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Absolute dependence on κB responsive elements for initiation and Tat-mediated amplification of HIV transcription in blood CD4 T lymphocytes

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The role of NF-κB-dependent signals in activating the transcriptional activity of the HIV regulatory region (LTR) was analyzed by systematic comparison of HIV LTR activity in human CD4 T cells purified from peripheral blood and a transformed lymphoblastoid T cell line. In normal CD4 T cells we also analyzed the role played by the viral κB responsive elements in HIV replication. Analysis of nuclear extracts of resting, normal T lymphocytes revealed the presence of the p50, but not the p65, NF-κB subunit and the induction by phorbol esters of *bona fide* (p50–p65) NF-κB complexes. In parallel, we observed clear enhancer-dependent HIV LTR transactivation comparable in intensity with that observed in lymphoblastoid cells. We show that unstimulated CD4 T lymphocytes offer a cellular environment of very low permissivity to HIV LTR functioning. This was in sharp contrast to the high spontaneous LTR activity observed in lymphoblastoid T cells, where LTR activity was essentially independent of κB-responsive elements. Due to the low basal LTR activity in resting T lymphocytes, NF-κB-dependent transactivation was a *sine qua non* event for induction of the HIV LTR. Surprisingly, even the function of HIV Tat in resting CD4 T lymphocytes was found to be absolutely dependent on LTR κB responsive elements. The relevance of these observations obtained in transient transfections was confirmed by the incapacity of blood CD4 T lymphocytes infected with an HIV infectious provirus carrying critical point mutations in the κB responsive elements to show any detectable transcriptional activity upon cell activation and prolonged culture *in vitro*. Our observations emphasize the importance of analyzing the functioning of HIV regulatory domains in the natural environment provided by normal CD4 T lymphocytes for HIV infection, and demonstrate an absolute requirement for NF-κB responsive elements for Tat-dependent and Tat-independent HIV transcription in blood CD4 T lymphocytes.

Key words: HIV/NF-κB/resting lymphocytes/Tat/viral transcription

Introduction

In lymphoid organs from infected patients, HIV remains in a quiescent state in most infected T cells (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993), thus suggesting that HIV transcription *in vivo* is not ongoing in CD4 lymphocytes. This notion is strengthened by findings *in vitro* showing that no HIV replication is obtained in resting peripheral blood lymphocytes (PBL). However, activation of infected PBL with mitogens results in transcription of the HIV genome and production of infectious viral progeny (Barré-Sinoussi *et al.*, 1983; Popovic *et al.*, 1984; McDougall *et al.*, 1985; Folks *et al.*, 1986a; Zack *et al.*, 1990; Bubrinsky *et al.*, 1991; Saksela *et al.*, 1993). Therefore, these data suggest that in the cellular environment of resting T lymphocytes, HIV remains in a quiescent state and HIV reactivation from this state of latency is absolutely dependent upon T cell activation (reviewed in Virelizier, 1990).

It is generally accepted that initiation of HIV transcription is under the control of cellular factors that interact with sequences located in the HIV long terminal repeat (LTR) (reviewed in Cullen and Greene, 1989; Jones, 1989; Gaynor, 1992). Among the multiple regulatory elements described in the HIV LTR, the main inducible regulatory domain is constituted by the core enhancer element, which binds and responds to the rel/κB family of transcription factors (Nabel and Baltimore, 1987; Nolan *et al.*, 1991; Schmitz and Bauerle., 1991; Schmid *et al.*, 1991; Bours *et al.*, 1992; Fujita *et al.*, 1992; Ryzeck *et al.*, 1992; Franzoso *et al.*, 1993) that are induced by a number of mitogens, cytokines and specific T cell activators, like antigen presentation through the TCR/CD3 receptor (Israel *et al.*, 1989; Osborn *et al.*, 1989; Tong-Starsken *et al.*, 1989; Hazan *et al.*, 1990; Beg *et al.*, 1993). Bona fide NF-κB is a heterodimer composed of p50 and p65 subunits (reviewed in Liou and Baltimore, 1993), where p65 is responsible for the transcriptional activity of the complex (Schmitz and Bauerle, 1991; Ballard *et al.*, 1992) and represents the major rel/κB transcriptional factor detected in the nuclei of T lymphocytes upon activation (Hazan *et al.*, 1990; Franzoso *et al.*, 1992).

Mutation of critical nucleotides in HIV enhancer sequences involved in the binding of induced NF-κB renders the HIV LTR unresponsive to activation signals capable of inducing nuclear translocation of these

transcription factors (Nabel and Baltimore, 1987; Folks *et al.*, 1989, Hazan *et al.*, 1990).

After HIV transcription is initiated, Tat, the autologous viral transactivator, greatly increases the expression of the HIV genome by elongation of nascent viral transcripts through interaction with TAR, a 59 nt RNA stem−loop structure located immediately proximal to the viral mRNA cap site (reviewed in Sharp and Marciniak, 1989; Cullen, 1993; Jones, 1993). In addition to Tat−TAR interactions a collaborative transcriptional effect between Tat and various cellular factors binding to upstream sequences in the HIV LTR, including NF-κB and SP1, has been described in different cell types (Berkhout *et al.*, 1990; Harrich *et al.*, 1990; Bachelerie *et al.*, 1991; Berkhout and Jeang, 1992; Kamine and Chinnadurai, 1992; Liu *et al.*, 1992; Taylor *et al.*, 1992). NF-κB can therefore increase HIV replication as a major element, not only driving the initiation of HIV transcription, but also cooperating with the Tat protein.

In contrast to what is observed in blood CD4 T lymphocytes, HIV infection of lymphoblastoid T cell lines results in intense replication in the absence of additional stimuli (Adachi *et al.*, 1986; Harrich *et al.*, 1990). Consequently, requirements for HIV replication in normal and transformed cells are very different, emphasizing the need to analyze mechanisms of viral transcription in cellular models relevant to HIV infection *in vivo*. Until now, the majority of studies analyzing the control mechanisms of HIV transcription have been performed in lymphoblastoid T cell lines (Folks *et al.*, 1989; Israel *et al.*, 1989; Lu *et al.*, 1990; Li *et al.*, 1991; Sakaguchi *et al.*, 1991; Liu *et al.*, 1992) and very little is known about the regulation of LTR-driven transcription and HIV reactivation in normal, resting peripheral blood CD4 T lymphocytes or about the respective roles of the HIV enhancer and Tat in the environment provided by one of the natural cell targets of HIV. Furthermore, the observation that HIV proviruses deleted of the enhancer region replicate to the same extent as wild-type proviruses in PHA-activated lymphocytes and lymphoblastoid cells (Leonard *et al.*, 1989) raises the question of whether the HIV enhancer is a critical regulatory element for both transcription initiation and replication of HIV in normal CD4 T lymphocytes.

These apparent contradictions prompted us to systematically compare the requirements for κB-dependent activation of the HIV LTR in blood CD4 T lymphocytes and lymphoblastoid T cells using transient transfections. Additionally, we analyzed whether NF-κB-dependent LTR transactivation was obligatory for both initiation and maintenance of viral transcription in blood CD4 T lymphocytes infected with either κB-mutated or wild-type HIV proviruses.

