Tat is required for efficient HIV-1 reverse transcription

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The ability of human immunodeficiency virus-1 (HIV-1) to undergo efficient reverse transcription is dependent on a number of parameters. These include the binding of the tRNA 3Lys to the HIV-1 primer binding site and the subsequent interaction with the heterodimeric reverse transcriptase. Recently, we demonstrated that TAR RNA was also necessary for efficient HIV-1 reverse transcription. Given the fact that the Tat protein is involved in the activation of HIV-1 gene expression in conjunction with TAR, we wished to determine whether Tat might also be involved in the control of HIV-1 reverse transcription. HIV-1 virions deleted in the tat gene were unable to initiate reverse transcription efficiently upon infection of peripheral blood mononuclear cells (PBMCs). This defect was not due to decreased amounts of genomic RNA, reverse transcriptase or other HIV-1 proteins which were incorporated into the virion. Following transfection of wild-type but not mutant tat genes into cell lines producing HIV-1 lacking tat, the virions produced could be complemented for defects in reverse transcription upon subsequent infection of PBMCs. In contrast, the defect in reverse transcription seen with HIV-1 lacking the tat gene could not be complemented when the target cells rather than the producer cells contained tat. Viruses lacking tat were also defective in endogenous assays of reverse transcription, although these viruses contained similar levels of reverse transcriptase. These results indicate that the Tat protein, in addition to regulating the level of gene expression, is also important for efficient HIV-1 reverse transcription.

Keywords: HIV-1/reverse transcription/Tat protein

Introduction

Reverse transcription is a process used by retroviruses in which the combined activities of the virus-encoded heterodimeric reverse transcriptase results in the synthesis of proviral DNA from its RNA genome (Baltimore, 1970; Temin and Mitaizumi, 1970). Reverse transcription is initiated by reverse transcriptase which, in conjunction with a cell-derived tRNA, binds to retroviral RNA sequences known as the primer binding site (PBS) (Panet et al., 1975). The binding of tRNA to the PBS in the retroviral genome results in the ability of reverse transcriptase to initiate DNA synthesis from the U5 and R elements, resulting in the synthesis of minus strand (--) strong stop DNA (Varmus and Brown, 1989; Goff, 1990; Whitcomb and Hughes, 1992). A full-length double-stranded DNA molecule is generated in a multiple step process that is initiated following a jump of the (--) strong stop DNA to the 3’ end of the RNA genome (Hu and Temin, 1990a,b; Hu et al., 1990). A potential role for additional viral and/or cellular proteins in regulating the efficiency of this process remains a distinct possibility.

Human immunodeficiency virus-1 (HIV-1) reverse transcription is dependent on the binding of the tRNA 3Lys to the PBS (Rhim et al., 1991; Li et al., 1994; Wakefield et al., 1994, 1995; Das et al., 1995). Specific interactions between the heterodimeric p66/51 HIV-1 reverse transcriptase and the tRNA 3Lys (Barat et al., 1993) can induce conformational changes in the heterodimeric reverse transcriptase (Robert et al., 1990; Zakhara et al., 1995). The combination of reverse transcriptase and tRNA 3Lys binds to the PBS to form a specific reverse transcription initiation complex that is assembled prior to its transition to a reverse transcription elongation complex (Isel et al., 1993, 1995, 1996). Extended interactions between the anticodon loop of tRNA 3Lys and a conserved A-rich loop located upstream of the PBS (Isel et al., 1993, 1995) facilitate this transition (Isel et al., 1996). Studies with other retroviruses such as Rous sarcoma virus (RSV) also indicate that complex RNA stem–loop structures flanking the PBS are critical for efficient reverse transcription (Cobrinik et al., 1988, 1991; Aiyar et al., 1992, 1994). The sequence and/or the structure of the PBS and flanking RNA sequences, the properties of the reverse transcriptase and the nature of the specific tRNA primer contribute to the specificity of this process for different retroviruses (Aiyar et al., 1994).

Other factors or regulatory elements in HIV-1 which are required for viral replication have the potential either directly or indirectly to regulate the efficiency of reverse transcription. For example, both the Tat protein (Dayton et al., 1986; Fisher et al., 1986; Huang et al., 1994) and TAR RNA (Harrich et al., 1994, 1995, 1996; Klaver and Berkhour, 1994) are required for efficient HIV-1 replication. Mutations of the tat gene decrease HIV-1 replication several thousand-fold (Dayton et al., 1986; Fisher et al., 1986; Huang et al., 1994). This defect has been attributed to the critical role that Tat plays in activating HIV-1 gene expression (Kao et al., 1987; Berkhour et al., 1989; Laspa et al., 1989, 1990; Feinberg et al., 1991; Marciak and Sharp, 1991; Kato et al., 1992). Tat activation is dependent upon a stable RNA stem–loop structure, that extends from the transcription initiation site to +57, known as TAR (Rosen et al., 1985; Muesing et al., 1987; Feng and Holland, 1988; Garcia et al., 1989; Selby et al., 1989)
which serves as the binding site for Tat (Dingwall et al., 1990). Tat, in conjunction with TAR RNA, results in marked stimulation of the elongation properties of RNA polymerase II (Kao et al., 1987; Laspi et al., 1989, 1990; Feinberg et al., 1991; Marciniak and Sharp, 1991; Kato et al., 1992) and these effects are probably mediated by association of Tat with RNA polymerase II or other components of the HIV-1 transcriptional elongation complex (Keen et al., 1996; Mavankal et al., 1996). Alterations in TAR RNA structure markedly decrease Tat activation, and viruses containing these TAR RNA mutations exhibit several thousand-fold decreases in replication upon infection of peripheral blood mononuclear cells (PBMCs) or T-cell lines (Harrich et al., 1994, 1995, 1996; Klaver and Berkhout, 1994). TAR revertant viruses arising from the original HIV-1 TAR mutant viruses were isolated and exhibited nearly wild-type levels of gene expression due to partial restoration of TAR RNA stem base pairing and their ability to bind Tat (Harrich et al., 1995). However, subtle base pair changes that alter TAR RNA structure were still present in these revertant viruses and they were found to be defective for reverse transcription following infection of PBMCs (Harrich et al., 1996). Analysis of the original HIV-1 TAR mutant viruses further demonstrated that an intact TAR element was required for efficient reverse transcription (Harrich et al., 1996). Whether the effect of TAR on modulating the efficiency of reverse transcription was mediated entirely by RNA secondary structure or by the binding of cellular or viral factors remains unclear.

