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Role of Tumor Necrosis Factor Alpha in Activation and Replication of the *tat*-Defective Human Immunodeficiency Virus Type 1

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Transcription of human immunodeficiency virus type 1 (HIV-1) depends on the function of the virus-encoded regulatory protein Tat, which interacts with the specific Tat response (TAR) element present in the leader sequence of all HIV-1 RNAs. In this study, we examined whether tumor necrosis factor alpha (TNF-α) can replace the requirement for a functional Tat protein. We found that TNF-α can induce expression of a latent, *tat*-defective virus and support its replication both in T cells and in primary mononuclear cells. Analysis of the transcriptional rate of the *tat*-defective HIV-1 transcriptional unit indicates that TNF-α stimulates the initiation of transcription but, in contrast to Tat protein, does not significantly reduce transcriptional polarity. Interestingly, we found that the processing of viral precursor proteins is altered in the absence of Tat. We propose that TNF-α-mediated induction of HIV-1 plays an essential role in the early stages of the virus life cycle and in viral latency.

Transcription of the human immunodeficiency virus type 1 (HIV-1) is regulated by virus-encoded Tat protein (38) and by cellular factors that interact with enhancer and promoter regions of the HIV-1 long terminal repeat (LTR) (14, 28, 36). Since the *tat*-defective mutant of HIV-1 is transcriptionally inactive, Tat-mediated transactivation represents one of the limiting steps in the HIV-1 replication cycle. It was shown recently that the interaction between the Tat and cellular factors binding either to the Tat response (TAR) region or to the Sp-1 and NF-κB binding sites, as well as to the TATA box of the HIV-1 promoter region, is essential for Tat-mediated transcriptional activation (1, 7, 15, 22, 39). Whereas in acute HIV-1 infection, Tat is the major transcriptional transactivator, the activation of the HIV-1 LTR and the latent endogenous HIV-1 provirus by different extracellular stimuli, such as tumor necrosis factor alpha (TNF-α), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (4, 9, 12, 42), or herpes simplex virus type 1 infection (8, 26, 27, 43), has been associated with an increased level of binding of NF-κB-specific proteins. Mutations in the NF-κB site that abolish its binding capacity prevent induction by TNF-α or TPA (28, 42). Furthermore, transcriptional activation of the HIV-1 LTR or of the endogenous HIV-1 provirus by extracellular stimuli such as TPA or herpes simplex virus type 1 infection can occur in the absence of the Tat protein (30, 31).

It has been shown that HIV-1 infection in vivo is associated with up-regulated synthesis of several cytokines, such as interleukin 1 (IL-1), TNF-α, and IL-6, which are able to transactivate expression of the HIV-1 LTR (3, 24, 29). In the present study, we used a T-cell line (CEMR7/neo) containing the integrated *tat*-defective HIV-1 provirus (5) to examine whether one of these cytokines, TNF-α, can replace the requirement for the functional Tat protein and asked two questions: (i) does TNF-α activation of the HIV-1 provirus increase both transcriptional initiation and processivity? and (ii) can TNF-α support replication of Tat-defective HIV-1 in T cells?

To determine whether TNF-α can activate expression of the *tat*-defective HIV-1 provirus, we treated the CEMR7/neo cells with TNF-α and analyzed the levels of viral transcripts by Northern (RNA) hybridization and viral proteins by Western blot (immunoblot) analysis at different times postinduction. Treatment of CEMR7/neo cells with TNF-α (100 U/ml) increased viral transcription as early as 4 h postinduction, and HIV-1 transcripts representing the viral unspliced (9.2-kb), singly spliced (4.2-kb), and doubly spliced (2.0-kb) RNAs could be detected by Northern hybridization (Fig. 1A). The increase in relative levels of HIV-1 RNAs was transient, and a significant decrease was observed at a later time postinduction (48 h). The TNF-α-mediated induction of *tat*-defective HIV-1 was not limited to CEMR7/neo cells, since enhanced expression of *tat*-defective HIV-1 was also observed in TNF-α-treated monocytes (U937) (data not shown). The increase in *tat*-defective HIV-1 transcription in TNF-α-treated cells was accompanied by an increased level of HIV-1 proteins (Fig. 1B). At both 24 and 48 h postinduction, we detected high levels of the glycoprotein precursor gp160, as well as the Gag precursor p55, p41, and low levels of p66 (reverse transcriptase) and p24 (Fig. 1B). p55 ($Pr55^{gag}$) and gp160 were also present in the untreated cells, but at much lower levels. The relative levels of $Pr55^{gag}$ in TNF-α-induced cells were high, and only low levels of p24 could be detected. In contrast, the levels of HIV-1 proteins in CEMR7/neo cells transfected with a Tat-expressing plasmid (expression of the *tat* gene was under the control of the simian virus 40 early promoter region) (Fig. 1C) showed a pattern similar to that observed in acutely infected T cells (37). The relative levels of $Pr55^{gag}$ were lower than the levels of p24, and p17 polypeptide could be detected. Furthermore, the gp160 and gp120 proteins were detected as a diffused band. In the presence of Tat, the expression of all structural HIV-1 proteins (Fig. 1C) was significantly higher than in TNF-α-induced cells. Since these data indicate different expression of viral proteins in TNF-

