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Repression of the Human Immunodeficiency Virus Type-1 Long Terminal Repeat by the c-Myc Oncoprotein

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Abstract The effect of *trans*-acting factors on *cis*-acting DNA elements on the HIV-1 promoter are the principal determinant regulating transcriptional activation and repression. Host factors that limit viral replication can contribute to the emergence and maintenance of proviral reservoirs. The current paradigm is that this sub-population of latently infected cells confers a biological advantage to the virus by facilitating evasion of immunologic responses and therapeutic strategies resulting in life-long and persistent infection. In this report, we show that ectopic expression of the nuclear phosphoprotein, c-Myc can inhibit HIV-1 gene expression and virus production in CD4+ T-lymphocytes. The effect exerted does not appear to involve other known functions of c-Myc such as proliferation, or apoptosis. The mechanism does implicate c-Myc in a direct role. We have found evidence that c-Myc can specifically recognize the HIV-1 initiator element surrounding the start site of transcription and linker scanning mutagenesis experiments confirmed a loss of c-Myc-mediated repression in the absence of this region. Moreover, we show that c-Myc can interact with the initiator binding proteins YY-1 and LBP-1 and can cooperate with these factors to synergistically repress HIV-1 LTR transcription. Taken together, these results indicate that c-Myc is an important regulator of HIV-1 transcription that potentially contributes to the latent proviral state. J. Cell. Biochem. 92: 400–413, 2004.

Key words: c-Myc; HIV-1; long terminal repeat; transcriptional repression; latency

The human immunodeficiency virus long terminal repeat (HIV-1 LTR) is subdivided into several discrete functional domains that can interact with cellular DNA binding transcription factors to regulate gene expression and viral replication [reviewed in Garcia et al., 1987; Levy, 1993; Antoni et al., 1994; Kingsman and Kingsman, 1996]. While a vast proportion of host factors exert a positive modulatory effect on the promoter, a few cellular proteins that down-regulate transcription have been identified including Myc Binding Protein-1 (MBP-1) [Ray and Srinivas, 1997], Leader Binding Protein-1 (LBP-1) [Parada et al., 1995], and Yin Yang-1 (YY-1) [Margolis et al., 1994]. YY-1 was first described to mediate transcription through initiator elements [Austen et al., 1997]. The ability of YY-1 to physically interact with a wide array of proteins contributes to recognition of multiple target genes and imparts this protein's multi-functionality. Thus, the promoter context and the pre-existing YY-1-protein complexes will dictate whether YY-1 behaves as an activator, repressor, or initiator. Indeed, it has been reported that YY-1 can cooperate with another initiator binding protein, LBP-1 to mutually repress HIV-1 gene expression and virus replication [Romerio et al., 1997] and that the mechanism involves recruitment of histone

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deacetylase [Coull et al., 2000]. In addition, YY-1 has also been demonstrated to associate with the c-Myc oncoprotein in a manner that depends on the cellular levels of c-Myc [Shrivastava et al., 1996].

c-Myc was first described as the cellular homologue to the transforming sequences of the avian myelocytomatosis retrovirus, MC29 [Vennstrom et al., 1982]. Through its function as a transcription factor, c-Myc is able to regulate such disparate biological activities that includes growth, cell cycle progression, differentiation, transformation, and apoptosis [reviewed in Oster et al., 2002]. c-Myc is a member of the basic-helix-loop-helix/leucine zipper class proteins that transactivates target genes by binding to canonical CACGTG elements termed E-box Myc sequences [reviewed in Luscher and Larsson, 1999] with its protein partner, Max [Blackwood and Eisenman, 1991]. In contrast, the inhibitory effect on transcription by c-Myc can occur in an initiator-independent [Gartel et al., 2001] or initiator-dependent manner [Li et al., 1994]. This is supported by the fact that c-Myc is capable of interacting with initiator binding proteins such as TFII-I [Roy et al., 1993], Myc interacting zinc finger protein-1 (Miz-1) [Peukert et al., 1997] and YY-1 [Shrivastava et al., 1996]. Despite intensive research, the relationship between c-Myc and YY-1 has remained obscure. It has been previously demonstrated that c-myc can inhibit the activation and repression functions of YY-1 in a manner that does not preclude YY-1 binding to its DNA recognition motif, but rather by competitively disrupting the ability of YY-1 to contact members of the basal transcriptional machinery [Shrivastava et al., 1993]. Interestingly, over-expression of YY-1 was found to activate the c-Myc P1 and P2 promoters [Riggs et al., 1993], whereas it was subsequently shown that YY-1 could inhibit c-Myc function in a manner that does not require direct interaction [Austen et al., 1998].

Thus, given the ability of YY-1 to negatively regulate HIV-1 transcription and the enigmatic relationship that exists between YY-1 and c-Myc, the potential modulatory role of c-Myc at this promoter raised interest. The results reported herein represent our findings which demonstrate repression of HIV-1 promoter expression and virus production in the presence of transfected, not endogenous introduced c-Myc.

MATERIALS AND METHODS

Expression Vectors

The plasmid, HIV-1-LTR-CAT contains coding sequences for the U3 and R regions from the 5′ long terminal repeat of the HXB2 clone of HIV-1. These regions are inserted upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene of pSVO-CAT. The HIV-1-LTR linker scanning mutants are derivatives of the wild type promoter in which 18 base pairs of the U3 region were sequentially replaced by a linker sequence corresponding to the restriction enzymes, *Nde*I-*Xho*I-*Sal*I [CATATGCTCGAGGTCGAC] in order to generate a panel of 26 mutants (NIH AIDS Research and Reference Reagent Repository, deposited by Dr. Steven Zeichner). pSV-Tat is an SV40 driven tat gene required for transactivation of the HIV-1–LTR. pEF–c-Myc is a plasmid construct containing the coding exons for the wild type c-Myc gene driven by the elongation factor promoter (a kind gift from Dr. Moulay Jamali, McGill University). The c-Myc amino terminal transactivation deletion mutants spanning amino acids 1–143 were cloned into the vector pMNBabeGFP-IRES and are driven by the Moloney murine leukemia virus promoter. The plasmids, pCMV-YY-1, and pCMV-LBP-1 represent the coding sequences for the genes driven by the cytomegalovirus promoter (a kind gift from Dr. Ulla Hansen, Boston University). pCMV-USF is a plasmid construct containing the USF gene cloned into the vector, pCDNA3.1(–) downstream of the cytomegalovirus promoter (a kind gift from Dr. Robert Roeder, Rockefeller University). Virus production studies were performed with pBH10, the infectious molecular cDNA clone of HIV-1 (NIH AIDS Research and Reference Reagent Repository). pCMV-GFP is a plasmid containing green fluorescent protein driven by the cytomegalovirus promoter and was employed as an internal transfection control. All plasmids were transformed in *E. coli* DH5α cells and selected for ampicillin resistance.

Cell Culture

Jurkat cells were grown in suspension in RPMI 1640 (Gibco, Grand Island, NY) containing 10% (v/v) fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), pyruvate (1 mM), and HEPES (10 mM). Cells were maintained at 37°C with

5% CO_2. Transfections were performed when densities reached 1 million cells/ml of culture volume.