Given the fact that TAR is critical for both the regulation of HIV-1 gene expression and reverse transcription, we wished to investigate whether the Tat protein was also involved in regulating both of these processes. Using HIV-1 deleted in the tat gene, we were able to demonstrate that virions that lacked Tat were unable to undergo efficient reverse transcription either following infection of PBMCs or in endogenous reverse transcription assays. However, transfection of tat expression vectors into cell lines producing viruses deleted in the tat gene resulted in the generation of viruses that were able to undergo efficient reverse transcription upon infection of PBMCs or in endogenous reverse transcription assays. No difference in the levels of reverse transcriptase, genomic RNA or other proteins incorporated into virions lacking tat were detected as compared with wild-type HIV-1. These studies demonstrate that the Tat protein either directly or indirectly is able to modify virion particles, to increase their ability to undergo efficient HIV-1 reverse transcription.

**Results**

**Tat is required for efficient HIV-1 reverse transcription**

Since we demonstrated that the HIV-1 TAR RNA was required for efficient reverse transcription (Harrich et al., 1996), we next addressed whether this process may be modulated by the Tat protein. TAR is necessary for mediating Tat activation of HIV-1 gene expression (Rosen et al., 1985; Muesing et al., 1987; Feng and Holland, 1988; Garcia et al., 1989; Selby et al., 1989; Dingwall et al., 1990), and a previous study suggested that Tat may function at other points in the HIV-1 life cycle in addition to regulating transcriptional activation (Huang et al., 1994). More specifically, although other viral transactivators could fully complement the transcriptional defects of viruses mutated in their tat genes, these viruses were unable to replicate efficiently or cause cytopathicity (Huang et al., 1994).

An HIV-1 provirus was constructed which contained a deletion in the tat gene such that only the first five amino acids could be expressed. This virus produced only low levels of gene expression (<10 pg/ml of p24 antigen) following transfection of a variety of different cell lines, indicating the lack of a functional Tat protein. In an attempt to isolate sufficient amounts of the virus for further studies, the puromycin resistance gene was inserted in place of nef, and stable cell lines containing tat-deleted viruses were isolated (Morgenstern and Land, 1990). A variety of T-lymphocyte cell lines were used, but only low levels of HIV-1 gene expression were obtained. However, 293 cells which contain the adenovirus E1A and E1B proteins are able to strongly activate HIV-1 gene expression and complement the defect in gene expression in viruses lacking tat (Graham et al., 1977; Kliewer et al., 1989; Harrich et al., 1994, 1995, 1996). Using puromycin selection of 293 cells transfected with HIV-1 deleted in their tat gene, we were able to obtain clonal 293 cell lines producing virus lacking the tat gene. We routinely obtained viral supernatant which contained 1–2 ng/ml of p24 antigen after 16 h of cell culture. These 293 isolates were expanded and analyzed using PCR to confirm the presence of intact 5’ and 3’ long terminal repeats (LTRs) and a defective tat gene.

The 293 cells containing the tat-defective provirus designated Δtat were expanded and transfected with either an RSV expression vector lacking the tat gene or a similar expression vector containing the wild-type tat gene (Garcia et al., 1988). HIV-1 supernatants containing similar amounts of reverse transcriptase activity were obtained from 293 cell lines producing either wild-type, Δtat or Δtat virus complemented with wild-type tat. A further biochemical characterization of these viruses was performed in Figures 3 and 4 (see below). These viruses were used to infect PBMCs, and the ability of these viruses to undergo reverse transcription was assayed by PCR analysis of low molecular weight DNA obtained at 2 and 24 h post-infection (Hirt, 1967). Reverse transcription products corresponding to (–) strong stop (Figure 1A), jump (Figure 1B) and full-length (Figure 1C) DNA were analyzed as described (Zack et al., 1990). As shown in Figure 1, the Δtat virus was very defective in reverse transcription at both 2 and 24 h post-infection, as reflected in the low amounts of (–) strong stop DNA (Figure 1A–C, lanes 9 and 14) compared with that seen with wild-type HIV-1 (Figure 1A–C, lanes 7 and 12). Transfection of an expression vector encoding tat onto 293 cells producing wild-type HIV-1 did not alter the ability of this virus to undergo reverse transcription upon subsequent infection of PBMCs (Figure 1A–C, lanes 8 and 13) as compared with wild-type HIV-1 alone (Figure 1A–C, lanes 7 and 12). In contrast, transfection of the tat expression vector onto 293 cells producing the Δtat virus resulted in virus that synthesized increased amounts of (–) strong stop DNA upon subsequent infection of PBMCs (Figure 1A, lanes 10 and 15). Similar increases in the levels of
HIV-1 virions lacking \( tat \) are defective in reverse transcription. Activated PBMCs were infected for 2 h with culture supernatant from 293 cells containing 6 ng of reverse transcriptase activity (5 U/μg) for either wild-type HIV-1 (A–D, lanes 7 and 12), wild-type HIV-1 produced from 293 cells following transfection with an RSV expression vector containing the 72 amino acid \( tat \) gene (A–D, lanes 8 and 13), \( \Delta tat \) virus (A–D, lanes 9 and 14), \( \Delta tat \) virus produced from 293 cells following transfection of a wild-type \( tat \) expression vector (A–D, lanes 10 and 15), mock supernatant (A–D, lanes 6 and 11) or heat-inactivated supernatants for mock, wild-type HIV-1, wild-type HIV-1 transfected with a wild-type \( tat \) expression vector, \( \Delta tat \) virus or \( \Delta tat \) virus transfected with a wild-type \( tat \) expression vector (A–D, lanes 1–5). At 2 h post-infection, residual virus was removed by washing and Hirt lysates were prepared from half of the infected cells while the remaining PBMCs were cultured for 24 h and Hirt lysates were prepared. The recovered nucleic acids were assayed for HIV-1 (A) strong stop (lanes 1–15), the first strand jump (B, lanes 1–15) and full-length DNA (C, lanes 1–15). Quantitative PCR analysis of cyt-oxy II content in Hirt lysates was used to standardize the DNA recovery (D, lanes 1–15). All PCR reactions were performed within the linear range as determined by assays of HIV-1 DNA copy number (0, 10, 50, 250 and 1000) or cell number (0, \( 2 \times 10^5 \), \( 2 \times 10^6 \), \( 1 \times 10^6 \) and \( 5 \times 10^6 \)). This analysis is representative of PCR reactions performed on three separate HIV-1 infections using independently prepared virus stocks.