Results

Resting CD4 blood T lymphocytes, in contrast to lymphoblastoid T cells, offer a poorly permissive cell environment for the spontaneous transcriptional activity of the HIV LTR

Using transient transfections assays, we analyzed the basal activity of a luciferase expression vector driven by the U3+R sequences of the HIV LTR (LTRWT−Luc) in normal CD4 lymphocytes and the lymphoblastoid cell line J-Jhan (Figure 1a). In normal CD4 lymphocytes, the spontaneous activity of the LTRWT−Luc vector was extremely low. These results were in clear contrast to the high level of enzymatic activity expressed from the same vector transfected into lymphoblastoid cells. Indeed, an 81-fold difference in relative luciferase activity (RLU) was observed in the lysates of J-Jhan cells (RLU/10^6 cells = 1221) as compared with enzymatic activity of lysates of normal CD4 T lymphocytes (RLU/10^6 cells = 15). The comparable levels of transcription activity of LTRWT− and LTRΔκB−Luc vectors in either J-Jhan cells or resting CD4 T lymphocytes indicate that the

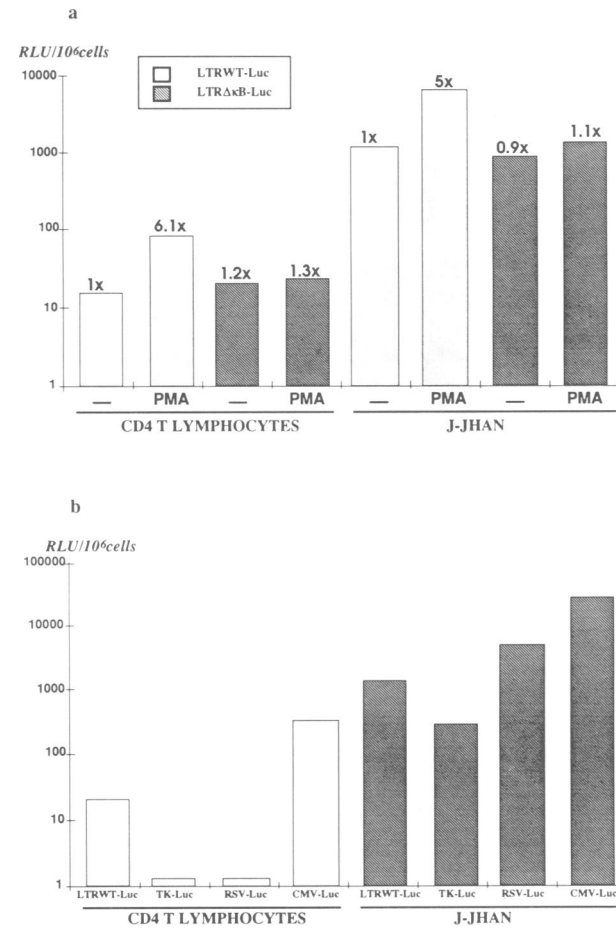

Fig. 1. (**a**) The HIV LTR is poorly expressed in normal CD4 T lymphocytes as compared with a lymphoblastoid cell line. CD4 lymphocytes (2×10^7) isolated from peripheral blood or J-Jhan cells were transfected by electroporation with 1 μg/10^6 cells of the indicated reporter plasmid. After transfection, cells were treated with 25 ng/ml PMA or were left untreated (−). Twenty four hours after transfection, cells were counted and luciferase activity measured. Results are expressed as relative luciferase units (RLU)/10^6 cells. RLU was calculated as the difference in light emission between the experimental sample and untransfected cells (background). In this particular experiment the background value was 51 RLU. Numbers above the bars indicate fold amplification of luciferase activity relative to the activity of the LTRWT−Luc plasmid transfected into the same unstimulated cell type. One of 15 representative experiments is shown. Note that a logarithmic scale is used. (**b**) Expression of different promoters in resting CD4 lymphocytes and a lymphoblastoid cell line. CD4 lymphocytes (2×10^7) isolated from peripheral blood or J-Jhan cells were transfected by electroporation with 1 μg/10^6 cells luciferase expression vectors placed under the control of several viral promoters. Twenty four hours after transfection, cells were counted and luciferase activity measured. Results are expressed as RLU/10^6 cells.

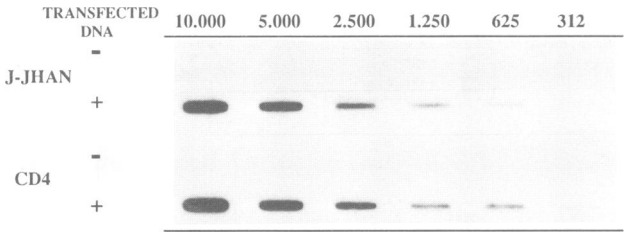

Fig. 2. Quantification of transfected DNA in resting CD4 lymphocytes and J-Jhan cells. CD4 T lymphocytes or J-Jhan cells were either transfected with 1 μg/10^6 cells LTRWT−Luc plasmid or were not transfected. Plasmid DNA was purified and serial dilutions of DNA obtained from each cell type were transferred onto a nylon membrane using a Dot-Blot apparatus and hybridized with an [α-^{32}P]dCTP - labeled wild-type HIV (LAI strain) LTR probe. The autoradiogram shown was exposed for 6 h. In the experiment shown, luciferase activity obtained in 10^6 cells was 1900 RLU in J-Jhan cells and was undetectable in CD4 lymphocytes.

absence of functional NF-κB binding sites does not affect the basal expression of the HIV LTR.

To rule out the possibility that, in the latter cells, a low uptake of transfected expression vectors was responsible for the weak luciferase activity detected, the amount of low molecular weight DNA present in the nuclear fraction of each cell type after transfection was quantified. Results from this analysis, shown in Figure 2, demonstrate that similar quantities of transfected LTRWT−Luc plasmid were loaded into the nuclei of J-Jhan and normal CD4 T lymphocytes.

To investigate whether the low HIV LTR expression level obtained in CD4 lymphocytes was a peculiarity of this promoter, we analyzed the basal activity of other viral promoters in both normal CD4 T lymphocytes and J-Jhan cells. As shown in Figure 1b, the promoters from both Rous sarcoma virus (RSV) LTR and thymidine kinase (TK) from herpes simplex virus were poorly expresed in normal lymphocytes. As compared with these vectors, the HIV LTR expressed a slight activity that was about 20 times lower than the strong level obtained with the CMV IE promoter.

NF-κB-dependent inducibility of the HIV LTR is comparable in normal and transformed T lymphocytes.

The reactivity of the wild-type HIV LTR to cell activation was explored in normal and lymphoblastoid cells (Figure 1a). In both cell types, a clear increase in LTR activity was induced by treatment with phorbol myristate acetate (PMA). The transactivation of the HIV LTRWT−Luc vector observed in PMA-treated cells was mediated by induction of the NF-κB binding sites. Indeed, in both cell types, deletion of NF-κB consensus sequences (LTRΔκB−Luc) (Figure 1a) abrogated the capacity of the LTR to respond by increased transcription to PMA activation.