Transfections

Jurkat cells were transiently co-transfected by the DEAE-dextran method. Cells were subcultured one day prior and density was adjusted such that on the day of transfection, cells were growing in active log phase. A total of 15 million cells per transfection were suspended in 1 ml of freshly prepared Tris-buffered saline containing 100 μg/ml each of $CaCl_2$ and $MgCl_2$ and 10 mM glucose. The DNA mixture was prepared by combining 1.0 μg of HIV-1-LTR-CAT and 0.25 μg of pSV-Tat, 1.0 μg of pCMV-GFP, and 1.0 μg each of the transcription factors, pEF-c-Myc, pCMV-YY-1, pCMV-LBP-1, pCMV-USF in various combinations and 1mg/ml (v/v) of DEAE-dextran. In control transfections, 1.0 μg of the reporter construct is combined with 0.25 μg of pSV-Tat and the unmodified vector(s) for each gene of interest. All transfections were adjusted with an appropriate quantity of salmon sperm DNA. The cell suspension was then combined with the DNA–DEAE–dextran mixture and incubated for 60 min at 37°C with 5% CO_2. dimethylsulfoxide (10%) (v/v) was added for 3 min to enhance transfection efficiency followed by immediate dilution in phosphate-buffered saline (PBS) [Ca^{2+}/Mg^{2+} free]. Cells were washed twice with PBS [Ca^{2+}/Mg^{2+} free] before being resuspended in complete medium and incubated for 48 h at 37°C with 5% CO_2.

Reporter Assays

Cells were harvested 48 h post-transfection. Cells were washed once in 5 ml of PBS followed by a more stringent wash in TEN buffer (40 mM Tris at pH 7.5, 1 mM EDTA at pH 8.0, 15 nM NaCl). Cell pellets were recovered by high-speed centrifugation and resuspended in 0.25 M Tris at pH 7.5. Cells were then lysed by three consecutive cycles of freezing and thawing. The supernatants were collected and analyzed for protein content by the modified Lowry method. Approximately 100–150 μg of protein was incubated for 60 min at 37°C with 4 mM acetyl CoA, and 0.1 μCi of [1,2-^{14}C]chloramphenicol (ICN). The chloramphenicol metabolites were extracted with the organic solvent, ethyl acetate and then spotted on thin-layer chromatography silica gel membranes. The various metabolites were resolved by a 19:1 eluant of chloroform:methanol and analyzed by exposure to a Bio-Rad GS 250 phosphor-Image screen for 24 h. The results were visualized using the Bio-Rad GS-363 scanner and the chloramphenicol metabolites were then quantitated using Bio-Rad Molecular Analyst software (version 2.0.1). The level of CAT expression was calculated as the percentage of the two acetylated forms of chloramphenicol relative to the total amount of [1,2-^{14}C]chloramphenicol. Reporter assays were repeated with more or less cell lysate if the percent CAT activity was not within the acceptable linear range of 5–70%. Transfection efficiencies were normalized by flow cytometry using pCMV-GFP as an internal control. Experiments were repeated at least three times to estimate the variations of transfection efficiency.

Cellular Proliferation and Viability

A 48 h post-transfection, aliquots from cultures for each sample were obtained and counted to assess cellular proliferation while viability was determined by the Trypan blue exclusion method.

Virus Production

Jurkat cells were transiently co-transfected with 1.0 μg of the infectious molecular clone, BH10 and 1.0 or 2.0 μg of c-Myc or the control unmodified vector by lipofectamine according to manufacturer instructions. Culture supernatants were removed 72 h post-transfection. Cell suspensions were cleared by low-speed centrifugation, diluted appropriately prior to p24 Gag quantitation and processed for reactivity by using an enzyme immunoassay diagnostic kit (NIH AIDS Research and Reference Reagent Repository).

Apoptosis

The ApoAlert Caspase-3 Colorimetric Assay Kit (Clontech, Mississauga, ON) detects the presence of caspase-3 by its cleavage of a labeled substrate DEVD-pNA. Detection of the cleaved chromophore *p*-nitroanilide was performed using a spectrophotometer at 405 ηm. Jurkat cells were counted and 2×10^6 cells from transfected samples were centrifuged at 200 *g* for 10 min. Cell pellets were resuspended in 50 μl of cell lysis buffer and incubated on ice for 10 min. The cell lysates were cleared by high-speed centrifugation for 3 min at 4°C. The supernatant was

retrieved and assayed for protease activity by the addition of 50 μl of reaction buffer and 5 μl of the caspase-3 substrate (1 mM DEVD-pNA). Samples were incubated for 1 h at 37°C and subsequently measured spectrophotometrically at 405 nanometers (nm). As a positive control, apoptosis was induced in Jurkat cells by incubation with 50 μM of actinomycin-D for 15 h. Uninduced samples are those cells transfected with the HIV-1 LTR-CAT and pSV-Tat expression plasmids which served as the negative control. Also, induced samples were treated with DEVD-fmk (a synthetic tetrapeptide that irreversibly inhibits caspase-3 activity) as an additional negative control. Units of protease activity were quantitated using a standard curve with free chromogenic molecule (pNA).

Co-Immunoprecipitation

Ten million cells were collected by low speed centrifugation and lysed in 1 ml of RIPA buffer (50 mM Tris at pH 7.5), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM AEBSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3Va_4 and 1 mM NaF) for 15 min at 4°C. The lysate was cleared by centrifugation at 14,000 rpm for 30 min. For co-immunoprecipitation studies, the lysate was precleared with 100 μl of protein A sepharose (50% slurry; MBI Fermentas) at 4°C for 10 min on an orbital shaker. Proteins were quantified by the modified Lowry method and adjusted to a concentration of 1 μg/μl with PBS. Only 500 μg of whole cell extract was tumbled overnight at 4°C with the rabbit polyclonal antibodies for c-Myc (N-262; Santa Cruz Biotechnology, Santa Cruz, CA), YY-1, LBP-1, or a nonspecific rabbit IgG as a control. The antibody–protein complexes were precipitated by the addition of 100 μl of a 1:1 protein A sepharose slurry in PBS and allowed to tumble at 4°C for 3 h. The antibody–protein complexes were washed three times in PBS to remove any nonspecific proteins. A 40 μl of 2× Laemmli buffer (containing 0.2 M Tris-Cl at pH 6.8, 4% SDS, 20% glycerol, 0.008% bromophenol blue, and 8% β-mercaptoethanol) was added to the beads and incubated at 95°C for 5 min. Supernatants were recovered by pulse centrifugation. Proteins were applied to a 10% SDS–polyacrylamide gel (37.5:1) and electrophoresed in 1× Tris-Glycine buffer at 200 V for 40 min. The proteins were subsequently electrophoretically transferred for 1 h at 100 V to a 0.2 μM pore nitrocellulose membrane. The membrane was blocked with 0.1% PBS–Tween-20 and 5% non-fat milk for 1 h at RT or overnight at 4°C with rotation. The membrane is then incubated with a 1:1000 dilution of the primary antibody in the blocking reagent for 1 h at RT or overnight at 4°C. The membrane was washed in 0.1% PBS-T and then incubated with a 1:1000 dilution of the secondary antibody conjugated to horse-radish peroxidase in blocking reagent for 30 min at RT. The membrane is again washed prior to the addition of the chemiluminescence reagents (ECL, Amersham Pharmacia, Baie D'Urfe, PQ) and exposure to Kodak X-Omat Blue film for 1 min.