The reverse transcription products were seen with the \( \Delta tat \) virus produced following transfection with wild-type \( tat \) by analyzing jump DNA at 2 h (Figure 1B, lane 10) and 24 h (Figure 1B, lane 15) and full-length DNA at 24 h (Figure 1C, lane 15). Analysis of the magnitude of the defects in \( \Delta tat \) virus reverse transcription relative to wild-type HIV-1 was analyzed by Phosphorimager quantitation at 24 h post-infection and indicated that the \( \Delta tat \) virus exhibited a 10-fold defect in strong stop DNA, a 7-fold defect in jump DNA and a 30-fold defect in full-length DNA. These defects were not detected in virus produced following transfection of the wild-type \( tat \) gene into 293 cells containing the \( \Delta tat \) virus. No reverse transcription products were detected in infections performed using supernatants from uninfected 293 cells (Figure 1A–C, lane 6) nor following heat inactivation of these viruses (Figure 1A–C, lanes 1–5). An internal control using PCR analysis of the mitochondrial cytochrome oxidase II gene (cyt-oxy II) from each of the PBMC samples indicated equal DNA recovery (Figure 1D, lanes 1–15). These results indicate that the \( tat \) gene is involved in the ability of HIV-1 to undergo efficient reverse transcription.

**Tat-complemented virus is defective for subsequent replication**

Filtered 293 viral supernatants containing equal amounts of reverse transcriptase activity for wild-type, \( \Delta tat \) and \( \Delta tat \) virus produced following transfection with wild-type \( tat \) were again used to infect PBMCs (Figure 2). The PBMCs were assayed for the amount of p24 antigen by enzyme-linked immunosorbent assay (ELISA) during a 30 day period of culture. Thus, we could determine
HIV-1 reverse transcription requires Tat

Fig. 3. Biochemical analysis of HIV-1 tat mutant viruses in PBMCs. (A) Filtered culture supernatants containing ~300 ng of HIV-1 p24 antigen were subjected to centrifugation and the pelleted virus was assayed for reverse transcriptase activity and p24 antigen content as described in Materials and methods. The assays were performed in triplicate with two independently prepared virus stocks. (B) Western blot analysis was performed with a mock-infected sample or equal quantities of virions produced from 293 cells (25 ng of p24 antigen) for wild-type HIV-1, Δtat virus or Δtat virus produced following transfection of a wild-type tat expression vector. HIV-1-specific proteins were detected using either an affinity-purified human polyclonal IgG directed against HIV-1 (lower panel) or an affinity-purified monoclonal IgG directed against HIV-1 gp120 (IBII) (upper panel) followed by treatment with secondary HRP-conjugated antibodies and chemiluminescence detection. The molecular weight markers are indicated.

whether wild-type tat complementation of the reverse transcriptase defect seen with the Δtat virus produced in 293 cells resulted in the generation of virus that was able to replicate efficiently in PBMCs. Both Δtat virus and Δtat virus produced following transfection of the wild-type tat gene were unable to replicate efficiently following infection of PBMCs when assayed over a 30 day culture period (Figure 2). In contrast, wild-type HIV-1 gave high levels of p24 antigen expression (Figure 2). These results indicate that although wild-type tat can complement the defect in reverse transcription seen with the Δtat virus upon infection of PBMCs, the absence of continued tat production in the PBMCs resulted in low levels of gene expression and a failure to maintain subsequent replication. Furthermore, these results indicated that complementation of the defect in reverse transcription of the Δtat virus with wild-type tat did not result in the generation of wild-type HIV-1 recombinants.

HIV-1 wild-type and Δtat virions have similar biochemical properties

It was important to characterize the biochemical properties of wild-type HIV-1 and Δtat virions produced in 293 cells both before and after transfection of the wild-type tat gene to determine whether decreased synthesis of HIV-1 proteins may account for the defects noted in reverse transcription upon infection of PBMCs. To compare the biochemical properties of these viruses, filtered viral supernatant from 293 cells was harvested by centrifugation and analyzed for both reverse transcriptase activity and p24 antigen (Figure 3A). This analysis indicated that the reverse transcriptase activity and p24 antigen levels were similar with these different viruses with a ratio of reverse transcriptase/p24 ranging from ~3.2 to 2.6. Next, these viruses were analyzed by Western blot analysis using human IgG antibodies directed against HIV-1 or a monoclonal antibody directed against gp120. This analysis indicated that the amount of gp120 and other HIV-1
proteins were similar for wild-type HIV-1, Δtat virus and ΔΔtat virus produced following transfection of 293 cells with the wild-type tat expression vector (Figure 3B).

Finally, we determined whether the same amount of HIV-1 RNA was incorporated into the wild-type and Δtat virions produced in either the presence or absence of a wild-type tat gene transfected into 293 cells. Virus preparations containing equal amounts of p24 antigen were subjected to PCR analysis. Similar amounts of RNA were incorporated into HIV-1 wild-type (Figure 4A, lanes 3 and 4), Δtat virus (Figure 4A, lanes 5 and 6) and ΔΔtat virus produced following transfection with wild-type tat (Figure 4A, lanes 7 and 8). There was no RNA detected in mock-infected 293 supernatants (Figure 4A, lanes 1 and 2) and the amount of an internal control HIV-1 RNA included in each sample prior to PCR analysis was similar (Figure 4B, lanes 1–8). Thus no gross biochemical abnormalities were detectable in the tat mutant virus as compared with wild-type HIV-1.

Wild-type but not mutant tat genes prevent defects in reverse transcription of Δtat virions

Next, we determined whether transfection of expression vectors containing a variety of tat mutants was able to prevent the defect in reverse transcription seen with the Δtat virus. The 293 cells containing the Δtat virus were transfected with expression vectors containing either wild-type tat, a tat mutant in four critical cysteine residues (tat/cys), a tat deletion mutant that eliminated the basic domain (Δ53) or a tat deletion mutant that contained the first 60 amino acids of tat (Δ60) (Garcia et al., 1988). The tat/cys and Δ53 tat mutants are very defective for tat activation of HIV-1 gene expression while the Δ60 tat mutant has nearly wild-type tat function. A schematic of the position of these mutants in the tat gene is shown (Figure 5). Virus stocks were prepared from each of these transfected 293 cells and equal amounts of reverse transcriptase activity were used to infect PBMCs. The reverse transcription products isolated from each of these infections were analyzed using PCR at both 2 and 24 h post-infection. Only the PCR analyses from samples isolated at 24 h post-infection are shown in Figure 5. The