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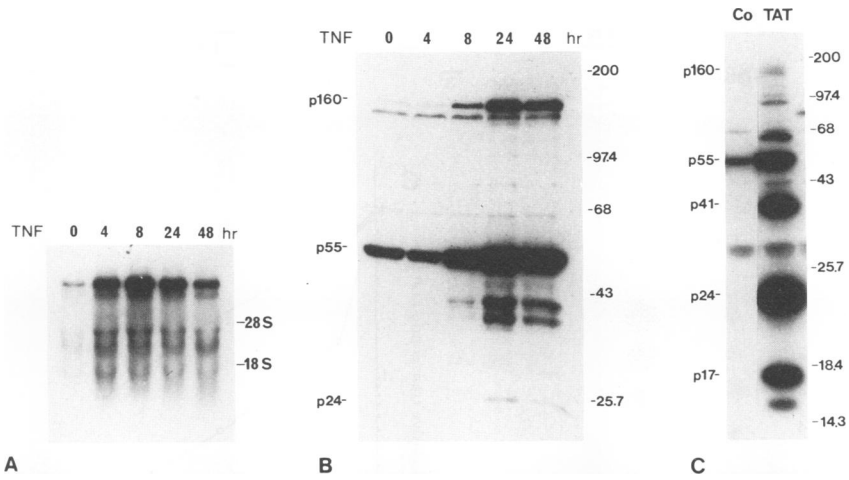

FIG. 1. Induction of expression of the *tat*-defective HIV-1 provirus in CEMR7/neo cells by TNF-α. (A) Time course of HIV-1 RNA induction by TNF-α. Total RNA was isolated from TNF-α (100-U/ml)-treated cells by a guanidine thiocyanate method (31) and analyzed by Northern blot hybridization. RNA (10 μg per lane) was separated on a 0.8% agarose formaldehyde gel and transferred to a nitrocellulose filter, and HIV-1-specific transcripts were detected by hybridization with a HIV-1 riboprobe, pJM105 (31). (B) Accumulation of HIV-1-specific proteins in CEMR7/neo cells stimulated with TNF-α. Cells were harvested at the indicated times, and cellular proteins (100 μg per lane) were resolved on an SDS–8% polyacrylamide gel and hybridized with a polyclonal serum from an HIV-1-seropositive patient as described previously (37). (C) HIV-1 proteins detected in CEMR7/neo cells transfected with 10 μg of a Tat-expressing plasmid, SV_2tat (lane TAT), and untransfected controls (lane Co). Cells were lysed 24 h after transfection and analyzed as described for panel B. Molecular size markers (in kilodaltons) are on the right.

α-treated and Tat-transfected cells, we used pulse-chase labelling experiment to determine whether the observed difference could be a result of different processing of viral precursor proteins. CEMR7/neo cells were induced with TNF-α or transfected with a *tat*-expressing plasmid and 24 h later were pulse-labeled with [^{35}S]methionine (250 μCi/ml) in methionine-free medium for 30 min at 37°C and then chased for 1 or 3 h in a complete medium. Viral proteins were collected by immunoprecipitation and identified by electrophoresis on sodium dodecyl sulfate (SDS)–8% polyacrylamide gel as described recently (37). It can be seen in Fig. 2 that in the presence of Tat, Gag precursor p55 ($Pr55^{gag}$) is effectively processed into the major core protein, p24, while in TNF-α-treated cells, only very little $Pr55^{gag}$ was processed during the 3-h chase period. The levels of Gag intermediate p41 were decreased at 3 h postlabelling only in Tat-transfected cells and not in TNF-α-induced cells. Similarly, processing of gp160 into gp120 was delayed in TNF-α-treated cells. These results suggest that in TNF-α-treated cells, the processing of the viral precursor into mature virion proteins is inhibited. This inhibition is not seen in the presence of the *tat* gene, since the induction of provirus in ACH-2 cells that contain the wild-type HIV-1 genome by TNF-α led to the expression of all structural proteins (42). Further studies are in progress to characterize the nature of this posttranscriptional difference in TNF-α-induced and Tat-expressing cells.