Electrophoretic Mobility-Shift Assay

Ten million cells were collected by low-speed centrifugation for 5 min. The cell pellet was washed with PBS, centrifuged, and resuspended in pre-chilled buffer A containing 10 mM HEPES at pH 7.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF. The supernatant was cleared by centrifugation and the cell pellet was resuspended in buffer A/0.1% NP-40 and incubated on ice for 10 min to permit cell lysis. The cytoplasmic fraction was collected by a 10 min centrifugation at 14,000 rpm. The pellet was further resuspended in chilled buffer B (containing 20 mM HEPES at pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 0.5 mM spermidine) and incubated for 15 min on ice. The nuclear proteins were subsequently collected by high-speed centrifugation and diluted in 75 μl of cold buffer C (20 mM HEPES at pH 7.0, 20% glycerol, 0.2 mM EDTA, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF). Protein content was determined by the Bradford assay. The double-stranded DNA probe used in the binding reactions represents the −19 to +26 region of the HIV-1-LTR initiator element 5′-TGCTTTTTGCCTGT-ACTGGGTCTCTCTGGTTAGACCAGATCTGA-G-3′ (Canadian Life Technologies, Canada) and contains binding sites for the transcription factors, USF, LBP-1, and YY-1. The oligonucleotide was end-labeled with [γ-^{32}P] and T4 polynucleotide kinase (MBI Fermentas) for 1 h at 37°C. The labeled oligonucleotides were purified on a G-25 sephadex column (Amersham Pharmacia). Protein–DNA binding reactions consisted of 5 μg of nuclear extract, 0.2 ng of radioactively end-labeled double stranded probe,

2.5 μg of poly(dI-dC) (Amersham Pharmacia) to reduce nonspecific interactions and DNA binding assay (DBA) buffer (10 mM Tris at pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol). In competition reactions, nuclear extracts were pre-incubated with a 100-fold excess of unlabelled oligonucleotide. In antibody depletion experiments, DNA binding reactions were supplemented with anti-c-myc antibody, a nonspecific antibody (anti-GST), or pre-immune serum. The reactions were incubated for 30 min at RT. The DNA–protein complexes were resolved on a 5% non-denaturing polyacrylamide gel (60:1). Electrophoresis was performed using the Protean II xi from Bio-Rad in 0.5× Tris-borate-EDTA (TBE) at 200 V for 4 h in the cold. The gel was blotted on to 3 MM Whatman filter paper and dried for 30 min at 80°C before being exposed to Kodak X-Omat Blue film overnight at −70°C.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism version 2 software. Results were analyzed by one-way analysis of variance (ANOVA) or the paired *t*-test in which the lowest level of significance was set at $^*P < 0.05$ with a 95% confidence level.

RESULTS

c-Myc Can Inhibit Tat-Activated HIV-1 LTR-Directed CAT Expression

To examine the role of c-Myc on HIV-1 LTR-directed transcriptional regulation, Jurkat cells were transiently co-transfected with 1.0 or 2.0 μg of pEF-c-Myc expression plasmid and a constant level of the HIV-1 LTR-CAT reporter construct in the presence of pSV-tat. As demonstrated in Figure 1, the population of cells that were treated with 1.0 μg of c-Myc expression vector exhibited a 31% reduction of HIV-1 LTR-CAT expression. Similarly, cells that received 2.0 μg of c-Myc showed a 43% reduction with respect to the control indicating that over-expression of the c-Myc protein is able to negatively regulate HIV-1 transcription in a dose-dependent manner in a CD4+ T-lymphocyte cell line. Importantly, comparable levels of repression in transfected 293-T cells were also observed (data not shown). Moreover, these levels of repression are consistent with numerous reports showing typically a 2–5-fold change in promoter activity [Li et al., 1994; Gartel et al., 2001; Staller et al., 2001]. The effects of c-Myc on basal transcriptional activity could not be evaluated as HIV-1 LTR-CAT expression was undetectable demonstrating the essential role of tat on HIV-1 transactivation (data not shown).

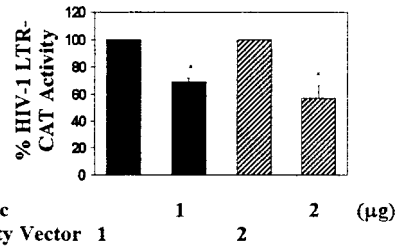

Fig. 1. c-Myc represses the HIV-1 LTR promoter in a dose-dependent manner. Jurkat cells were transfected with 1.0 μg of the HIV-1 LTR-CAT reporter, 0.25 μg of pSV-tat and 1.0 μg or 2.0 μg of the c-myc expression vector or the unmodified vector as control. All transfections were supplemented with appropriate quantities of carrier DNA (salmon sperm). The histogram demonstrates the average percent CAT activity of each group with respect to the control (HIV-1 LTR-CAT, pSV-Tat, and unmodified vector). The results are expressed as +SEM for n = 5. Statistical significance was determined by a one-way ANOVA test ($^*P < 0.05$).

Effect of c-Myc on Cellular Viability and Apoptosis

It has been well-established that c-Myc plays an integral role in the regulation of a wide variety of cellular processes [Dang, 1999]. Consequently, the levels of c-Myc protein within a cell must be precisely controlled. Indeed, when c-Myc is inappropriately expressed or deregulated, it has been implicated in promoting cellular transformation. Under these circumstances, c-Myc can also drive an apoptotic program as a fail-safe mechanism to avoid neoplasia [reviewed in Claassen and Hann, 1999; Prendergast, 1999]. It was therefore important to investigate whether the repressive effect of c-Myc on the HIV-1 LTR was not simply due to death of the transfected cells. Cell viability was monitored by the trypan blue exclusion assay and apoptosis was examined by the Hoechst-propidium iodide staining method and the caspase-3 assay. Cellular proliferation (Fig. 2A) and viability (Fig. 2B) was assessed 48 h post-transfection. It was found that all groups of cells that expressed ectopic c-Myc did not differ significantly from cells transfected with the HIV-1 LTR reporter construct and tat alone. Hoechst-propidium iodide staining of c-Myc transfected cells did not exhibit apoptotic features compared to the

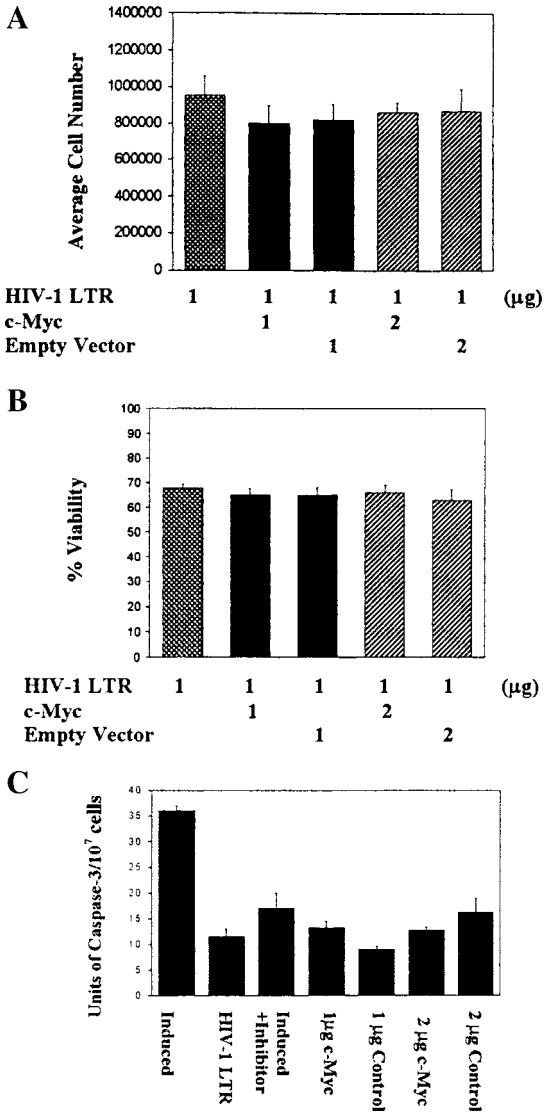

Fig. 2. The effect of c-Myc on cellular proliferation, viability and apoptosis in transfected Jurkat cells. **A**: Aliquots of cells were recovered 48 h post-transfection and counted. The results are expressed graphically as +SEM for n = 2–3. Statistical significance was determined by the one-way ANOVA test (*$P < 0.05$) with respect to the positive control. **B**: The effect of transfection on cellular viability was assessed by the trypan blue exclusion assay from aliquots of cells 48 h post-transfection. The results are depicted graphically as the average percent viability + SEM and is representative of 2–3 independently performed experiments. Statistical significance was assessed by the one-way ANOVA test (*$P < 0.05$). **C**: The effect of c-myc on apoptosis was determined by the caspase-3 assay. Cell lysates are prepared 48 h post-transfection and used to measure caspase-3 activity by a colorimetric assay. The actinomycin D treated cells represent the positive control, while negative controls included cells transfected with the promoter–reporter construct and tat alone as well as the actinomycin-D induced cells treated with a caspase-3 inhibitor. The results shown represent the average of 2–3 independent experiments where statistical significance was determined by the one-way ANOVA test (*$P < 0.05$).