Role of NF-κB consensus sequences in Tat-mediated transactivation of the HIV LTR in normal and lymphoblastoid T lymphocytes

The participation of NF-κB consensus sequences in Tat-mediated transactivation of the HIV LTR was comparatively analyzed in normal T lymphocytes and J-Jhan cells. Both cell types were co-transfected with either a LTRWT−Luc or a LTRΔκB−Luc vector and a Tat expression vector driven by the cytomegalovirus immediate early promoter (CMV−TAT). Data presented in Figure 3a show that in blood CD4 T lymphocytes and J-Jhan lymphoblastoid cells a pronounced increase in transcriptional activity of the LTRWT−Luc vector was induced by CMV−TAT.

In the lymphoblastoid cell line J-Jhan the transactivation level induced by Tat in the LTRΔκB−Luc vector was only sligthly reduced as compared with results obtained with the wild-type LTR-directed vector. In striking contrast, in resting blood T lymphocytes, deletion of κB responsive elements from the LTR completely prevented Tat-induced transactivation of the LTRΔκB−Luc expression vector (Figure 3a).

To exclude the possibility that the levels of Tat generated in blood CD4 T lymphocytes could be a limiting factor in induction of the LTRΔκB vector, increased amounts of CMV−TAT vector were co-transfected with either the wild-type or the κB-deleted counterpart. As is shown in Figure 3b, the transcription activity of the wild-type LTR was enhanced in parallel with the amount of CMV−TAT transfected, albeit no transcriptional activity was obtained from the κB-deleted LTR whatever the amount of CMV−TAT co-transfected. That indicates that the κB-dependent LTR unresponsiveness to Tat was not due to a limiting availability of the transactivator in CMV−TAT-transfected lymphocytes.

In an attempt to reproduce experimental conditions closer to HIV reactivation occurring in naturally infected CD4 T lymphocytes we performed experiments where expression of Tat was driven by the HIV LTR. Under these experimental conditions Tat-dependent transactivation of wild-type LTR was not observed in blood CD4 T lymphocytes, probably as a consequence of the low amount of Tat produced from either wild-type or ΔκB-directed LTR−Tat vectors in these cells (Figure 4). In J-Jhan cells, comparable Tat-mediated transactivation of LTRWT−Luc was observed independently of the presence or absence of κB responsive elements in the co-transfected LTR−Tat expression vectors, thus confirming the κB-independent activity of the HIV LTR in lymphoblastoid cells. When PMA and Tat synergistic transactivation of the LTRWT−Luc vector was analyzed, it was observed that comparable levels of luciferase activity were obtained in J-Jhan cells regardless of the LTR-driven Tat expression vector used. In contrast, the absence of κB responsive elements prevented the induction of Tat in normal T cells and the collaborative transactivation of LTRWT−Luc upon stimulation with PMA observed in J-Jhan cells (Figure 4).

Analysis of p50 and NF-κB in the nuclei of resting and activated normal CD4 T lymphocytes

Nuclear extracts from normal T lymphocytes were analyzed in an electrophoretic mobility shift assay (EMSA) (Figure 5a) using a radiolabeled, double-stranded oligonucleotide containing the two NF-κB consensus motifs representing the HIV enhancer. Nuclei from unstimulated T lymphocytes contained a DNA binding activity which specifically associated with the HIV enhancer oligonucleotide. These complexes were composed of p50 homodimers, as demonstrated by the fact that incubation of nuclear extracts from resting cells with IκB/MAD3 modified

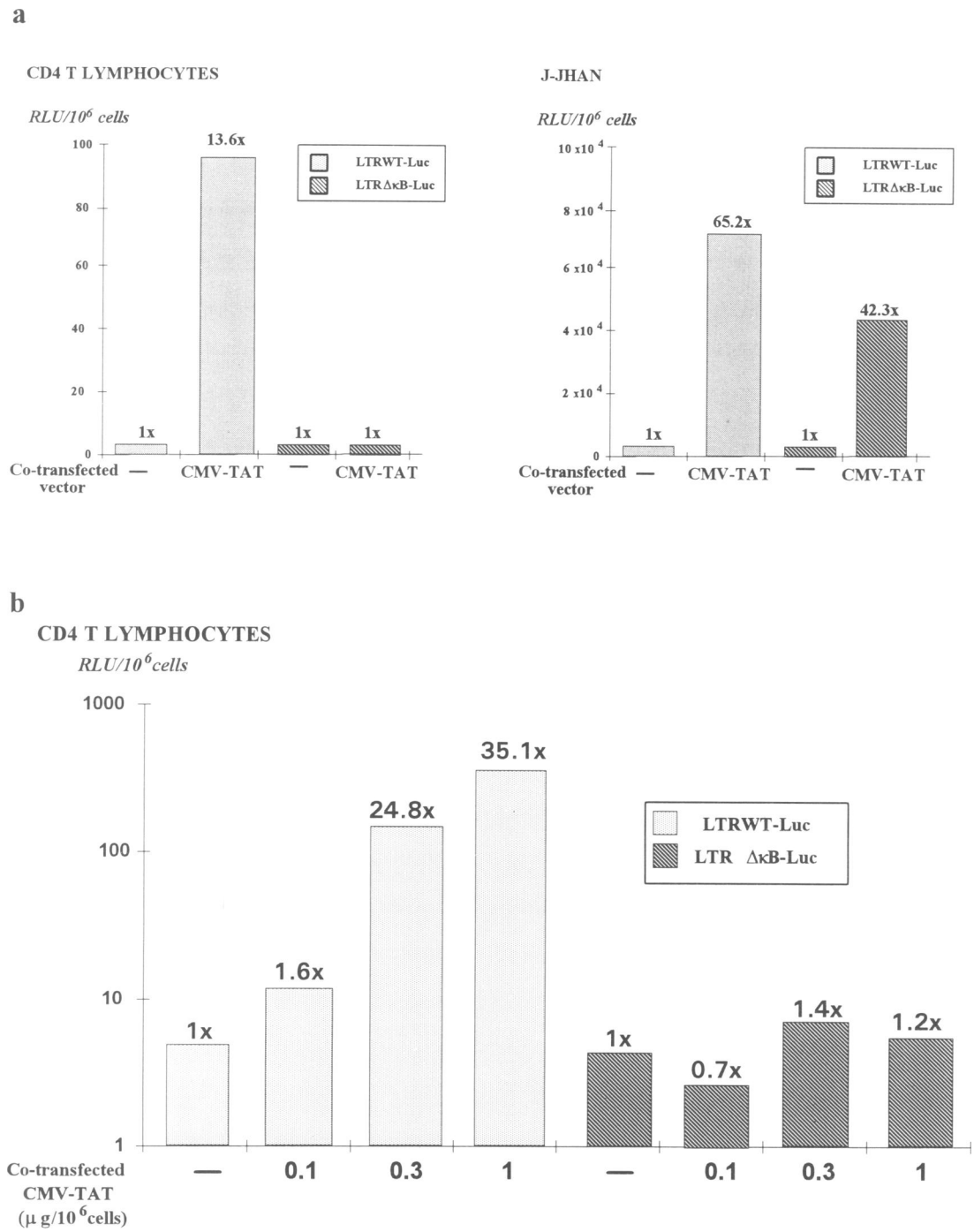

Fig. 3. (**a**) HIV LTR Tat-mediated transactivation in resting CD4 T lymphocytes is dependent on κB responsive elements. CD4 T lymphocytes or J-Jhan cells were transiently transfected by electroporation with 1 μg/10^6 cells of the indicated reporter plasmids alone or with a CMV−TAT expression vector (0.5 μg/10^6 cells). Twenty four hours after transfection, cells were counted and luciferase activity was measured in cell lysates. Results from one of 10 representative experiments are shown. Values are expressed as RLU/10^6 cells. Numbers above the bars indicate fold amplification of luciferase activity relative to the activity of the LTRWT−Luc plasmid transfected into the same unstimulated cell type. (**b**) Lack of transactivation of the LTRΔκB−Luc plasmid by CMV−TAT is not due to limited production of the Tat protein. Blood CD4 T lymphocytes were transiently transfected by electroporation with 1 μg/10^6 cells of the indicated reporter plasmids alone or with different doses of a CMV−TAT expression vector. Twenty four hours after transfection cells were counted and luciferase activity was measured in cell lysates. Values are expressed as RLU/10^6 cells. Numbers above the bars indicate fold amplification of luciferase activity relative to the activity of the same plasmid in the absence of CMV−TAT co-transfection.

neither its capacity to bind the HIV enhancer nor its mobility, but was displaced by a p50 antiserum.