In studies examining the mechanism of Tat-enhanced transcription of the HIV-1 LTR, it has been shown that Tat is required not only for the enhancement of transcriptional initiation but also for processivity of transcription and synthesis of full-length RNA (13, 16, 17, 19, 23). To examine whether the mechanism of TNF-α-mediated transactivation of HIV-1 provirus is different from that of Tat transactivation, we determined the effects of both types of transactivators on the HIV-1 transcriptional rate by the run-on assay (Fig. 3). The nuclear run-on assay was performed as described recently (31, 34). Nuclei were isolated from unstimulated cells (control) and CEMR7/neo cells treated with TNF-α (100 U/ml) for 6 h or from cells transfected with Tat-expressing plasmid at 24 h after transfection. The ^{32}P-labelled transcripts were then hybridized to RNA antisense probes complementary to different regions of HIV-1 RNA

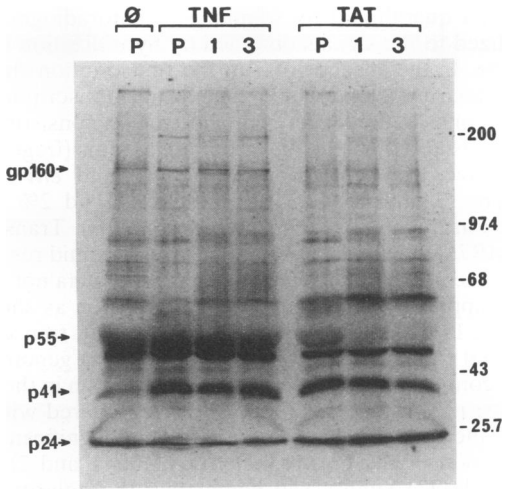

FIG. 2. Processing of HIV-1 proteins induced by TNF-α or Tat protein. CEMR7/neo cells were treated with TNF-α (100 U/ml) or transfected with 10 μg of a Tat-expressing plasmid, SV_2tat. Twenty-four hours later, cells were pulse-labelled with 250 μCi of [^{35}S]methionine in methionine-free medium for 30 min and then chased in the complete medium for 1 and 3 h. Cellular lysates were immunoprecipitated with anti-HIV-1 antibodies as described previously (37). Ø, uninduced, pulsed (P) cells. HIV-1-specific proteins are shown on the left. Molecular size markers (in kilodaltons) are on the right.