positive control (actinomycin D-treated; data not shown). As a further measure of c-Myc-induced apoptosis, we investigated the induction of caspase-3 activity in Jurkat cells transfected with 1.0 or 2.0 μg of c-myc or the unmodified vector. As a positive control, cells were induced with actinomycin-D, while cells treated with actinomycin-D and a caspase-3 inhibitor were employed as a negative control. It is evident from the data presented in Figure 2C that none of the groups whether transfected with c-myc or the unmodified vector exhibited a level of caspase-3 activity statistically different from the negative control. Furthermore, the levels of caspase-3 for all groups were significantly lower with respect to the positive control. Thus, the collective data assert that c-Myc represses tat-activated HIV-1 LTR-CAT expression and that this effect is not a consequence of toxicity or apoptosis.

Inhibition of Virus Production by c-Myc

To determine if c-Myc could influence virus production, Jurkat cells were co-transfected with the infectious molecular HIV-1 clone, BH10 and 1.0 or 2.0 μg of c-Myc expression plasmid or 1.0 or 2.0 μg of the unmodified vector as controls. Culture supernatants were recovered 72 h post-transfection for determination of p24 Gag antigen levels as a direct measure of virus production. Co-transfections with the vector expressing c-Myc inhibited virus production in a dose-dependent manner (Fig. 3).

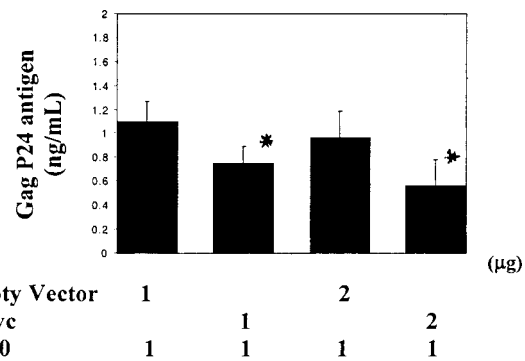

Fig. 3. Ectopic c-Myc expression reduces virus production. Jurkat cells were tranfected with 1.0 μg of an infectious molecular clone of HIV-1, BH10, and 1.0 or 2.0 μg of c-myc or the unmodified vector. Culture supernatants were recovered 72 h post-transfection to determine levels of virus production by the p24 antigen assay. The graph describes the average p24 antigen as ng/ml. The results are expressed as +SEM for n = 3–5. Statistical significance was determined by a two-tailed paired *t*-test (*$P < 0.05$).

Cooperative Inhibition of HIV-1 LTR Transcription by the Transcription Factors, c-Myc, YY-1, and LBP-1

Since it was previously shown that association with c-Myc inhibits the transcriptional activating and repressing abilities of YY-1 [Seto et al., 1991; Shrivastava and Calame, 1994] and that YY-1 and LBP-1 could synergistically repress the HIV-1 LTR [Romerio et al., 1997; Coull et al., 2000], we investigated the effect of ectopically introduced c-Myc on HIV-1 gene expression in the presence of YY-1 and/or LBP-1. A population of Jurkat cells was transiently co-transfected with the HIV-1 LTR-CAT reporter vector, tat, and various combinations of c-Myc, YY-1, and LBP-1. Figure 4A describes the average results obtained expressed as relative CAT activity. YY-1 inhibited the HIV-1 LTR as previously reported [Margolis et al., 1994]. Overall, cells that were co-transfected with any paired combination of transcription factors reduced HIV-1 promoter expression to a greater extent in comparison with any single protein. Indeed, we observed a 75% reduction in transcription in the presence of tat when c-Myc and LBP-1 were simultaneously ectopically expressed. When all three proteins were concurrently over-expressed, tat-activated HIV-1 LTR activity was reduced by as much as 86% indicating that c-Myc, YY-1, and LBP-1 are able to synergistically repress the HIV-1 promoter. While the repressive effect on the HIV-1 LTR in the presence of simultaneous ectopic expression of c-Myc, YY-1, and LBP-1 was not significantly different from co-expression of any pair of transcription factors, the tendency for greater transcriptional inhibition with all three proteins was consistently detected.

It has been previously reported that c-Myc can interact with YY-1 in mammalian cells [Shrivastava et al., 1996] via the c-Myc basic/helix-loop-helix/leucine zipper motif within the carboxyl terminal domain [Austen et al., 1998] and that YY-1 can interact with LBP-1 in vivo [Romerio et al., 1997; Coull et al., 2000]. We therefore wanted to investigate if the synergistic repression of the HIV-1 LTR that we observed was due to formation of a multi-protein complex between c-Myc, YY-1, and LBP-1 by performing co-immunoprecipitation studies. Jurkat whole cell extracts were immunoprecipitated with anti-YY-1, anti-c-Myc, anti-LBP-1 antibodies or a non-specific rabbit polyclonal antibody and then probed by western blot for c-Myc, YY-1, and LBP-1 expression. We confirmed that c-Myc and LBP-1 can interact with YY-1 in vivo in Jurkat cells (Fig. 4B). In addition, we found evidence that endogenous levels of c-Myc can co-immunoprecipitate with LBP-1. The results are specific as only trace amounts of protein were recovered by nonspecific antibody and mock immunoprecipitation. Thus, these

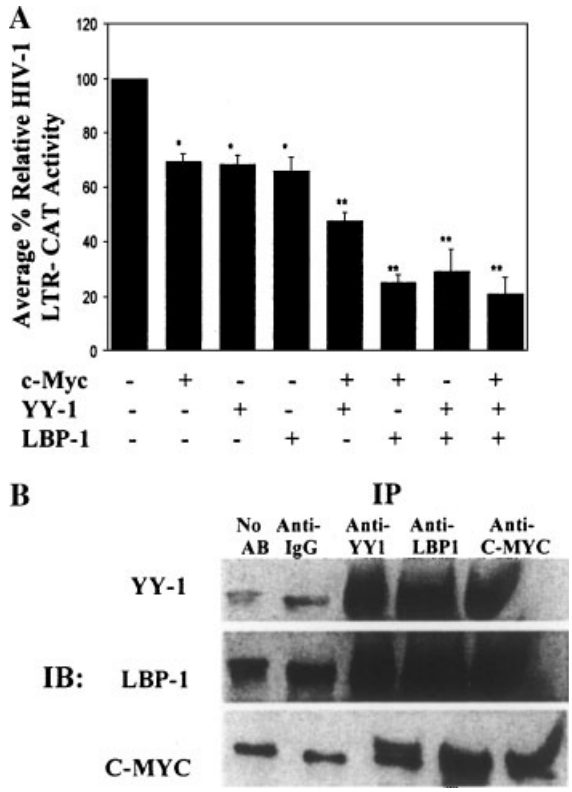

Fig. 4. **A**: Transcriptional repression of the HIV-1 LTR by concurrent ectopic expression of c-Myc, YY-1, and LBP-1. Jurkat cells were transiently co-transfected with 1.0 μg of the HIV-1 LTR-CAT plasmid construct, 0.25 μg of pSV-tat and various combinations of 1.0 μg of pEF–c-Myc, pCMV–YY-1, pCMV–LBP-1 or unmodified vectors. The graph shows the average percent CAT activity of each group with respect to the control empty vector. The results are expressed as +SEM and are representative of five independently conducted experiments. Statistical significance was determined by the one-way ANOVA test ($*P < 0.01$, $**P < 0.001$). **B**: c-Myc, YY-1, and LBP-1 can interact with each other in vivo and in the absence of the HIV-1 LTR. Whole cell extracts were prepared from Jurkat cells and immunoprecipitated with anti-c-Myc, anti-YY-1, anti-LBP-1, irrelevant rabbit IgG antibodies, or left untreated. The resulting complexes were captured with protein A sepharose beads, boiled and the supernatants were loaded on to a 10% SDS–polyacrylamide gel. The results are shown as immunoblots probing for (i) YY-1 expression, (ii) LBP-1 expression, and (iii) c-Myc expression. The results shown are representative of two independently performed experiments.