Δtat virus was very defective for reverse transcription when analyzed for DNA synthesis, including (--) strong stop (Figure 6A, lane 4), jump (Figure 6B, lane 4) and full-length (Figure 6C, lane 4), as compared with wild-type HIV-1 (Figure 6A–C, lane 2). This defect was not seen in ΔΔtat virions produced following transfection of
HIV-1 reverse transcription requires Tat

Fig. 7. Tat expressed in target cells does not increase Δtat virus reverse transcription. Equivalent cell numbers of either HeLa, HeLa/CD4 or HeLa/CD4/tat were infected for 2 h with either mock supernatant (A–D, lanes 1, 8 and 15) or 15 μg of reverse transcriptase (5 U/μg) activity obtained from 293 cells for either wild-type HIV-1 (A–D, lanes 5, 12 and 19), HIV-1 Δtat virus (A–D, lanes 6, 13 and 20) or HIV-1 Δtat virus produced following transfection with a wild-type tat expression vector (A–D, lanes 7, 14 and 21). Similarly, heat-inactivated viruses including wild-type HIV-1 (A–D, lanes 2, 9 and 16), HIV-1 Δtat virus (A–D, lanes 3, 10 and 17) or Δtat virus produced following transfection of a wild-type tat expression vector (A–D, lanes 4, 11 and 18) were used to infect each cell line. The residual virus was removed by washing, and Hirt lysates were prepared from the infected cells at 24 h post-infection. The recovered nucleic acids were assayed for HIV-1 (–) strong stop (A, lanes 1–21), first strand jump (B, lanes 1–21) and full-length DNA (C, lanes 1–21). Quantitative PCR analysis of cyt-ox II content in Hirt lysates (D, lanes 1–21) was used to normalize the input in all PCR reactions. This analysis is representative of three independent infections and subsequent PCR reactions. All PCR reactions were performed within the linear range of the assay as indicated by the HIV-1 DNA standards (0, 10, 50, 250 and 1000) and the cell number (0, 4×10^5, 1×10^6, 3×10^6 and 1×10^7).

the wild-type tat gene into the 293 cells, since the amounts of the PCR products were similar to that seen with wild-type HIV-1 (Figure 6A–C, lanes 2 and 3). In contrast, Δtat virus produced in 293 cells following transfection of the tat mutant tat/cys exhibited marked defects in reverse transcription upon infection of PBMCs (Figure 6A–C, lane 5). A tat mutant, Δ53, when transected into 293 cells containing the Δtat virus, resulted in virus that was still partially defective in the synthesis of reverse transcription products upon infection of PBMCs (Figure 6A–C, lane 6). Finally, transfection of the 293 cells into tat mutant Δ60 restored the ability of the Δtat virus produced to undergo reverse transcription upon infection of PBMCs (Figure 6A–C, lane 7). Analysis of these PCR assays using Phosphorimager quantitation indicated that relative to wild-type HIV-1 there were defects in strong stop, jump and full-length products of 8-, 10- and 30-fold for Δ tat virus alone; 12, 16- and 100-fold for Δtat virus complemented with tat/cys; 5-, 6- and 50-fold for Δtat virus complemented with Δ53 and of 2-fold or less for Δtat virus complemented with Δ60. There were no detectable PCR products following mock infection of PBMCs (Figure 6A–C, lane 1) and there was equal recovery of DNA from the PBMCs as judged by equivalent PCR products seen with the control cyt-ox II (Figure 6D, lanes 1–7). These results indicate that the ability of tat to complement reverse transcription of the Δtat virus was dependent on a functional tat gene.

**Tat expression in target cells does not increase the reverse transcription of Δtat virions**

A wild-type tat expression vector when transfected into 293 cells producing the Δtat virus was able to prevent the defect in reverse transcription with this virus following infection of PBMCs (Figures 1 and 6). Next, we addressed whether the presence of the tat gene in the target cells used for HIV-1 infection rather than in the HIV-1 producer cells could also complement the defect in reverse transcription seen with the Δtat virus. HeLa cells either lacking or possessing the CD4 receptor (Maddon et al., 1986) or HeLa CD4 cells stably expressing the tat gene were used in infection experiments with the different HIV-1 virus stocks. The HeLa CD4 cells containing the tat gene resulted in 50-fold levels of activation of a transfected HIV-1 LTR CAT plasmid compared with HeLa CD4 cells (data not shown). The different HeLa cell lines were infected with equal amounts of reverse transcriptase produced from the 293 cells containing wild-type HIV-1, Δtat virus or Δtat virus complemented with wild-type tat. The levels of HIV-1 reverse transcription products were assayed at 24 h post-infection.

No reverse transcription products were detected with any of the viruses when they were used to infect HeLa cells lacking the CD4 receptor (Figure 7A–C, lanes 5–7). In contrast, reverse transcription products were detected when 293 supernatants containing either the wild-type, Δtat virus or the Δtat virus complemented with wild-type tat were used to infect HeLa CD4 cells (Figures 7A–C, lanes 12–14). The Δtat virus was very defective in the synthesis of reverse transcription products when used to infect HeLa CD4 cells (Figure 7A–C, lane 13). No defects in reverse transcription were seen with Δtat produced in 293 cells following transfection of the wild-type tat gene (Figure 7A–C, lane 14), with similar levels of reverse transcription products detected as compared with wild-type HIV-1 (Figure 7A–C, lane 12). When each of these
viruses produced in 293 cells was used to infect HeLa CD4 cells containing the tat gene, the Δtat virus was again very defective for reverse transcription, with defects ranging from 20-fold for strong stop and jump DNA to 100-fold for full-length DNA (Figure 7A–C, lane 20). In contrast, the Δtat virus complemented with wild-type tat (Figure 7A–C, lane 21) and wild-type HIV-1 (Figure 7A–C, lane 19) gave similar levels of reverse transcription products. Neither mock infection or heat-treated viruses gave detectable levels of reverse transcription products when used to infect any of the HeLa cell lines (Figure 7A–C, lanes 1–4; lanes 8–11; lanes 15–18). There was equal recovery of DNA samples from the HIV-1-infected HeLa cells as determined from PCR analysis of the cyt- oxy II gene (Figure 7D, lanes 1–21). These results indicate that the expression of wild-type tat in the producer cells but not the target cells was able to reverse the defects in reverse transcription seen with the Δtat virus.