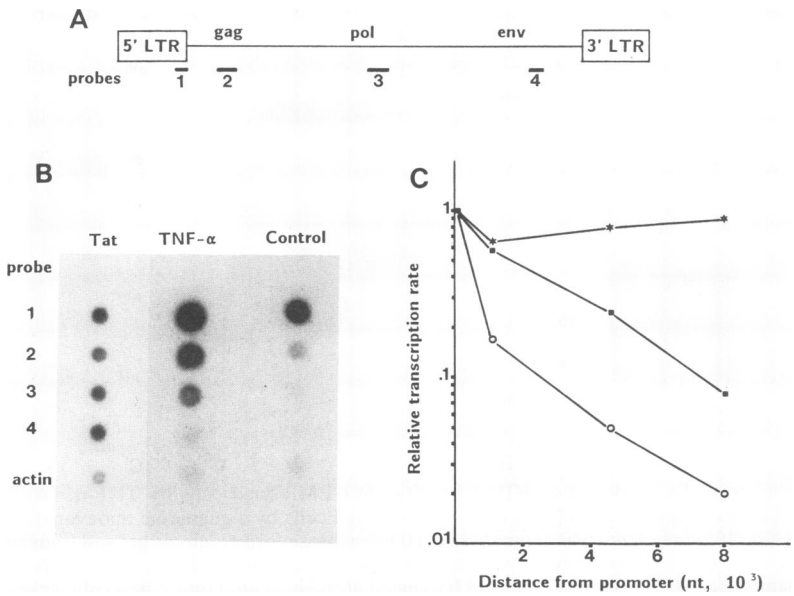

FIG. 3. Nuclear run-on analysis of HIV-1 provirus transcription in CEMR7/neo cells. (A) Schematic of RNA probes used in the nuclear run-on assays. RNA antisense probes were synthesized by using Gem 4 plasmids containing cloned DNA fragments from the HIV-1 (HXBc2 clone) genome. The subcloned HIV-1 DNA fragments probes were numbered as follows: 1, −116 to +80 (LTR); 2, +747 to +1084 (*gag*); 3, +4153 to +4646 (*pol*); 4, +7782 to +8050 (*env*). Human γ-actin riboprobe (10) was used as an internal control for transcription. (B) Nuclear run-on assay. Nuclei were isolated from unstimulated CEMR7/neo cells (Control), cells stimulated for 6 h with TNF-α (100 U/ml) (lane TNF-α), or cells transfected with Tat-expressing plasmid (the *tat* gene was under the control of the simian virus 40 early promoter region) (lane Tat). Lane Tat shows a shorter exposure (2 days, independent experiment) than lanes TNF-α and Control (5 days). (C) Relative transcription rate of the HIV-1 genome. The transcription rate of each fragment is expressed as a fraction of the transcription of fragment 1 (HIV-1 LTR), which was assumed to be 1.0. *, *tat*-transfected CEMR7/neo cells; ■, TNF-α-treated cells; ○, control cells.

immobilized on nitrocellulose filters. HIV-1 DNA fragments used for the synthesis of RNA probes are shown in Fig. 3A. Hybridization to human γ-actin riboprobe was used as an internal control. To quantitate the rate of transcription, signals obtained in hybridization with HIV-1-specific probes were then quantitated by scanning of autoradiograms and normalized to the signals obtained by hybridization to γ-actin probe. In the absence of Tat, the transcription showed a strong polarity (5, 20), and significant transcription was detected only in the leader sequence region consisting of 80 nucleotides proximal to the promoter region (fragment 1); the relative transcription rates in *gag* and *env* regions (fragments 2 and 4) were only about 17 and 2% of that detected in fragment 1, respectively (Fig. 3B). Transfection of CEMR7/neo cells with Tat-expressing plasmid resulted in an increase in the initiation of transcription (data not shown) and in suppression of polarity of transcription as shown by others (5, 19) (Fig. 3C). In the presence of Tat, transcription proceeded efficiently through the entire HIV-1 genome (Fig. 3C). In contrast, TNF-α increased transcription in the leader sequence region only by about 2-fold (compared with 5- to 10-fold increase by Tat) and increased significantly the transcription of the *gag* region (fragments 1 and 2). However, while Tat suppressed completely the polarity of the transcription and increased the transcription of both *gag* and *env* regions with almost the same efficiency, TNF-α stimulated transcription through the *gag* region much more efficiently than through the *pol* and *env* region. Thus, while about 60% of the initiated complexes reached the *gag* region, only 25 and 8% reached the *pol* and *env* regions, respectively, indicating that TNF-α does not affect greatly the transcriptional polarity (Fig. 3C). These data indicate that while TNF-α stimulates HIV-1 transcription, it does not functionally substitute for the Tat effect. This is further demonstrated in Fig. 3C, which shows that the transcriptional rates (indicated by the slopes of the curves) are very similar in the presence and absence of TNF-α, suggesting that distribution of the RNA polymerase along the HIV-1 genome is not greatly altered in TNF-α-treated cells. Notably, the activation of proviral transcription by TNF-α, which is mediated by binding of NF-κB-specific proteins to the enhancer region of the HIV-1 LTR, resembles the activation of the HIV-1 promoter by adenovirus E1A protein. Laspia et al. (20) have observed that E1A, which interacts with the TATA element, increased slightly the transcriptional initiation and elongation of HIV-1 LTR-directed gene expression. However, cooperation between E1A and Tat resulted in a marked enhancement of the transcriptional elongation. Thus, it appears that stimulation of the HIV-1 LTR promoter by either cellular (NF-κB) or viral (E1A) transactivators increases the density of RNA polymerase in the vicinity of the promoter but that in contrast to Tat protein, these transactivators do not reduce efficiently the polarity of transcription.