Analysis by Western blot of the nuclear HIV enhancer binding proteins from normal T lymphocytes (Figure 5b) demonstrated the presence of p50, but not p65, proteins in the nuclei of resting cells. After induction with PMA, p65 became clearly detectable and the level of p50 was substantially increased in the nuclei of activated cells.

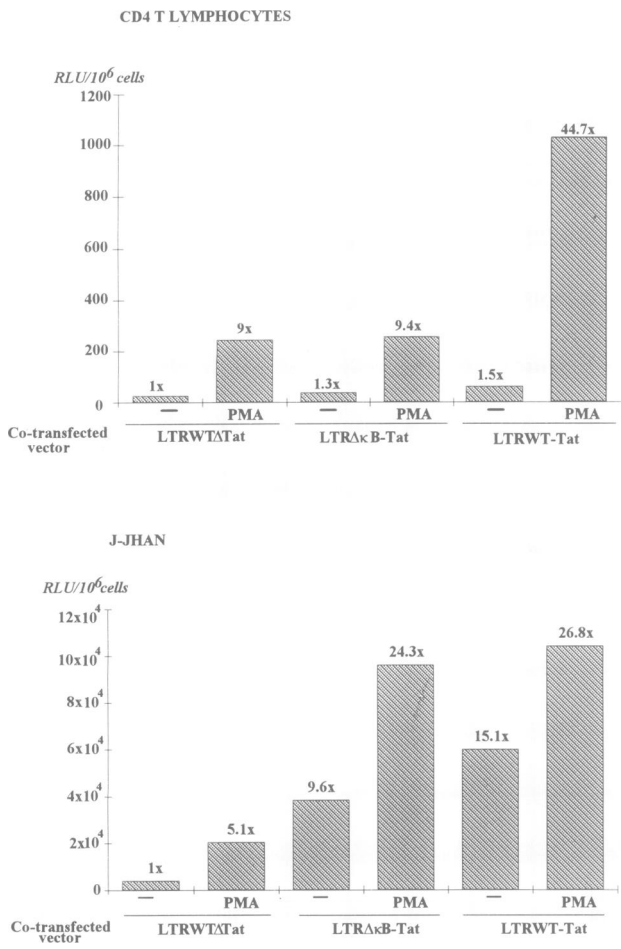

Fig. 4. HIV LTR-directed Tat expression is dependent on κB responsive elements. CD4 T lymphocytes or J-Jhan cells were transfected by electroporation with 1 μg/10^6 LTRWT−Luc reporter plasmid and different Tat expression vectors (0.5 μg/10^6 cells). Tat production was driven either by a wild-type LTR (LTRWT−Tat) or by a κB-deleted LTR (LTRΔκB−Tat). A truncated version of a Tat cDNA cloned under the control of a wild-type LTR was used as control (LTRWTΔTat). Cells were either left unstimulated (−) or stimulated with 25 ng/ml PMA. Luciferase activity was measured 24 h after transfection and results are expressed as RLU/10^6 cells. Numbers above the bars indicate fold amplification of luciferase activity relative to the luciferase activity of the LTRWT−Luc plasmid transfected into the same unstimulated cell type. One of four representative experiments is shown.

Point mutations in the viral enhancer which prevent binding of NF-κB abolish transcriptional activity of HIV proviruses in normal CD4 T lymphocytes

To study the relevance of κB sequences in the replication of a whole HIV virus, CD4 lymphocytes or PHA-induced primary T cells were infected with either wild-type or κB-mutated infectious viruses generated in J-Jhan cells by transfection of plasmids that encompass the complementary halves of an infectious HIV provirus (Figure 6a). Activation of HIV-infected resting CD4 T lymphocytes (Figure 6b) was induced through the CD3 complex with a specific monoclonal antibody 24 h after incubation with wild-type or κB-mutated viral preparations. Virus replication in both HIV-infected resting (Figure 6b) and PHA-induced T lymphocytes (Figure 6c) were assessed by detection of p24 antigen in the supernatants of cell

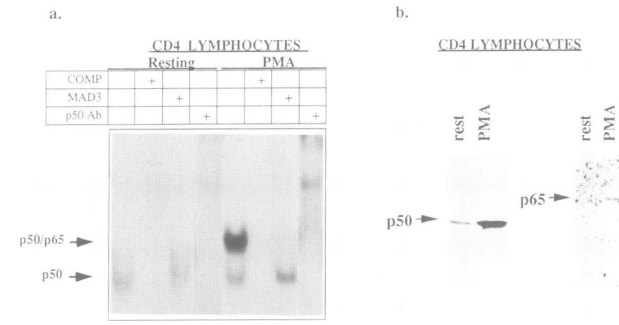

Fig. 5. High levels of p50 NF-κB subunit are detected in the nuclei of resting CD4 T lymphocytes. (**a**) Electrophoretic mobility shift assay. Three micrograms of protein from a nuclear extract were incubated with a [α-^{32}P]dCTP-labeled double-stranded oligonucleotide containing the two HIV LTR NF-κB consensus motifs. Specificity of the binding was assessed by competition with a 40 times molar excess of cold oligonucleotides (COMP). Twenty nanograms of recombinant IκBα (MAD3) or a rabbit polyclonal antibody against p50 (p50 Ab) were used to block DNA binding of NF-κB complexes. (**b**) Western blot. Ten micrograms of nuclear protein were electrophoresed in a SDS−PAGE gel, transferred to nitrocellulose membranes and probed with rabbit polyclonal antibodies directed against recombinant p50 or p65. Antigen−antibody interactions were revealed with a horseradish peroxidase-conjugated donkey anti-rabbit antibody, using a chemiluminescence detection kit (Amersham).

cultures collected at different times throughout the culture period. In clear contrast to the wild-type provirus, which was able to replicate in both activated cell populations, no p24 protein was detected either at early or late times in cell cultures infected with the κB-mutated counterpart. To rule out the possibility that a lower infectivity of the κB-mutated clone could explain the differences observed, we analyzed the amount of proviral DNA in PHA-induced T cell blasts. As is shown in Figure 6D, the amount of proviral DNA in cells infected with either the wild-type or the κB-mutated virus were comparable until day 12, thus excluding the possibility that the lack of replication observed with the viral clone was due to a low input of infectious particles. Since ELISA detection of p24 antigen was not sensitive enough to detect a low expression of the κB-mutated provirus, we decided to analyze the eventual production of viral RNA transcripts by reverse transcription (RT)-PCR. Using PHA-induced T cell blasts, which offer a more favorable environment for HIV replication than T lymphocytes infected in the resting state, we observed that no HIV RNA was detected in cells infected with the HIV κB-mutated virus, despite the fact that proviral DNA was still detectable at day 20. In contrast, large amounts of viral RNA were obtained in samples from cells infected with the HIV wild-type clone collected at days 20 and 30. In keeping with the intense production of viral RNA obtained in the lysates from these cells, a dramatic and parallel increase in the amount of HIV wild-type proviral copies is evidence of robust replication and propagation of this viral clone in cell culture.