experiments demonstrate that c-Myc is able to interact with both YY-1 and LBP-1 in vivo and in the absence of the HIV-1 LTR. It further provides suggestive evidence that this tertiary complex pre-assembles in the cell and is recruited to the promoter, although the exact nature of these interactions requires further investigation.

Effect of Ectopic USF on c-Myc-Mediated Repression of HIV-1 Transcription

Since it has been shown that the basic-helix-loop-helix protein, USF stimulates transcription through an E box motif located in proximity to the initiation region of the HIV-1 LTR [Du et al., 1993], it was important to investigate if the ectopic expression of c-Myc could outcompete USF and thus antagonize USF-mediated stimulation of HIV-1 transcription. This hypothesis was tested by co-transfecting increasing concentrations of c-Myc with constant levels of ectopic USF and the HIV-1 LTR. As demonstrated in Figure 5, c-Myc inhibited, while USF stimulated tat-activated HIV-1 LTR-CAT expression. However, when c-Myc and USF were co-expressed, USF-mediated activation of the HIV-1 LTR was not overcome by increasing amounts of c-Myc. This provides suggestive evidence that the mechanism of c-Myc repression of the HIV-1 LTR occurs by interactions with YY-1 and LBP-1 and not by out-competition of USF at the E box element at the core promoter.

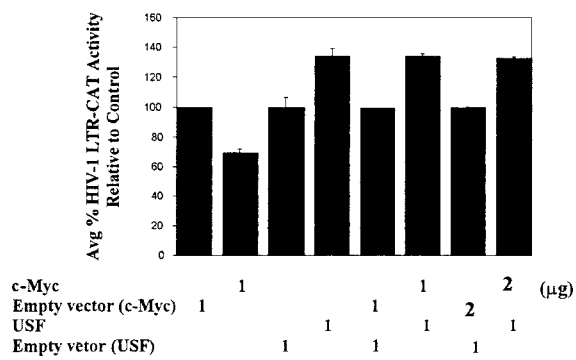

Fig. 5. c-Myc does not antagonize USF-mediated stimulation of the HIV-1 LTR. Jurkat cells were transiently transfected with 1.0 μg of the HIV-1 LTR-CAT plasmid, 0.25 μg of pSV-tat, and 1.0 μg of pEF–c-myc and/or 1.0 μg of pCMV–USF. The results are presented graphically as the average percent CAT activity relative to the appropriate empty vector control and are expressed as +SEM for n = 3. Statistical significance was determined by one-way ANOVA (*$P < 0.05$).

Recognition of the HIV-1 LTR by c-Myc

The mechanism of c-Myc-facilitated repression was further investigated by the electrophoretic mobility-shift assay. Nuclear extracts prepared from Jurkat cells were treated with a [γ-^{32}P]-end-labeled probe corresponding to nucleotides −19 to +26 of the HIV-1 initiator, which includes binding sites for YY-1, LBP-1, and USF. In competition gel-shift experiments, nuclear extracts were pre-incubated with a 100-fold excess of unlabeled probe while in antibody depletion studies, the binding reactions were supplemented with a nonspecific antibody (anti-GST), pre-immune serum, or an anti-c-Myc antibody. The effect of c-Myc antibody on electrophoretic mobility-shift complexes formed with the initiator oligonucleotide provided evidence that c-Myc was involved in complex formation (Fig. 6, lane 5). Preimmune serum or an

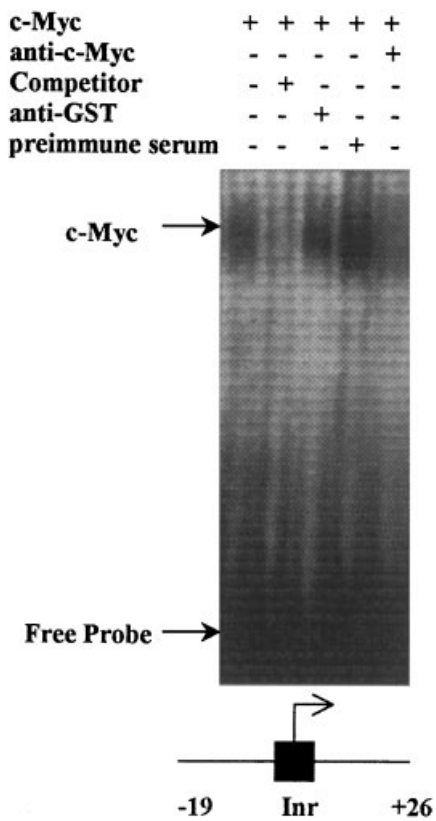

Fig. 6. A c-Myc-specific complex forms on the HIV-1 LTR. Jurkat cells were transfected as previously described and nuclear extracts were probed with a radioactively labeled oligonucleotide corresponding to the −19 to +26 region of the HIV-1 LTR with respect to the transcriptional start site. The lanes are designated as follows: (**1**) c-Myc; (**2**) Competition; (**3**) c-Myc + 2.0 μg nonspecific antibody (anti-GST); (**4**) c-Myc + 2.0 μg preimmune serum; (**5**) c-Myc + 2.0 μg anti-c-Myc antibody.

irrelevant antibody had no effect on complex formation (Fig. 6, lanes 3 and 4), while the competition reaction demonstrates that the complex formed was sequence-specific since it could be abrogated by the inclusion of the unlabeled probe (Fig. 6, lane 2). These results indicate that c-Myc can specifically recognize the initiator element and therefore exerts a direct effect on HIV-1 LTR transcription.

Characterization of the c-Myc Responsive Element of the HIV-1 LTR

To lend further support to the results of the electrophoretic mobility-shift assay, the c-Myc responsive site on the HIV-1 LTR was characterized by structure-function studies performed with a series of linker scanner mutants spanning the entire U3 region (Fig. 7A). Jurkat cells were transfected with tat, the panel of HIV-1 LTR reporter mutants in the presence or absence of c-Myc and analyzed for relative CAT expression. As demonstrated in Figure 7B, c-Myc retained the ability to repress transcription in a vast proportion of HIV-1 LTR mutants that were surveyed to a comparable extent as the wild-type promoter. However, the linker scanner mutants −363/−346, −183/−166, and −3/15 exhibited a more profound repressive effect indicating that these regions must participate in activation. In the complete absence of the TATA motif (−39/−22), reporter activity was abrogated to nearly undetectable levels rendering the role of c-Myc at this particular region of the LTR uninterpretable. However, c-Myc lost the ability to repress in the absence of the −21/−4 domain implicating this site as the minimal responsive element on the HIV-1 LTR. This region is known to correspond to the upstream portion of the initiator, which includes a proximal E box motif flanking the TATA element, the YY-1 binding site and the low affinity LBP-1 binding site. Importantly, these results are consistent with the electrophoretic mobility-shift where we show c-Myc binding to the initiation region as well as the co-immunoprecipitation studies where we demonstrated that c-Myc, YY-1, and LBP-1 could interact with each other in vivo.