The question which remained was whether TNF-α transactivation has any biological significance. We asked, therefore, (i) whether TNF-α treatment induced production of virions in CEMR7 cells and (ii) whether the *tat*-defective HIV-1 virions were infectious and, if so, whether they could replicate in the presence of TNF-α. Figure 4 shows that the levels of reverse transcriptase (RT) activity were increased by about 8- and 14-fold at 24 and 48 h after induction of CEM7R/neo cells by TNF-α, respectively, compared with the RT levels detected in the medium from untreated controls, indicating that TNF-α can induce production of *tat*-defective HIV-1 virions in T cells. However, this induction

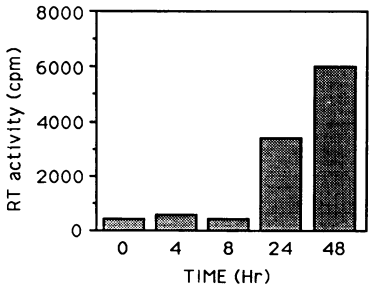

FIG. 4. Release of HIV-1 virions in TNF-α-stimulated CEMR7/neo cells. Medium from TNF-α (100-U/ml)-treated cells was collected at the indicated times, clarified by low-speed centrifugation, and assayed for the presence of virion-associated RT activity as previously described (37). RT activity is expressed as counts per minute per 10 μl of the assayed sample and represents an average of two independent measurements. The levels of RT activity in cells transfected with *tat*-expressing plasmid SV_2tat were 130,000 ± 30,000 at day 2 after transfection.

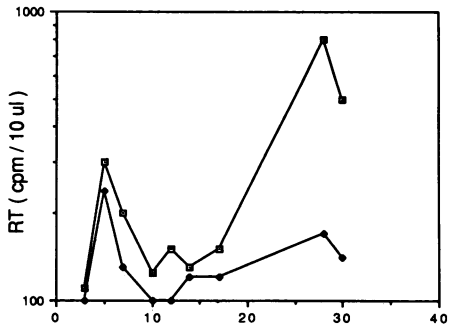

FIG. 5. Effect of TNF-α on infectivity of *tat*-defective HIV-1. The purified nonadherent PBMC were stimulated with phytohemagglutinin (5 μg/ml) for 72 h and then infected with *tat*-defective HIV-1 (100,000 cpm by RT assay). Cultures were incubated in the presence (□) or absence (◆) of TNF-α (50 U/ml), and virus replication was monitored by the presence of RT activity in the culture medium at different times after infection (*x* axis; numbers are days postinfection).

was about 20-fold lower than that produced in the cells transfected with Tat-expressing plasmid (130,000 cpm in *tat*-transfected cells versus 6,000 cpm in TNF-α-induced cells).

The infectivity of *tat*-defective HIV-1 produced in TNF-α-treated CEMR7/neo cells was measured in C8166 cells, which produce significant levels (133 U/ml) of TNF-α constitutively. Cells were infected with *tat*-defective HIV-1 (induced in CEMR7/neo cells by TNF-α treatment) and cultured in the presence and absence of anti-TNF-α antibodies. The *tat*-defective virus was able to replicate in C8166 cells. The presence of anti-TNF-α antibodies in the medium suppressed virus replication but did not abort it completely (data not shown). These results suggest that the antibodies were not able to neutralize completely the effect of autocrine TNF-α. The inability of the antibodies to neutralize completely the effect of autocrine TNF-α was also seen previously with the other cytokines produced in an autocrine manner (41). These data indicate that *tat*-defective HIV-1 can infect and replicate in cells that produce TNF-α constitutively. However, we cannot exclude at this point the possibility that other cellular factors present in C8166 cells may be able to complement TNF-α stimulation.