Discussion

To study the role of NF-κB in LTR transactivation in a cellular environment relevant to HIV infection, we have developed efficient systems of infection and transfection of normal CD4 T lymphocytes derived and purified

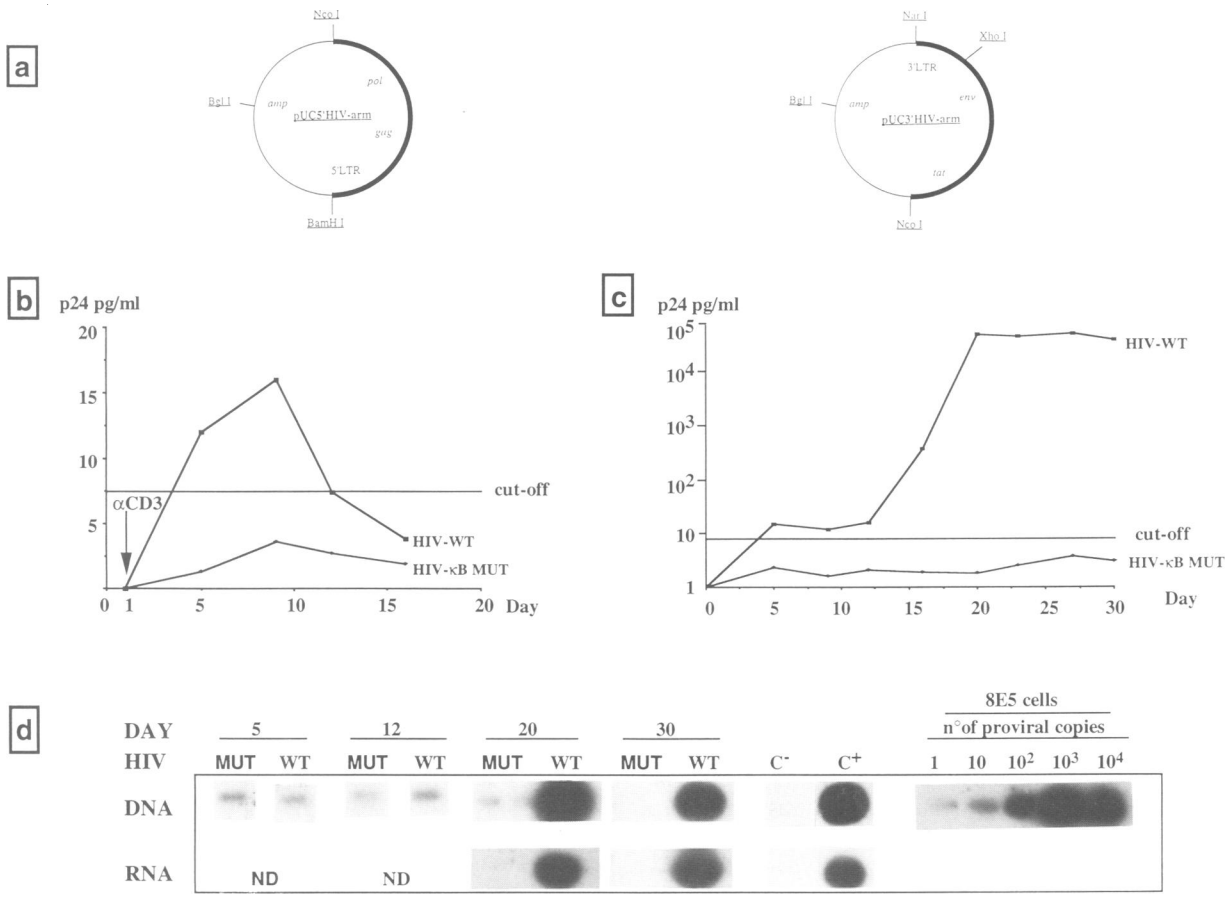

Fig. 6. HIV replication in CD4 T lymphocytes is absolutely dependent on LTR κB responsive elements. (**a**) Schematic representation of complementary HIV proviral vectors co-transfected in J-Jhan lymphoblastoid cells to generate infectious viruses. (**b**) Infection and replication in blood CD4 T lymphocytes. Purified, resting blood CD4 T lymphocytes were infected with wild-type (HIV-WT) or κB-mutated (HIV-κB MUT) HIV infectious viruses. After infection, cells were stimulated with anti-CD3 antibodies and maintained in culture medium suplemented with recombinant IL2. Viral replication was assessed by detection of HIV p24 antigen in culture supernatants. Cut-off line indicates the threshold of p24 detectability. (**c**) Infection of PHA-induced T cell blasts. PBLs were activated with PHA for 24 h and infected with either wild-type or κB-mutated viruses. Cells were maintained in culture medium supplemented with recombinant IL2. Viral replication was assessed by detection of HIV p24 antigen in culture supernatants. Cut-off line indicates the threshold of p24 detectability. (**d**) Assessment of proviral DNA copies and viral RNA production in long-term cultures of PHA-induced T cell blasts by PCR amplification of a 115 bp sequence of the HIV-1 *gag* gene. DNA and RNA from 10^5 8E5 (HIV-infected) cells were used as positive controls (C+). A standard curve of HIV DNA copies from 8E5 cells is shown. Uninfected CD4 T lymphocytes were used as a negative control (C−). In each point, DNA and RNA extracted from 10^5 lymphocytes were amplified. MUT, HIV κB-mutated virus. WT, wild-type virus. ND, not done.

from peripheral blood lymphocytes. We systematically compared HIV LTR activity in normal cells with that in a lymphoblastoid cell line (J-Jhan). Our results show that both basal LTR expression and the requirements for transactivation are radically different in the two cell types, thus stressing the relevance of studying the regulation of HIV transcription in an appropriate normal cell context.

Basal activity of the HIV LTR was found to be strikingly different in both cell types. In normal, resting CD4 T lymphocytes transcriptional activity was very low, in contrast to the high levels of spontaneous LTR activity found in unstimulated J-Jhan cells. However, in both cell types in the absence of PMA induction, the basal activity of the LTRWT and ΔκB vectors were strictely comparable, thus suggesting that other regions in the LTR account for the basal transcription activity of the HIV LTR.

The low transcriptional activity of the HIV LTR in blood CD4 T lymphocytes is shared by other viral promoters (herpes simplex virus TK and RSV LTR). The lack of gene product expression or, in the case of the HIV LTR, of post-translational modifications of NF-κB factors which occur in stimulated blood T lymphocytes could account for the poor transcriptional environment provided by quiescent blood CD4 T lymphocytes for these viral promoters.