**HIV-1 virions lacking tat exhibit defects in endogenous reverse transcription**

Finally, we assayed endogenous reverse transcription (Arts et al., 1994) using virions produced in 293 cell lines expressing either wild-type HIV-1, Δtat virus or Δtat virus complemented following transfection with wild-type tat or different tat mutants. Virus stocks containing equal quantities of reverse transcriptase were treated with DNase I to remove potential contaminating chromosomal DNA. PCR analysis was then performed on a portion of these virions with specific primers to detect pre-existing endogenous DNA (Figure 8A and B, lanes 1, 3, 5, 7, 9 and 11). The other portion of the virions was incubated for 60 min at 37°C in the presence of either 12 μM deoxynucleotides (dNTPs) (Figure 8A, lanes 2, 4, 6, 8, 10 and 12) or dNTPs lacking dTTP (Figure 8B, lanes 2, 4, 6, 8, 10 and 12). The dTTP was omitted from these reactions to demonstrate that the DNA detected by PCR was the product of the endogenous reverse transcription reaction rather than pre-existing endogenous DNA. These virions were incubated in a stop solution (10 mM Tris pH 8.0, 10 mM EDTA, 20 μg/ml herring sperm DNA and 50 μg/ml protease K) at 50°C then at 95°C for 10 min and assayed by PCR for the presence of HIV-1 (−) strong stop DNA. The amount of (−) strong stop DNA synthesized in the endogenous reverse transcription assay was similar for wild-type HIV-1 (Figure 8A, lane 2). Δtat virus produced following transfection of 293 cells with wild-type tat (Figure 8A, lane 6) and Δtat virus produced following transfection with the ΔA60 tat mutant (Figure 8A, lane 12). In contrast, there was a marked decrease in the amount of (−) strong stop DNA synthesized with the Δtat virus (Figure 8A, lane 4) or the Δtat virus produced following transfection of 293 cells with either the tat/cys mutant (Figure 8A, lane 8) or the tat mutant ΔS3 (Figure 8A, lane 10). Phosphorimager quantitation of the PCR assays indicated that Δtat virus exhibited a 10-fold defect relative to wild-type HIV-1. The Δtat virus produced following transfection of 293 cells with wild-type tat was 2-fold higher than wild-type virus, while the Δtat virus produced following transfection of 293 cells with tat/cys exhibited a 16-fold defect, the Δtat virus produced following transfection with ΔS3 tat exhibited a 5-fold defect and the Δtat virus produced following transfection with ΔA60 tat exhibited no defect. DNase I treatment was sufficient to remove potential contaminating chromosomal DNA or cell nuclei as indicated by the near complete

**Fig. 8.** Endogenous reverse transcription assays of HIV-1 wild-type and tat mutants. (A) and (B) Either wild-type HIV-1 (lanes 1 and 2), Δtat virus (lanes 3 and 4) or Δtat virus produced following transfection with either wild-type tat designated Δtat/WT (lanes 5 and 6) or the tat mutants tat/cys designated Δtat/cys (lanes 7 and 8), ΔS3 designated Δtat/ΔS3 (lanes 9 and 10) or ΔA60 designated Δtat/ΔA60 (lanes 11 and 12) were assayed in triplicate for reverse transcriptase activity. Culture supernatants (~150 μl) containing activity equivalent to 20 pg of recombinant reverse transcriptase were treated with 10 mM MgCl₂ and 100 U of DNase I for 20 min at 30°C. To detect endogenous viral DNA, 50 μl of the treated viral supernatant were lysed and subjected directly to PCR analysis (A and B, lanes 1, 3, 5, 7, 9 and 11). The remaining viral supernatants were supplemented with either 12 μM dNTPs (A, lanes 2, 4, 6, 8, 10 and 12) or 12 μM dNTPs minus dTTP (B, lanes 2, 4, 6, 8, 10 and 12). DNase I activity was confirmed by the addition of 10⁶ copies of an HIV-1 LTR plasmid to wild-type HIV-1 supernatant not supplied with dNTPs (lane 13). The presence of endogenous HIV-1 DNA was measured in wild-type HIV-1 supernatant not supplemented with dNTPs (lane 14). All reactions were incubated at 37°C for 60 min and terminated with 200 μl of stop solution at 50 then 95°C for 10 min at each temperature. All lysed supernatants were assayed by PCR for the presence of an HIV-1 (−) strong stop DNA as described in Materials and methods. All PCR reactions were performed within the linear range of the assay.
degradation of 10^6 copies of an HIV-1 plasmid containing DNA sequences detected by PCR that was added to viral supernatant not supplemented with dNTPs (Figure 8A, lane 13). No HIV-1 DNA was detected in DNase I-treated wild-type HIV-1 supernatant not supplied with dNTPs (Figure 8B, lane 14). Finally, there was little synthesis of (∼) strong stop DNA when dTTP was omitted from the endogenous reaction (Figure 8B, lanes 2, 4, 6, 8, 10 and 12), indicating that virions required exogenous pools of deoxynucleotides to initiate measurable levels of (∼) strong stop DNA. These results indicate that the levels of endogenous reverse transcription for the wild-type and Δtat-deleted viruses correlated with the in vivo reverse transcription analysis for these viruses.

Discussion

Recently, we demonstrated that a number of HIV-1 mutants in TAR were defective for reverse transcription upon infection of PBMCs (Harrich et al., 1996). Since TAR RNA is involved in the control of HIV-1 reverse transcription, we investigated whether the tat gene which requires TAR RNA to activate gene expression might also be required for efficient reverse transcription. Previous data have demonstrated that HIV-1 virions containing deletions in the tat gene were very defective for viral replication (Dayton et al., 1986; Fisher et al., 1986; Huang et al., 1994). This defect has been attributed primarily to a failure of tat-mediated increases in HIV-1 gene expression (Dayton et al., 1986; Fisher et al., 1986). However, the fact that heterologous viral transactivators inserted into HIV-1 proviruses lacking tat could complement defects in HIV-1 gene expression but not defects in viral replication and cytopathicity suggested potential effects of tat in regulating other steps in the viral life cycle (Huang et al., 1994).