Characterization of c-Myc Functional Domains

It has been previously reported that the transcriptional activation and repression activities of c-Myc can be segregated from each other and linked to precise domains of the protein [Lee et al., 1996; Xiao et al., 1998; Chang et al., 2000; Oster et al., 2003]. Therefore, to address which region of c-Myc is involved in repressing transcription of the HIV-1 LTR, we employed a series of deletion and point mutants of the amino terminal portion of the c-Myc protein (amino acids 1–143) (Fig. 8A). Jurkat cells were transiently transfected with the wild-type HIV-1 LTR reporter construct and various c-Myc mutants and then assayed for levels of expression compared to wild-type c-Myc. As demonstrated in Figure 8B, repression was enhanced between 35% and 79% for the panel of mutants that we tested. Contrary to previous reports that link Myc Box II to transcriptional repression of the Adenovirus Major Late promoter [Li et al., 1994], we were unable to map this particular domain, nor any amino terminal mutant surveyed to repression of the HIV-1 LTR. Moreover, it provides suggestive evidence that a region within the carboxyl terminal domain of c-Myc is involved in facilitating repression of the HIV-1 LTR.

DISCUSSION

Throughout the viral lifecycle, HIV-1 uses multiple strategies to exploit the host cell machinery to facilitate replication and enhance its pathogenicity. Once integrated into the host genome, the provirus is subject to transcriptional control through dynamic interactions with cellular proteins. While the vast majority of host factors exert a positive regulatory effect that enhances virion production, only a few cellular proteins have been identified to inhibit HIV-1 transcription. The observations presented in this article indicate that the multifunctional c-Myc oncoprotein can negatively influence tat-activated HIV-1 transcription. Tat is an early gene product that is essential for optimal HIV-1 transcription and viral replication. The mechanism by which tat mediates transcriptional activation involves recruitment of a complex of host factors referred to as P-TEFb, which significantly enhances the rate of initiation [Romano et al., 1999]. Indeed, c-Myc has also been shown to interact with components of the PTEF-b complex and transactivate the cad promoter [Eberhardy and Farnham, 2002], however, it remains unclear whether c-Myc interacts with PTEF-b at the HIV-1 LTR. While this mechanism is inconsistent with the results of our transcription

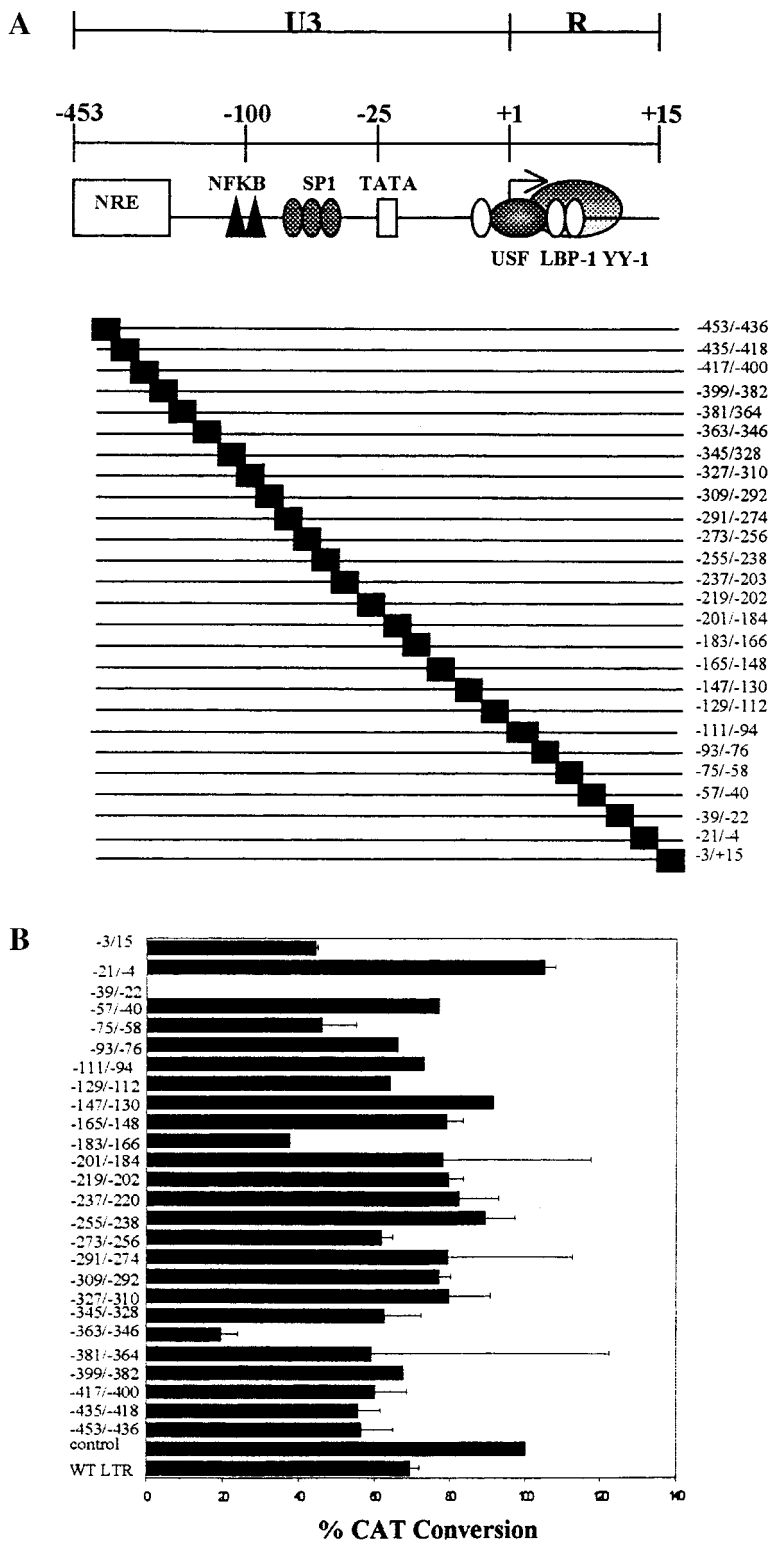

Fig. 7. Identification of the c-Myc responsive element on the HIV-1 LTR by linker scanner mutagenesis. **A**: Schematic representation of the wild-type structure of the HIV-1 LTR including known protein binding sites and their relative positions. Shown below is an illustration of the linker scanning mutants used in these experiments. **B**: Jurkat cells were transiently cotransfected with linker scanner mutants linked to the CAT reporter, tat, and c-myc. Control experiments were performed with the mutant promoter reporter construct, tat, and the unmodified vector. The results are presented graphically as the average percent CAT activity with respect to the control expressed as +SEM for $n = 1-3$. For simplicity, only one control is shown. Statistical significance was determined by the one-way analysis of variance test, $^*P < 0.05$.