To rule out the possibility that the low basal transcription activity observed in resting CD4 T lymphocytes was due to inefficient transfection of the HIV LTR as compared with that achieved in J-Jhan cells, the amount of plasmid uptake was analyzed using low molecular weight DNA extraction (Hirt, 1967). Our results show that the amounts of plasmid loaded by transfection in the nuclear compartment were similar in both cell types. The low luciferase activity detected in blood CD4 T lymphocytes thus truly reflected an extremely low activity of the HIV LTR in this cellular environment.

Although PMA stimulation induced a κB-dependent transactivation of the HIV LTR comparable in both cell types in terms of fold induction, the relative importance of this induction was very different depending on the

cell type considered. In J-Jhan cells, NF-κB activation increased an already ongoing transcription, whereas in CD4 lymphocytes cell activation and induction of the enhancer function was a *sine qua non* event in the initiation of HIV transcription. This is in keeping with the well-known difference in permissiveness to HIV replication of these two cell types. Indeed, in lymphoblastoid cell lines HIV infection results in active replication in the absence of other stimuli, whereas replication is undetectable in HIV-infected, resting CD4 lymphocytes, where T cell activation is essential for triggering of viral replication.

Tat-mediated transactivation of the HIV LTR was strictly dependent on the HIV enhancer in normal T lymphocytes, but not in lymphoblastoid cells, even when they were transfected with a potent Tat expression vector (CMV−TAT). The lack of a dose-dependent effect of Tat in the induction of the κB-deleted LTR argues against the possibility that the amount of Tat generated from the CMV-driven expression vector is a limiting factor which may explain the unexpected inability of CMV−TAT to transactivate the κB-deleted HIV LTR.

When the HIV LTR was used to drive Tat expression in order to mimic transcription conditions occuring in HIV-infected lymphocytes, no LTR transactivation was observed in resting CD4 lymphocytes. This is in keeping with the low transcriptional activity of the LTR in these cells, which would be unable to generate the amount of Tat required for LTR transactivation. In contrast, in J-Jhan cells, in keeping with the high basal activity of the HIV LTR, Tat generated either from the wild-type or the κB-deleted LTR leads to a potent transactivation of the HIV promoter independently of the κB sites.

The precise mechanisms accounting for this absolute dependence of Tat function on κB responsive elements in resting T lymphocytes remain unknown. The comparable low levels of transcription obtained with either wild-type or enhancer-mutated HIV LTR make it unlikely that quantitative differences in the rate of viral RNA elongated by Tat from each type of LTR account for the absence of Tat-mediated transactivation of the enhancer-deleted promoter.

The failure of Tat to amplify the transcription activity of κB-deleted promoters in resting lymphocytes suggests some indirect interaction of Tat with the viral enhancer. Such a possibility has been previously proposed (Berkhout *et al.*, 1990; Harrich *et al.*, 1990; Liu *et al.*, 1992; Taylor *et al.*, 1992) to explain the synergistic effect of Tat and the NF-κB complexes on transcription initiation of the HIV LTR. Some physical interaction of Tat, direct or mediated by other cell factors, with the structural complex formed by the viral enhancer and its specific DNA binding proteins could provide the molecular basis for the observed Tat−enhancer collaboration. The TBP−Tat association recently described could be an example of the interaction of Tat with cellular transcription factors (Kashanchi *et al.*, 1994).

Although in our experimental model the constitutive p50 homodimers seem to be devoid of any significant transcriptional activity on their own, occupancy of LTR κB responsive sequences by these protein complexes might be required for LTR transactivation induced by Tat in resting T lymphocytes. In agreement with this hypothesis, our preliminary results (data not shown) indicate that Tat and p50 expressed from CMV-directed vectors in resting T lymphocytes synergize to transactivate the HIV LTR, despite the inability of p50 alone to modify basal activity of the LTR.

The κB-dependent Tat transactivation of the LTR we describe in resting T lymphocytes is in agreement with recently published data by Moses *et al.* (1994) obtained in unstimulated human primary monocytes/macrophages co-transfected with Tat and HIV LTR−CAT vectors carrying critical point mutations in the viral enhancer. Thus, it should be stressed that CD4 T lymphocytes and macrophages, the two major natural target cells of HIV infection, display similar requirements in terms of LTR expression and Tat transactivation, in striking contrast to T lymphoblastoid and myelomonocytic cells (Arenzana-Seisdedos and Alcamí, unpublished results)

In order to validate our findings in a more relevant experimental model to natural HIV infection, we analyzed HIV replication in blood CD4 T lymphocytes using viruses encompassing either a wild-type or a κB-mutated LTR. In contrast to previous studies, our results show that viral transcription was absent when κB-mutated viruses were used.

Our results show that enhancer point mutations of κB responsive sequences in a HIV provirus do not modify the infectivity, since equivalent numbers of proviral copies were detected in wild-type and κB-mutated provirus-infected cells up to 12 days after virus adsorption. HIV replication assessed by p24 production was detected exclusively in cells infected with wild-type but not with mutated κB viruses in both PHA-induced T cell blasts and T cells infected in a resting state and then activated by an anti-CD3 antibody. The inability of the κB-mutated virus to replicate, even in the higly permissive cellular environment of PHA-induced T cell blasts, was confirmed by the lack of viral RNA detected using the sensitive RT−PCR technique. Correlated with the intense viral replication, a strong increase in the number of proviral copies was detected after infection with the wild-type but not the κB-mutated viral clone. Together these results are evidence that the presence of κB sites in the HIV LTR is critical for viral replication and propagation in human blood CD4 T lymphocytes.

Altogether, the above results underline the importance of using non-transformed cells for the analysis of HIV transcription and replication in T lymphocytes and indicate how much the use of lymphoblastoid cell lines may mislead our interpretation of the molecular events regulating HIV latency and persistence. As opposed to T lymphoblastoid cells, the environment of normal human lymphocytes, the natural target cell of the virus, does not provide the LTR with any detectable, constitutive activity and is not spontaneously permissive for HIV genome transcription. The phenomenon of T cell activation modifies this environment through NF-κB activation which initiates viral transcription from an apparently absolute quiescence and further amplifies transcription by interacting functionally with the viral transactivator Tat. Such notions lead to a better understanding of the basic strategy of long-term quiescence observed in the T lymphocytes of infected patients.

Materials and methods

Cells

J-Jhan is a human lymphoblastoid cell line derived from Jurkat cells. 8E5 is a clone derived from A3.01 cells which contains a single copy of HIV (Folks *et al.*, 1986b). Human PBL from healthy donors were isolated by Ficoll–Hypaque gradient from leukocytes. The $CD4^+$ T cell population was obtained by negative selection using magnetic beads (Dynabeads, Dynal) coated with monoclonal antibodies directed against CD8, CD19, CD14 and CD11c. After purification, >90% of cells expressed the CD4 marker and <3% of other cell types were detected by fluorocytometry. All cells were grown in RPMI 1640 medium enriched with 10% fetal calf serum (FCS).