A role for tat in early steps in the HIV-1 life cycle has not been demonstrated due at least in part to difficulty with obtaining sufficient quantities of HIV-1 virions lacking tat. The adenovirus E1A and E1B proteins in the 293 cells (Graham et al., 1977) complement the defects in HIV-1 gene expression seen with both TAR and tat mutants because the primary effect of E1A on activating gene expression is by interaction with upstream cellular transcription factors (Kliewer et al., 1989). Clonal populations of 293 cells containing HIV-1 deleted in the tat gene were isolated and the quantity of this virus was sufficient to analyze early steps in the HIV-1 life cycle. Surprisingly, we found that HIV-1 lacking the tat gene was very defective in the initiation of reverse transcription as reflected in defects in the synthesis of (∼) strong stop DNA. There was a corresponding decrease in the amount of first strand synthesis and a further defect in the synthesis of full-length DNA, suggesting that the processivity of the reverse transcriptase might also be affected in virus lacking tat. It will be important to determine whether the defects in reverse transcription seen with virus lacking tat are entirely due to a defect in initiation or whether additional effects of reverse transcriptase processivity are also present.

The defects in reverse transcription seen with HIV-1 lacking tat could be prevented by transfection of wild-type but not a variety of mutant tat expression vectors into the 293 cells containing the Δtat virus. These studies indicate the requirement for a functional Tat protein to complement the defects seen with virions lacking tat. However, the defect in reverse transcription of the Δtat virus upon infection of PBMCs was not due to detectable differences in the amount of HIV-1 proteins such as gp120. Furthermore, the amount of pd24 antigen, reverse transcriptase and intravirion RNA was standardized carefully for each of the viral stocks produced from the 293 cell lines so that any potential differences between the stocks could be identified. The Δtat virus produced in 293 cells complemented with wild-type but not mutant tat genes can undergo efficient reverse transcription both in vivo and in vitro in the presence of an exogenous dNTP pool. Although it is possible that subtle changes in HIV-1 virion structure occur in the absence of tat, electron microscopy did not detect ultrastructural abnormalities of viruses lacking tat which were produced under conditions where their gene expression was complemented with heterologous viral transactivators (Huang et al., 1994).

A variety of studies indicate that reverse transcription is subject to complex regulation by both viral and cellular proteins. For example, the cellular milieu is critical for the efficiency of HIV-1 reverse transcription as reflected in the marked defects in replication seen in quiescent lymphocytes (Zack et al., 1990, 1992; O’Brian et al., 1994). Mutants in several viral proteins including integrase (Masuda et al., 1995), Nef (Schwartz et al., 1995) and Vif (von Schwedler et al., 1993) result in viruses that exhibit defects in reverse transcription. In addition, the nucleocapsid which facilitates strand transfer may also increase the efficiency of reverse transcription (Lapadat-Tapolsky et al., 1993, 1995; Allain et al., 1994; Peliska et al., 1994). Cellular proteins including DNA topoisomerase I (Priel et al., 1990) and cyclophilin A (Franke et al., 1994; Thali et al., 1994) are also present in HIV-1 virions. DNA topoisomerase I has been suggested to stimulate the elongation properties of reverse transcriptase (Priel et al., 1990; Jardine et al., 1993; Takahashi et al., 1995). Cyclophilin A is incorporated into the HIV-1 particle via its interactions with the Gag protein (Franke et al., 1994; Thali et al., 1994). Mutations in critical proline residues in Gag which bind cyclophilin A result in virions which exhibit defects in replication that may be due in part to defects in reverse transcription (Braaten et al., 1996). These results suggest that HIV-1 reverse transcription is subject to complex regulation and that viral and cellular proteins in addition to reverse transcriptase are probably critical in regulating the efficiency of this process.

Recent studies suggest that reverse transcription may have at least three defined stages of regulation, including initiation, a transition between initiation and elongation, and elongation (Isel et al., 1996). This pattern of regulation is dependent on modified nucleosides in the tRNA_Lys, which facilitate interactions with the HIV-1 PBS and with an A-rich loop located 12–17 nucleotides upstream of the PBS (Isel et al., 1993, 1996). This complex pattern of RNA interactions may be critical for the regulation of the transition between the initiation and elongation of reverse transcription which is seen with HIV-1 reverse transcriptase but not with several other viral reverse transcriptases (Arts et al., 1996b). Data indicating that tRNA_Lys DNA chimeras can lead to the synthesis of (∼)
strand strong stop DNA synthesis with a reverse transcriptase mutant that is defective for the initiation of reverse transcription with authentic tRNA\(^{3\prime}\)\(^{5\prime}\) further indicate a role for multiple RNA interactions in the reverse transcription process (Arts et al., 1996a). Our previous finding that TAR RNA is involved in the regulation of (–) strand strong stop DNA synthesis would be consistent with a role for TAR RNA in these multiple RNA interactions to regulate an early step in the synthesis of (–) strand strong stop DNA synthesis.

The question arises as to how tat is able to stimulate efficient reverse transcription. One possibility is that tat is incorporated into HIV-1 virion particles via binding to TAR RNA (Dingwall et al., 1990; Calnan et al., 1991; Weaks and Crothers, 1991). Tat binding to TAR RNA may alter TAR structure such that the initiation of reverse transcription is enhanced. For example, NMR analysis indicated that Tat binding induces a conformational change which forms a more stable TAR RNA structure (Aboul-ela et al., 1995). It is possible that Tat may facilitate interactions between TAR RNA and other RNA elements including tRNA\(^{3\prime}\)\(^{5\prime}\) and U5 RNA which are involved in the reverse transcription process. Whether these RNA interactions may lead to direct association of Tat with either reverse transcriptase or nucleocapsid remains to be determined. The initial interactions of Tat with TAR RNA and subsequent direct interactions with reverse transcriptase could result in stimulation of the initiation of reverse transcriptase with secondary effects of Tat on the processivity of reverse transcriptase. However, using Western blot analysis with rabbit polyclonal antibodies directed against Tat, we have been unable to detect Tat protein in concentrated preparations of HIV-1 virus. This could be due to the fact that the detection limits of our Western blot analysis using polyclonal Tat antibodies fail reproducibly to detect Tat if only four or less molecules are present in each virion particle.

Alternatively, Tat could have an indirect effect on stimulating reverse transcription by altering either virion structure or the association of viral or cellular factors. For example, Tat could alter intravirion nucleotide pools which are critical for efficient reverse transcription (Zack et al., 1990, 1992; O’Brian et al., 1994). However, our analysis using sensitive techniques (Sherman and Fyfe, 1989) did not demonstrate differences in the nucleotide levels in HIV-1 produced in the presence or absence of Tat (D.Harrich and R.B.Gaynor, unpublished observation). Further studies will be required to elucidate the mechanisms by which Tat enhances the efficiency of HIV-1 reverse transcription. Such studies could potentially identify novel transdominant inhibitors as well as yield a better understanding of potential cellular and viral factors that regulate this key step in the HIV-1 life cycle.

