Flow Systems). (B) Cells were pulsed with 0.5 μCi of [³H]thymidine for 16 h before harvesting at day 3 and incorporated [³H]thymidine was monitored by beta counter. One of three representative experiments is shown.

that of the full-length c-Myc. Neither the *c-myc* sense nor non-sense PS-ODN induced accumulation of these two proteins (Fig. 2, top). These data are consistent with previous studies showing that expression of c-Myc short (c-MycS) proteins in some tumor cell lines arised from two translational initiation sites downstream of the full-length c-Myc start codon (40–45). These downstream-initiated c-MycS proteins lack most of the NH₂-terminal transactivation domain; they are produced through a leaky scanning mechanism, since optimization of the traditional initiation codon for full-length c-Myc results in less synthesis of the c-MycS proteins (45). Because the *c-myc* antisense oligonucleotide we used corresponds to the initiation site of full-length c-Myc mRNA, and the two smaller proteins we detected are about the same size as c-MycS isoforms, it seemed likely that the 46- and 50-kD proteins are produced through the same mechanism leading to deletion of the NH₂-terminal region. This possibility was substantiated by the fact that antibodies specific to either NH₂-terminal phosphorylated Thr58/Ser62 or the whole NH₂ terminus region of c-Myc failed to recognize 46- and 50-kD proteins (Fig. 2, middle and bottom). However, both antibodies were able to recognize p64 and p67 full-length c-Myc, which did not change expression in cells treated with different PS-ODNs (Fig. 2). The same result was obtained in a CD4⁺ lymphoid cell line, CEM (data not shown). Thus, the *c-myc* antisense oligonucleotides, but not control PS-ODNs, selectively in-

PS-ODN most likely is mediated by these NH_2-terminally truncated c-Myc proteins.

Finally, we tested whether *c-myc* antisense PS-ODN could inhibit the entry of cell cycle and proliferation induced in primary $CD4^+$ T cells after TCR and CD28 ligation. Treating $CD4^+$ T cells with 6 μM of *c-myc* antisense oligonucleotide, which efficiently blocked HIV-1 LTR circle formation, could not inhibit cell cycle progression (Fig. 3 A). Similarly, *c-myc* antisense, sense, and non-sense PS-ODN had no effects on $CD4^+$ T cell proliferation induced by CD3 plus CD28 mAbs (Fig. 3 B). These data are consistent with previous findings that NH_2-terminally truncated c-Myc proteins do not interfere with cell growth (45, 49).

The study presented here reveals a novel function of c-Myc for regulation of HIV-1 nuclear import. Blocking of HIV-1 DNA nuclear import by *c-myc* antisense PS-ODN appeared to be mediated through the presence of 46- and 50-kD NH_2-terminally truncated c-Myc proteins, which do not affect cell cycle progression or cell proliferation (Fig. 3, A and B). Our data imply that the mechanism by which c-Myc controls HIV-1 DNA nuclear import is distinct from those controlling cell cycle progression. However, precisely where and how c-Myc is required for HIV-1 DNA nuclear import in proliferating $CD4^+$ T cells remains to be discovered. NH_2-terminal–defective c-Myc proteins are able to heterodimerize with Max, translocate to nucleus, repress gene expression, stimulate cellular proliferation, and induce cell apoptosis (49). However, c-MycS proteins are not able to activate gene transcription (49). It is probable that c-Myc regulates HIV-1 DNA nuclear import through its transactivation activity by regulation downstream gene expression. The ability of HIV-1 to infect nondividing cells, such as monocytes, terminally differentiated macrophages, mucosal dendritic cells, or γ-irradiated cells, is believed to be a unique feature since oncoretroviruses only can establish infection when the cells undergo mitosis (50–57). The ability of HIV-1 to infect nondividing cells is presumably related to the fact that its PIC can be recognized by the cell nuclear import machinery (58–61) and actively transported through nucleopores (62). Moreover, a cellular serine/threonine protein kinase, mitogen-activated protein kinase (MAPK), can associate with HIV-1 PIC to facilitate nuclear targeting of viral DNA (63–66). It is unclear whether HIV-1 DNA nuclear import in proliferating CD4 T cells is regulated through the identical pathway seen in nondividing cells. A reasonable possibility is that c-Myc affects the expression of genes encoding cellular proteins involved in nuclear transport. Further elucidation of the role of c-Myc in regulation of expression of cellular nuclear importing molecules might help us to understand how c-Myc regulates HIV-1 DNA nuclear import.

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