Plasmids

The LTRWT–Luc and LTRΔκB–Luc expression vectors have been described previously (Bachelerie *et al.*, 1991). Briefly, they carry U3+R regions of the HIV LTR (LAI strain) from nucleotide −644 (*Xho*I) to +78 (*Hin*dIII), except that the tandem κB responsive elements have been deleted in LTRΔκB–Luc and replaced by the consensus sequence for *Bcl*I (Du *et al.*, 1989). LTRWT and LTRΔκB were cloned in the pC-luciferase plasmid (Schwartz *et al.*, 1990). LTRWT– and LTRΔκB–Tat contain full-length Tat cDNA placed under the control of the wild-type or the enhancer-deleted HIV LTR regions described above. LTRWT–ΔTat was constructed using a Tat cDNA coding for a truncated protein lacking the first 30 N-terminal amino acids. Plasmid pUC5′HIV-arm contains 5′ sequences of the wild-type and its κB-mutated HIV counterpart provirus (Harrich *et al.*,1990) derived from SF2 (*Bam*HI–*Sph*I fragment) and HTLVIIIB pBH10 (*Sph*I–*Nco*I fragment). pUC3′HIV-arm carries 3′ wild-type and κB-mutated HIV sequences (*Nco*I–*Nar*I fragment) derived entirely from SF2. Isolated plasmids were not infectious, but could produce infectious HIV particles if upon digestion with *Bgl*I and *Nco*I restriction enzymes they were co-transfected with their complementary construct.

Enhancer-mutated 5′ and 3′ LTR sequences were (−106)CTTTAA-ACTTTCCGCTTTAAACTTTCCAG(−77)G, where mutated nucleotides are underlined.

The CMV–Luc and TK–Luc plasmids containing the human CMV immediate early promoter and the TK promoter from herpes simplex virus respectively have been previously described (Schwartz *et al.*, 1990; Bachelerie *et al.*,1991). The plasmid RSV–Luc contains the LTR from Rous sarcoma virus (Invitrogen) cloned in the pC-luciferase plasmid.

Electrophoretic mobility shift assays

Nuclear extracts were obtained and analyzed as described (Bachelerie *et al.*, 1991) using a [α-^{32}P]dCTP-labeled double-stranded synthetic wild-type HIV enhancer oligodeoxynucleotide. DNA binding competition was assessed by pre-incubating the extract with a 40-fold excess of unlabeled oligonucleotide. When indicated, 20 ng recombinant IκBα or specific polyclonal antibodies directed against recombinant p50 (Arenzana-Seisdedos *et al.*, 1993) were added to samples 10 min before addition of the radiolabeled probe.

Western blot

Ten micrograms of nuclear protein were electrophoresed in a SDS–PAGE gel, transferred to nitrocellulose membranes (PVDF, Sigma) and probed with rabbit polyclonal antibodies directed against recombinant p50 or p65. Antigen–antibody interactions were revealed with a horseradish peroxidase-conjugated donkey anti-rabbit antibody, using a chemiluminescence detection kit (Amersham).

Transfection assays

Cells were resuspended in RPMI supplemented with 10% FCS and electroporated using a Celljet electroporator (Eurogentec). J-Jhan cells were transfected at 280 V, 1500 μF and resistance ∞. CD4 T cells were transfected at 320 V, 1500 μF and resistance ∞. After transfection, cells were incubated in RPMI with 10% FCS at 37°C, activated or not with PMA (25 ng/ml) and harvested 24 h later. Luciferase activity was measured in a luminometer (Berthold). Data are expressed in terms of relative luciferase activity units (RLU), calculated as (light emission from experimental sample−light emission of untransfected cells)/10^6 cells.

Quantitative analysis of low molecular weight DNA

Cell nuclei were isolated by centrifugation after incubation for 5 min at 4°C in lysis buffer (10 mM HEPES, pH 8, 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA and 0.2% Triton X-100). Low molecular weight DNA was purified according to a procedure modified from the original method (Hirt, 1967), as previously described (Bachelerie *et al.*, 1991). Serial dilutions of DNA obtained from both cell types were transferred onto a nylon membrane and hybridized with a ^{32}P-labeled DNA probe containing the wild-type HIV (LAI strain) LTR sequence.

HIV infection

Plasmids containing complementary sequences of wild-type and κB-mutated HIV viruses respectively were digested with *Bgl*I and *Nco*I restriction enzymes and transfected into J-Jhan cells. Culture supernatants from infected cells were clarified by low speed centrifugation and virus was pelleted by ultracentrifugation. Quantification of virion-associated p24 antigen in viral pellets was performed using an enzyme-like immunoassay (Coulter). Resting CD4 T lymphocytes or PHA-induced T cell blasts were infected with a dose of 500 ng HIV p24 protein/10^6 cells. CD4 lymphocytes were co-cultured in 24-well plates with autologous macrophages, previously isolated by adherence. Both purified CD4 lymphocytes and T cell blasts were cultivated in RPMI 1640 medium supplemented with 10% FCS and 60 ng/ml recombinant IL2 (Cetus). Blood CD4 T lymphocytes were stimulated with CD3 antibodies (T28) at a dose of 1:1000 of ascitic fluid. Every week, PHA-activated blasts (5×10^5 cells/well) were added to cell cultures. Viral replication was measured by quantifying the levels of HIV p24 protein using an enzyme-like immunoassay (Coulter).

Detection of proviral DNA and mRNA transcripts in infected cells

For detection of proviral copies, total DNA from 10^5 HIV-infected CD4 lymphocytes or serial dilutions of 8E5 cells was extracted as described (Higuchi,1989). To analyze viral DNA, PCR amplification was performed using SK38 and SK39 primers (Kellogg and Kwok, 1990), which amplify a 115 bp fragment corresponding to sequences in the HIV-1 *gag* gene. Thirty five cycles of amplification were performed as previously described (Kellogg and Kwok, 1990). RNA from 5×10^5 HIV-infected or uninfected CD4 lymphocytes or 8E5 cells was extracted using the RNAzol method (Chomczynski and Sacchi, 1987). After treatment with an excess of RNase-free DNase (Boehringer Manheim), viral RNA was reverse transcribed (Kellogg and Kwok, 1990) and subjected to PCR amplification using primers SK38 and SK39. Afer amplification, PCR products were electrophoresed, transferred to nylon membranes (Zeta Probe, Bio-Rad) and hybridized with 10^6 c.p.m. of a [α-^{32}P]dCMP-labeled SK19 probe encoding an internal sequence of the *gag* gene (Kellogg and Kwok, 1990).

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References

Adachi,A., Gendelman,H.E., Koenig,S., Folks,R., Willey,R., Rabson,A. and Martin,M.A. (1986) *J. Virol.*, **59**, 284–289.

Arenzana-Seisdedos,F., Fernández,B., Domínguez,I., Jacqué,J.M., Thomas,D., Díaz-Meco,M.T., Moscat,J. and Virelizier,J.L. (1993) *J. Virol.*, **67**, 6596–6604.

Bachelerie,F., Alcamí,J., Arenzana-Seisdedos,F. and Virelizier,J.L. (1991) *Nature*, **350**, 709–712.

Ballard,D.W., Dixon,E.P., Peffer,N.J., Bogerd,H., Doerre,S., Stein,B. and Greene, W.C. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 1875–1879.

Barré-Sinoussi,F. *et al.* (1983) *Science*, **220**, 868–871.

Beg,A.A., Finco,T.S., Nantermet,P.V. and Baldwin,A.S. (1993) *Mol. Cell. Biol.*, **13**, 3301–3310.

Berkhout,B. and Jeang,K.T. (1992) *J. Virol.*, **66**, 139–149.