### Materials and methods

#### Plasmids

The HIV-1 proviral construct pBRDH2puro was made by inserting the puromycin resistance gene (Morgenstern and Land, 1990) into the proviral construct pBRDH1 which was derived from HIV-1 pBlHo (Hahn et al., 1984). The tat deletion construct pBRDH2puroΔtat was derived from pBRDH2puro that was linearized with the restriction enzyme BamH1 and treated with mung bean nuclease. The digested plasmid was incubated with T7 polymerase in the presence of deoxy-nucleosides and circularized with T4 DNA ligase. A 14 bp deletion from +5430 to +5443 within the tat gene was identified by DNA sequencing that created a frame shift in a region that did not alter other HIV-1 genes. The first five amino acids of tat (MEPVD) were translated followed by eight amino acids translated from the n –1 alternate reading frame (LEASRRKSA) followed by a stop codon.

Both the wild-type and mutant eucaryotic vectors expressing tat were described previously (Garcia et al., 1988). For these vectors, the HIV-1 tat gene encoding either the first 72 amino acids (wild-type), 60 amino acids (Δ60), 53 amino acids (Δ53) or serine substitutions of cysteine at residues 22, 27, 31 and 34 (tat(Δcys)) were placed downstream of the RSV promoter in a vector containing the SV40 splice acceptor and polyadenylation sequences (Garcia et al., 1985).

#### Cells and cell lines

A 100 mm plate of 50% confluent 293 cells (Graham et al., 1977) was transfected with 20 μg of Moloney-murine pBRDH2puro-Δtat (Harrich et al., 1994). The transfected 293 cells were split 1:25, 3 days post-transfection and grown in modified Iscove’s medium supplemented with 5% newborn calf serum (NCS), 2.5% fetal bovine serum (FBS), 1% penicillin–streptomycin and 1 μg/ml puromycin. In 4–6 weeks, individual cell foci were removed from the culture dish using cloning wells (Bellco, Inc.), expanded, and cell free culture supernatant was assayed for p24 antigen by ELISA (DuPont). A 293 cell line designated 5250 produced 1–2 ng/ml of HIV-1 p24 antigen in 16 h. Both the HIV-1 LTR and tat genes were obtained from chromosomal DNA by PCR and subjected to DNA sequencing.

A HeLa cell line that stably expressed the human CD4 receptor was obtained from the NIH AIDS reference and reagents program (Maddon et al., 1986) (NIH ARRP #154). The HeLa CD4 cells were transfected using lipofectamine (Gibco-BRL) with a pBabe expression vector containing the wild-type tat gene (Morgenstern and Land, 1990). At 3 days post-transfection, cells were split 1:50 and maintained in Iscove’s medium supplemented with 5% NCS, 2% FBS, 1% penicillin–streptomycin, 1% glutamine, 0.5 mg/ml G418 (Gibco) and 0.2 mg/ml hygromycin B (Boehringer Mannheim). Individual foci were isolated, expanded and assayed for expression of Tat protein using a HIV-1 LTR CAT transactivation assay (Garcia et al., 1989) prior to use in HIV-1 infection experiments.

PBMCs were obtained from HIV-1 seronegative donors and grown in RPMI supplemented with 20% FBS (Gibco-BRL), 1% penicillin–streptomycin (Gibco-BRL) and 5 μg/ml phytohemagglutinin (Boehringer Mannheim) for 3 days (Harrich et al., 1994). The PBMCs were then placed in RPMI culture medium supplemented with 20 U/ml interleukin-2 (BTI, Inc.) and used immediately for HIV-1 infection experiments.

#### Virus stocks

All HIV-1 virus stocks including wild-type and Δtat mutants were produced in stable 293 cell lines (Graham et al., 1977; Harrich et al., 1994, 1996). The 293 cell lines were grown in 100 mm dishes until they were 75% confluent and the culture medium was replaced with RPMI 1640 supplemented with 10% FBS, 1% penicillin–streptomycin and 1% glutamine (Gibco-BRL) and cultured for 16 h. To complement 293 S250 cells with either wild-type or mutant tat genes, 100 mm plates of 40% confluence were transfected using lipofectamine with 5 μg of expression vectors for RSV-tat wild-type, RSV-tatΔN0, RSV-tatΔ53 or RSV-tatΔcys expression plasmids (Gibco-BRL). Three days post-transfection, the culture medium was changed to RPMI 1640 supplemented with 10% FBS, 1% penicillin–streptomycin and 1% glutamine (Gibco-BRL) and cultured for 16 h. All virus supernatants were collected, passed through a 0.45 μm filter and stored in aliquots at –80°C. All virus stocks were assayed for p24 antigen by ELISA and reverse transcriptase activity using a non-radioactive assay that utilized a synthetic template–primer hybrid poly(A) oligo(dT)\(_{12}\) and digoxigenin–biotin-labeled dUTP, which was detected by an ELISA protocol (Boehringer Mannheim) and incorporated into the newly synthesized DNA.

#### Immunoblot analysis of virions

Virus stocks were pelleted by centrifugation at 22 000 g for 2 h at 4°C and suspended in phosphate-buffered saline/1% bovine serum albumin (PBS/BSA). The virus suspensions were assayed for reverse transcriptase activity and p24 antigen content. Each virus suspension was diluted and normalized to 0.35 ng of p24 antigen/ml in 4% Laemmli buffer. Viral lysates were incubated at 95°C for 10 min and 25 ng of p24 antigen was loaded in each lane. Samples were subjected to electrophoresis on 10% SDS–polyacrylamide gels. The proteins were transferred to nitrocellulose and probed with either 1:100 human anti-HIV-1 IgG.
HIV-1 reverse transcription requires Tat

Viral stocks prepared from 293 cell lines expressing either wild-type or tat mutant HIV-1 virus were used to infect 107 activated PBMCs in 100 mm tissue culture plates. Cells (5 g/ml proteinase K), then incubated at 2°C for 60 min. A visible pellet was washed with 70% ethanol and resuspended in 0.5 ml of Tris buffer (10 mM Tris pH 7.0, 1 M NaCl). The cell lysates were harvested and Hirt lysates were prepared. The DNA amounts were normalized using PCR with the cyt-oxy II gene as an internal control, and PCR analysis was performed to analyze HIV-1 reverse transcription products.