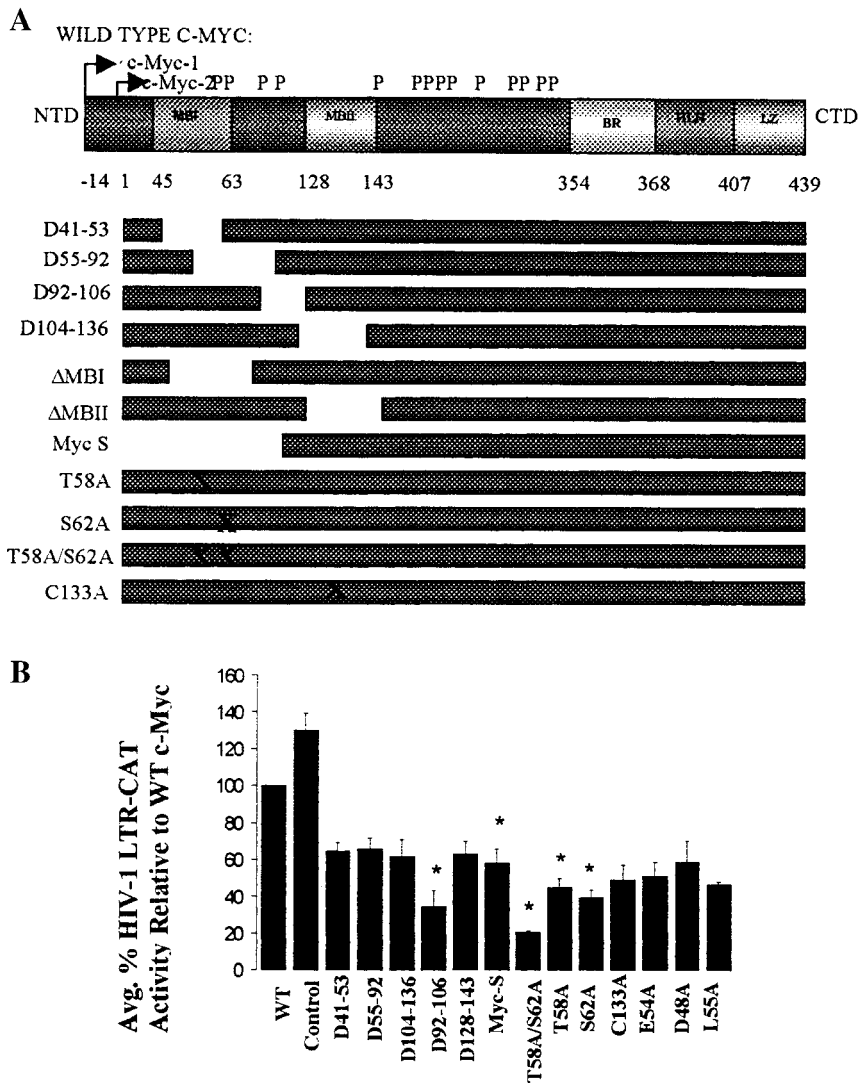

Fig. 8. Characterization of the c-Myc functional domain(s) involved in repression of the HIV-1 LTR. **A**: Schematic illustration of the structure of the wild type c-Myc protein structure and the panel of c-Myc deletion and point mutants employed in the analysis for transregulatory domain(s) involved in repression. The amino terminus spans residues 1–143 and includes the transactivation domain and two highly evolutionary conserved elements termed Myc Box I (MBI) and Myc Box II (MBII). The carboxyl terminal domain is comprised of a basic region (BR) and a helix-loop-helix/leucine zipper (HLH/LZ) motifs. Phosphorylation sites are designated as **P**. The panel of c-Myc mutants tested span the amino terminal region and are illustrated below the wild-type figure. Deletion mutants are represented as a discontinuity in the diagram, while point mutations are illustrated as **X**. **B**: The series of c-Myc mutants were transfected with the HIV-1 LTR promoter-reporter construct as previously described. In control experiments, Jurkat cells are transfected with the reporter plasmid, tat, and the unmodified empty vector instead of c-Myc. The results are illustrated graphically as the average percent CAT activity relative to wild type c-Myc and are expressed as +SEM for n = 3. Statistical significance was determined by one-way ANOVA test (*$P < 0.05$).

assays, we do not exclude the possibility that c-Myc may compete with tat for interaction with PTEF-b. Additional studies are required to address the relationship between c-Myc and tat at the HIV-1 promoter.

Previous studies have shown that YY-1 can interact with c-Myc and LBP-1 [Shrivastava et al., 1993; Coull et al., 2000]. Indeed, the results of our co-immunoprecipitation studies confirmed that these interactions can occur in Jurkat cells as well as demonstrate a novel interaction between c-Myc and LBP-1. Collectively, the co-immunoprecipitation and gene expression studies demonstrate that c-Myc participates in complex formation with the nuclear factors YY-1 and LBP-1 resulting in synergistic repression of HIV-1 transcription. However, the exact natures of these inter-

actions are yet to be fully elucidated. The functional significance of this multiprotein complex may be to reduce initiation by preventing the recruitment of members of the basal transcriptional machinery. Given that both c-Myc and YY-1 can bind TBP [Maheswaran et al., 1994; reviewed in Thomas and Seto, 1999] is consistent with the possibility that c-Myc sequesters TBP thereby preventing productive interactions between TBP and YY-1. Indeed, this idea is not without precedent. Previously, it was shown that c-Myc could interact with an initiator binding protein, TFII-I and obstruct TFII-I-TBP complex formation [Roy et al., 1993]. More recently, at the p21ink4b promoter it was demonstrated that c-Myc could sequester the initiator binding protein, Miz-1 resulting in transcriptional repression by interfering with productive interactions between Miz-1 and the p300 co-activator [Staller et al., 2001]. Alternatively, c-Myc may contribute to HIV-1 transcriptional repression by recruitment of ATPase-dependent chromatin remodeling enzymes via its ability to interact with INI-1 [Cheng et al., 1999], a component of the SWI/SNF multiprotein complex. In this manner, the coupling of YY-1 mediated histone deacetylase recruitment [Coull et al., 2000] with c-Myc mediated recruitment of the chromatin remodeling machinery could account for the enhanced transcriptional repression that we observed.

The HIV-1 LTR initiation region possesses an E box motif which is recognized by the basic-helix-loop-helix/leucine zipper protein, USF. It has been previously shown that USF can stimulate transcription when it binds to this region [Du et al., 1993]. Since other helix-loop-helix proteins can recognize E box sequences, we sought to examine an alternative mechanism whereby c-Myc outcompetes USF for binding to this element thereby interfering with USF-mediated activation. However, we were unable to detect reduced gene expression in the presence of increasing levels of c-Myc and constant levels of USF indicating that repression by c-Myc does not occur by direct DNA binding. These results are consistent with a previous report at the rat prothymosin-alpha promoter demonstrating that a mechanistic specificity exists for binding by different helix-loop-helix proteins to E box elements [Desbarats et al., 1996].

Previously, it was demonstrated that c-Myc can inhibit transcription in an initiator-dependent manner [Li et al., 1994]. Given that the YY-1 and LBP-1 binding sites encompass the initiation region and since c-Myc can interact with these factors in vivo, we investigated whether c-Myc could recognize the HIV-1 initiator by electrophoretic mobility-shift assay. Our findings reveal that c-Myc formed a specific protein–DNA complex which could be abrogated in the presence of c-Myc antibody. Importantly, studies with HIV-1 LTR linker scanner mutants confirmed that c-Myc lost the ability to repress transcription in the absence of the −21 to −4 domain of the initiator, thus implicating this site as the minimal c-Myc responsive element.

We also sought to identify the precise c-Myc domain involved in transcriptional repression by performing structure-function studies with deletion mutants spanning the amino terminal domain. This region is known to possess two highly conserved motifs termed Myc Box I and Myc Box II which are believed to be critical for gene regulation and biological activity [reviewed in Oster et al., 2002]. Although investigations of the Adenovirus Major-Late promoter determined that Myc Box II was involved in repression [Li et al., 1994], mutations at this site neither abolished nor attenuated expression of the HIV-1 LTR in Jurkat cells. Indeed, no single domain within the amino terminus appeared to be critical for c-Myc-mediated repression of the HIV-1 promoter. This suggests that c-Myc-mediated regulation of the HIV-1 LTR occurs by a novel mechanism. Additional structure–function studies may reveal the c-Myc site involved and offer valuable insights into the mechanism of transcriptional repression of the HIV-1 LTR.