Berkhout,B., Gatignol,A., Rabson,A.B. and Jeang,K.-T. (1990) *Cell*, **62**, 757–767.

Bours,V., Burd,P.R., Brown,K., Villalobos,J., Park, S., Ryzeck,R.P., Kelly,B.R.K. and Siebenlist,U. (1992) *Mol. Cell. Biol.*, **12**, 685–695.

Bubrinsky,M.I., Stanwick,T.L., Dempsey,M.P. and Stevenson, M. (1991) *Science*, **254**, 423–427.
Chomczynski,P. and Sacchi,N. (1987) *Annls Biochem.*, **162**, 156–159.
Cullen,B.R. (1993) *Cell*, **73**, 417–420.
Cullen,B.R. and Greene W.C. (1989) *Cell*, **58**, 423–426
Du,E.J., Maury,W.J., Folks,T.M., Fauci,A.S. and Rabson,A.B. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 5974–5979.
Embretson,J., Zupancic,M., Ribas,J.L., Burke,A., Racz,P., Tenner-Racz,K. and Haase,A.T. (1993) *Nature*, **362,** 359–362.
Folks,T., Kelly,J., Benn,S., Kinter,A., Justement,J., Gold,J., Redfield,R., Sell,K.W. and Fauci,A.S. (1986a) *J. Immunol.*, **136**, 4049–4053.
Folks,T. *et al.* (1986b) *J. Exp. Med.*, **164**, 280–290.
Folks,T.M., Clouse,K.A., Justment,J., Rabson,A., Due,E., Kehrl,J.H. and Fauci,A.S. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 2365–2369.
Franzoso,G., Bours,V., Park,S., Tomita-Yamaguchi,M., Kelly,K. and Siebenlist,U. (1992) *Nature*, **359**, 339342.
Franzoso,G., Bours,V., Azarenko,V., Park,S., Tomita-Yamaguchi,M., Kanno,T., Brown,K. and Siebenlist,U. (1993) *EMBO J.*, **12**, 3893–3901.
Fujita,T., Nolan,G.P., Ghosh,S. and Baltimore,D. (1992) *Genes Dev.*, **6**, 775–787.
Gaynor,R. (1992) *AIDS*, **6**, 347–363.
Harrich,D., García,J., Mitsuyasu,R. and Gaynor,R. (1990) *EMBO J.*, **9**, 4417–4423.
Hazan,U., Thomas,D., Alcamí,J., Bachelerie,F., Israel,N., Yssel,H., Virelizier,J.L. and Arenzana-Seisdedos,F. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 7861–7865.
Higuchi,R. (1989) *Amplifications*, **2**, 1–3.
Hirt,R. (1967) *J. Mol. Biol.*, **26**, 365–369.
Israel,N., Hazan,U., Alcamí,J., Munier,A., Arenzana-Seisdedos,F., Bachelerie,F., Israel,A. and Virelizier,J.L. (1989) *J. Immunol.*, **143**, 3956–3960.
Jones,K.A. (1989) *Nature New Biol.*, **1**, 127–135.
Jones,K. (1993) *Curr. Opin. Cell Biol.*, **5**, 461–468.
Kamine,J. and Chinnadurai,G. (1992) *J. Virol.*, **66**, 3932–3936.
Kashanchi,F., Piras,G., Radonovich,M.F., Duvall,J.F., Fattaey,A., Chiang,C., Roeder,R.G. and Brady,J.N. (1994) *Nature*, **367**, 295–299.
Kellogg,D. and Kwok,S. (1990) In Innis,M.A., Gelfand,D.H., Stinsky,J.J. and White, T.J.(eds), *PCR Protocols. A Guide to Methods and Applications*. Academic Press, San Diego, CA, pp. 337–347.
Leonard,J., Parrott,C., Buckler-White,A., Turner,W., Ross,E.K., Martin,M.A. and Rabson,A.B. (1989) *J. Virol.*, **63**, 4919–4923.
Li,Y., Ross,J., Scheppler,J.A. and Franza,B.R.,Jr (1991) *Mol. Cell. Biol.*, **11**, 1883–1893.
Liou,H.C. and Baltimore,D. (1993) *Curr. Opin. Cell Biol.*, **5**, 477–487.
Liu,J., Perkins,N.D., Schmid,R.M. and Nabel,G.J. (1992) *J. Virol.*, **66**, 3883–3887.
Lu,Y, Touzjian,N., Steuzel,M., Dofrman,T., Sodroski,J.G. and Haseltine,W.A. (1990) *J. Virol.*, **64**, 5226–5229.
McDougal,J.S., Mawle,A., Cort,S.P., Nicholson,J.K., Cross,G.D., Scheppler-Campbell,J.A., Hicks,D. and Sligh,J. (1985) *J. Immunol.*, **135**, 3151–3162.
Moses,A.V., Ibañez,C., Gaynor,R., Ghazal,P. and Nelson,J.A. (1994) *J. Virol.*, **68**, 298–307.
Nabel,G.J. and Baltimore,D. (1987) *Nature*, **326**, 711–718.
Nolan,G.P., Ghosh,S., Liou,H.-C., Tempst,P. and Baltimore,D. (1991) *Cell*, **64**, 961–969.
Osborn,L., Kunkel,S. and Nabel,G.J. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 2336–2340.
Pantaleo,G., Graziosi,C., Demarest,J.F., Butini,L., Montrini,M., Fox,C.H., Orenstein,J.M., Kotler,D.P. and Fauci,A.S. (1993) *Nature*, **362**, 355–358.
Popovic,M., Sarngadharan,E., Read,E. and Gallo,R.C. (1984) *Science*, **224,** 497–500.
Ryzeck,R.P., Bull,P., Takamida,M.,Bours,V., Siebenlist,U., Dobrzanski,P. and Bravo.R. (1992) *Mol. Cell. Biol.*, **12**, 674–684.
Sakaguchi,M., Zenzie-Gregory,B., Groopman,J.E., Smale,S.T. and Kim,S. (1991) *J. Virol.*, **65**, 5448–5456.
Saksela,K., Muchmore,E., Girard,M., Fultz,P. and Baltimore,D. (1993) *J. Virol.*, **67**, 7423–7427.
Schmid,R., Perkins,N.D., Duckett,C.S., Andrews,P.C. and Nabel,G.J. (1991) *Nature*, **352**, 733–736.
Schmitz,M.L. and Bauerle,P.A. (1991) *EMBO J.*, **10**, 3805–3817.
Schwartz,O., Virelizier,J.L., Montagnier,L. and Hazan,U. (1990) *Gene*, **88**, 197–205.
Sharp,P.A. and Marciniak,R.A. (1989) *Cell*, **59**, 229–230.
Taylor,J.P., Pomerantz,R., Bagarra,O., Chowdhury,M., Rappaport,J., Khalili,K. and Amini,S. (1992) *EMBO J.*, **9**, 3395–3403.
Tong-Starsksen,S., Luciw,P.A. and Peterlin,B.M. (1989) *J. Immunol.*, **142**, 702–707
Virelizier,J.L. (1990) *Curr. Opin. Immunol.*, **2**, 409–413.
Zack,J.A., Arrigo,S.J. Weitsman,S.R., Go,S.A., Haislip,A. and Chen,I.S.Y. (1990) *Cell*, **61**, 213–223.

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