Since the induction of TNF-α synthesis by HIV-1 infection was observed both in vitro and in vivo (2, 3, 24, 29), we examined whether the low levels of exogenous TNF-α (50 U/ml) could facilitate the replication of *tat*-defective HIV-1 in primary mononuclear cells (peripheral blood mononuclear cells [PBMC]). Leukocytes were separated on a Ficoll gradient, and the adherent cells were removed by attachment to a plastic dish for 12 h and stimulated with 5 μg of phytohemagglutinin per ml for 72 h. The stimulated cells were then infected with the *tat*-defective HIV-1 mutant in the presence of 50 U of TNF-α per ml. At 24 h postinfection, cells were washed and further cultured in the medium containing IL-2 (50 U/ml) in the presence or absence of TNF-α (50 U/ml). Medium was replaced every 3 to 4 days, and replication of *tat*-defective HIV-1 was measured by assaying the levels of virion-associated RT in the culture medium for 30 days. For comparison, cells were also infected with the pNL43 clone of HIV-1 (data not shown). While the replication of pNL43 HIV-1 reached its maximum at 5 to 10 days postinfection and then ceased because of cell death, the replication of *tat*-defective provirus in the presence of TNF-α proceeded at a much lower rate, reaching the peak at 28 days postinfection (Fig. 5). Even at this point, the levels of released virions were significantly lower than the levels of virions released in the cells acutely infected with pNL43 HIV-1. However, in the absence of TNF-α, *tat*-defective HIV-1 showed a low level of replication at the initial stage of infection (possibly due to the presence of various cytokines released by the phytohemagglutinin-stimulated PMBC) but ceased to replicate at later times postinfection. In contrast to the acute pNL43 HIV-1 infection, no cytopathic effect was observed in the TNF-α-treated cultures during the 30-day course of infection but a small cytopathic effect could be seen in infected, TNF-α-treated cultures after 40 days of culturing in vitro. It was shown recently that the replication of the NF-κB-site-deleted HIV-1 provirus was associated with the mutation in TATA box of HIV-1 LTR (35). It is unlikely that the alteration of the regulatory sequences is required for the replication of the *tat*-defective HIV-1 provirus, since this virus replicates efficiently without any lag period in the TNF-α-producing cell line C8166; however, additional analysis is needed to determine whether the replication in TNF-α-treated peripheral blood lymphocytes, which occurs at late time postinfection, is also associated with specific changes in the regulatory sequences.

The data presented in this study indicate that HIV-1 has developed mechanisms that allow it to replicate even in the absence of the virus-encoded Tat protein. However, this alternative pathway is much less efficient than Tat-mediated transactivation. Surprisingly, we have also observed that processing of the precursors of viral polypeptides is inhibited in the absence of Tat. Further experiments are needed to determine the molecular basis of this posttranscriptional effect of Tat. However, the low levels of viral structural proteins in the TNF-α-treated cells can explain the striking difference between the levels of proviral transcripts induced by TNF-α and the amount of virions released from these cells. These data indicate that while TNF-α can effectively induce proviral transcription, it does not functionally substitute for Tat and it is able to support effectively virus replication only in the presence of the *tat* gene product. We suggest, therefore, that during the virus life cycle, Tat-independent transactivation plays an important role in the initiation of proviral transcription that occurs before the

biologically significant levels of viral transactivator Tat are synthesized. The increase in rate of transcription allows the transcription to proceed through the *env* gene and facilitate the expression of HIV-1 regulatory genes such as *tat* and *rev*. The observation that TNF-α and other cytokines with transactivating potential (IL-1 and IL-6) are induced during the HIV-1 infection (24, 29) indicates that cytokines, induced upon binding of HIV-1 to the host cell, may play an essential role in the initiation of HIV-1 replication. Interestingly, HIV-1-specific $CD4^+$ cytotoxic T lymphocytes were shown to release TNF-α in concentrations sufficient to up-regulate latent HIV-1 provirus expression (21), and many of the commonly used T-cell lines that are highly permissive to HIV-1 replication (e.g., C8166) produce TNF-α constitutively.

The HIV-1 isolates defective in the functionally active *tat* gene were isolated from HIV-1-infected patients (25). These viruses show restricted replication in vitro but can be propagated in Tat-expressing cell lines (6). Our results indicate that the restricted replication of *tat*-defective HIV-1 provirus may be facilitated in vivo by the presence of cytokines. Although the TNF-α facilitated replication of the *tat*-defective HIV-1 provirus in PBMC, it is much less efficient than the replication of pNL43 HIV-1. However, the fact that *tat*-defective viruses may be able to establish noncytopathic, persistent viral infection suggests that *tat*-defective HIV-1 may contribute to the pathogenicity of the disease. The ability of TNF-α to complement partially the Tat-mediated genome expression of HIV-1 indicates that whereas the Tat protein enhances significantly the efficiency of HIV-1 replication, HIV-1 can also replicate, although less efficiently, in its absence. This observation may have important implications for the possible clinical use of *tat*-directed inhibitors as antiviral drugs. It has recently been shown that Tat-sequestering systems such as TAR decoy RNA (40) and the drug Ro5-3335 (11) are able to inhibit HIV-1 replication in vitro. The question, however, remains whether these inhibitors will also be effective in vivo, where the presence of transactivating cytokines (18, 44) and the presence of opportunistic infections (32, 33) are common cofactors of HIV-1 infection.

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