The observations reported in this article support a model whereby c-Myc interacts with the DNA binding transcription factors, YY-1 and LBP-1 at the HIV-1 initiator element to synergistically suppress transcription. Moreover, the mechanism does not appear to involve other known functions of c-Myc such as proliferation and apoptosis, nor direct DNA binding to noncanonical E Box motifs or competition with the transcriptional activator, USF. Taken together, these studies implicate a critical role for c-Myc in repression of HIV-1 transcription and viral replication and may be essential for the establishment and maintenance of proviral latency.

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REFERENCES

Antoni BA, Stein SB, Rabson AB. 1994. Regulation of human immunodeficiency virus infection: Implications for pathogenesis. Adv Virus Res 43:53–145.

Austen M, Luscher B, Luscher-Firzlaff JM. 1997. Characterization of the transcriptional regulator YY-1. The bipartite transactivation domain is independent of interaction with the TATA box-binding protein, transcription factor IIB, TAFII55, or cAMP-responsive element-binding protein (CBP)-binding protein. J Biol Chem 272:1709–1717.

Austen M, Cerni C, Luscher-Firzlaff JM, Luscher B. 1998. YY1 can inhibit c-myc function through a mechanism requiring DNA binding of YY1 but neither its transactivation domain nor direct interaction with c-Myc. Oncogene 17:511–520.

Blackwood EM, Eisenman RN. 1991. Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 4998:1211–1217.

Chang DW, Claassen GF, Hann SR, Cole MD. 2000. The c-Myc transactivation domain is a direct modulator of apoptotic versus proliferative signals. Mol Cell Biol 20:4309–4319.

Cheng SW, Davies KP, Yung E, Beltran RJ, Yu J, Kalpana GV. 1999. C-MYC interacts with INI1/hSNF5 and requires the SWI/SNF complex for transactivation function. Nat Genet 1:102–105.

Claassen GF, Hann SR. 1999. Myc-mediated transformation: the repression connection. Oncogene 18:2925–2933.

Coull JJ, Romerio F, Sun J-M, Volker JL, Galvin KM, Davie JR, Shi Y, Hansen U, Margolis DM. 2000. The human factors YY-1 and LSF repress the human immunodeficiency virus type-1 long terminal repeat via recruitment of histone deacetylase 1. J Virol 74:6790–6799.

Dang CV. 1999. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 19: 1–11.

Desbarats L, Gaubatz S, Eilers M. 1996. Discrimination between different E-box binding proteins at an endogenous target gene of c-myc. Genes Dev 10:447–460.

Du H, Roy AL, Roeder RG. 1993. Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. EMBO J 12:501–511.

Eberhardy SR, Farnham PJ. 2002. Myc recruits PTEF-b to mediate the final step in the transcriptional activation of the cad promoter. J Biol Chem 277:40156–40162.

Garcia JA, Wu FK, Mitsuyasu R, Gaynor RB. 1987. Interactions of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. EMBO J 6:3761–3770.

Gartel AL, Ye X, Goufman P, Shianov P, Hay N, Najmabadi F, Tyner AL. 2001. Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. PNAS 98:4510–4515.

Kingsman SM, Kingsman AJ. 1996. The regulation of human immunodeficiency virus type-1 gene expression. Eur J Biochem 240:491–507.

Lee LA, Dolde C, Barrett J, Wu CS, Dang CV. 1996. A link between c-Myc-mediated transcriptional repression and neoplastic transformation. J Clin Invest 97:1687–1695.

Levy JA. 1993. Pathogenesis of human immunodeficiency virus infection. Microbiol Rev 57:183–289.

Li LH, Nerlov G, Prendergast G, MacGregor D, Ziff EB. 1994. C-myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II. EMBO J 13:4070–4079.

Luscher B, Larsson LG. 1999. The basic region/helix-loop-helix/leucine zipper domain of myc proto-oncoproteins: Function and regulation. Oncogene 18:2955–2966.

Maheswaran S, Lee H, Sonenshein GE. 1994. Intracellular association of the protein product of the c-myc oncogene with the TATA-binding protein. Mol Cell Biol 14:1147–1152.

Margolis DM, Somasundaran M, Green MR. 1994. Human transcription factor YY-1 represses human immunodeficiency virus type-1 transcription and virion production. J Virol 68:905–910.

Oster SK, Ho CS, Soucie EL, Penn LZ. 2002. The myc oncogene: Marvelousl Y complex. Adv Cancer Res 84:81–154.

Oster SK, Mao DY, Kennedy J, Penn LZ. 2003. Functional analysis of the N-terminal domain of the Myc oncoprotein. Oncogene 13:1998–2010.

Parada CA, Yoon JB, Roeder RG. 1995. A novel LBP-1 mediated restriction of HIV-1 transcription at the level of elongation in vitro. J Biol Chem 270:2274–2283.

Peukert K, Staller P, Schneider A, Carmichael G, Hanel F, Eilers M. 1997. An alternative pathway for gene regulation by Myc. EMBO J 16:5672–5686.

Prendergast GC. 1999. Mechanisms of apoptosis by c-myc. Oncogene 18:2967–2987.

Ray RB, Srinivas RV. 1997. Inhibition of human immunodeficiency virus type-1 replication by a cellular transcriptional factor MBP-1. J Cell Biochem 64:565–572.

Riggs K, Saleque S, Wong KK, Merrell KT, Lee JS, Shi Y, Calame K. 1993. Yin-Yang 1 activates the c-myc promoter. Mol Cell Biol 13:7487–7495.

Romano G, Kasten M, De Falco G, Micheli P, Khalili K, Giordano A. 1999. Regulatory functions of Cdk9 and cyclin T1 in HIV tat transactivation pathway gene expression. J Cell Biochem 75:357–368.

Romerio G, Gabriel MN, Margolis DM. 1997. Repression of human immunodeficiency virus type-1 through the novel cooperation of human factors YY-1 and LSF. J Virol 71: 9375–9382.

Roy AL, Carruthers C, Gutjahr T, Roeder RG. 1993. Direct role for myc in transcription initiation mediated by interactions with TFII-I. Nature 365:359–361.

Seto E, Shi Y, Shenk T. 1991. YY-1 is an initiator sequence-binding protein that directs and activates transcription in vitro. Nature 354:241–245.

Shrivastava A, Calame K. 1994. An analysis of genes regulated by the multi-functional transcriptional regulator Yin Yang-1. Nucleic Acids Res 22:5151–5155.

Shrivastava A, Saleque S, Kalpana GV, Artandi S, Goff SP, Calame K. 1993. Inhibition of transcriptional regulator Yin Yang-1 by association with c-Myc. Science 262:1889–1892.

Shrivastava A, Yu J, Artandi S, Calame K. 1996. YY-1 and c-Myc associate in vivo in a manner that depends on c-Myc levels. Proc Natl Acad Sci USA 93:10638–10641.

Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Moroy T, Bartek J, Massague J, Hanel F, Eilers M. 2001. Repression of p15INK4b expression by Myc through association with Miz-1. Nat Cell Biol 4:392–399.

Thomas MJ, Seto E. 1999. Unlocking the mechanism of transcription factor YY-1: Are chromatin modifying enzymes the key? Gene 236:197–208.

Vennstrom B, Sheiness D, Zabielski J, Bishop JM. 1982. Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J Virol 42:773–779.

Xiao Q, Claassen G, Shi J, Adachi S, Sedivy J, Hann SR. 1998. Transactivation defective c-Myc-S retains the ability to regulate proliferation and apoptosis. Genes Dev 12: 3803–3808.