Endogenous reverse transcription assay

Virus stocks prepared from 293 cell lines expressing either HIV-1 wild-type, Δtat or ΔΔtat complemented with either wild-type tat or tat mutants Δcys, Δ53 and Δ60 were assayed for reverse transcription activity (Boehminger Mannheim). For each endogenous reverse transcription reaction, 150 μl of viral supernatant was added to 200 μl of stop solution (10 mM Tris pH 8.0, 10 mM EDTA, 20 μg/ml sheared herring sperm DNA, 50 μg/ml proteinase K), then incubated at 50 and 95°C for 10 min at each temperature. The remaining supernatants were subjected to centrifugation (5 g/ml each DATP, dCTP, dGTP and dTTP) or 12 μM dNTPs minus dTTP and incubated at 37°C for 60 min. The reactions were terminated with 400 μl of stop solution then incubated at 50 and 95°C for 10 min at each temperature. Assays of 10 μl of each of the endogenous reverse transcription reactions were performed for (+) strand stop DNA using ‘hot’ PCR as described, except for the addition of 2 mM MgCl2.

PCR analysis of HIV-1 DNA

Chromosomal DNA from 293 cell lines expressing either the wild-type or tat mutant HIV-1 virus was isolated as previously described (Harrich et al., 1996). Exactly 10 ng of reverse transcriptase activity equal to 6 ng (5 U/μl) of recombinant reverse transcriptase was supplemented with 10 mM MgCl2 and 300 μl of DNase I (Worthington Biochemical, New Jersey) and then incubated at 30°C for 30 min. DNase I-treated viral supernatants were heat inactivated at 60°C for 20 min for use in control infections. For each infection, 2×10^6 activated PBMCs were infected with the appropriate DNase I-treated virus stock in a 150 mm tissue culture plate at a multiplicity of infection equal to 6 ng (5 U/μl) of recombinant reverse transcriptase. For each endogenous reverse transcription reaction 10 μg of purified chromosomal DNA and 50 ng of the oligonucleotide primers (±436–415) 5′-CCCAAC/AAGACAAAGAGAT-TGA-3′, sense) and (+242+219) 5′-CTCGTGCCGAGAGCTCC-TCTGG-3′, antisense) (5′ LTR) were supplemented with 2×10^6 activated PBMCs in a 150 mm tissue culture plate with gentle reciprocal shaking at 37°C for 2 h. The heat-inactivated virus stocks were used to infect 1×10^6 activated PBMCs in 100 mm tissue culture plates. Cells used in the infection were washed three times with RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin and 1% glutamine (Gibco-BRL). All cells infected with heat-inactivated virus or one half of the infected cells were removed and total cytoplasmic nucleic acids were obtained by modified Hirt lysis. Briefly, the cell pellet was washed in 1× phosphate-buffered saline, pelleted at 400 g for 10 min, and gently resuspended in 0.5 ml of Tris buffer (10 mM Tris pH 7.0, 10 mM EDTA). The cell lysate was subjected to centrifugation at 15,000 g for 20 min. The nucleic acids were precipitated with two volumes of ethanol overnight at −20°C. The nucleic acids were pelleted by centrifugation at 15,000 g for 4°C for 1 h and the alcohol was carefully removed, leaving a visible pellet that was washed with cold 70.0% ethanol, air dried, and resuspended in 500 μl of PCR quality TE (10 mM Tris pH 7.8, 0.1 mM EDTA). The remaining products were separated by electrophoresis on a 6% polyacrylamide gel as described and cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 1% glutamine (Gibco-BRL) and 2×10^6 activated PBMCs.

 Approximately 10^5 HeLa/CD4 and HeLa/CD4/Tat cells were incubated with DNase I-treated viral supernatants produced from 293 cells expressing either wild-type HIV-1, Δtat or ΔΔtat complemented with wild-type bat tat viral-containing activity equal to 15 ng of reverse transcription (5 μg/μl) or heat-inactivated viruses for 2 h at 37°C with constant shaking. Cells were washed three times with media. At 24 h post-infection, cells were harvested and Hirt lysates were prepared. The DNA fragments were ligated into TA vector (Invitrogen) and analyzed by DNA sequencing.

To determine viral replication kinetics, 2×10^6 activated PBMCs were infected with either HIV-1 wild-type or tat mutant viral supernatants that contained reverse transcriptase activity equal to 6 ng (5 U/μl) of recombinant reverse transcriptase. The cells were washed as previously described and cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 1% glutamine (Gibco-BRL), 1× glucose, 1 U/ml of interleukin-2 (BTI, Inc.). The cells were sampled every 3 days, and either split 1:2 or supplemented with culture media as needed. Approximately 10^5 HeLa/CD4 and HeLa/CD4/Tat cells were incubated with DNase I-treated viral supernatants produced from 293 cells expressing either wild-type HIV-1, Δtat or ΔΔtat complemented with wild-type bat tat viral-containing activity equal to 15 ng of reverse transcription (5 μg/μl) or heat-inactivated viruses for 2 h at 37°C with constant shaking. Cells were washed three times with media. At 24 h post-infection, cells were harvested and Hirt lysates were prepared. The DNA fragments were ligated into TA vector (Invitrogen) and analyzed by DNA sequencing.

For each experiment, an internal control, and PCR analysis was performed to analyze HIV-1 reverse transcription products.

Quantitative RT-PCR was performed using HIV-1 virion-associated RNA isolated from pelleted virus particles. DNase I-treated viral supernatants were used for either the wild-type or Δmutant virus particles were used to centrifugation at 22 000 g for 90 min and resuspended in 1× PBS/BSA. The viral suspensions were assayed for p24 antigen and reverse transcriptase activity. Exactly 100 ng of p24 antigen were suspended in 1× PBS/BSA (Gibco-BRL) according to the in vitro reverse transcription conditions. During the purification, an in vitro synthesized HIV-1 RNA (I.C. RNA) was added to monitor RNA recovery and in vitro reverse transcription efficiency (Harrich et al., 1996). Nucleic acids were precipitated overnight (−20°C) and recovered by centrifugation at 15 000 g at 2°C for 60 min. A visible pellet was washed with 70% ethanol, centrifuged as before and the pellet was resuspended in 30 μl of PCR TE (10 mM Tris pH 7.8, 0.1 mM EDTA). Duplicate reactions that contained 5 μl of each viral RNA, 50 ng of the oligonucleotide primers (+242+219) 5′-CTCGTGCCGAGAGCTCC-TCTGG-3′, antisense) and 5 μl of dimethylsulfoxide were incubated at 65°C for 10 min. In vitro reverse transcription reactions were performed in the presence and absence of M-MLV reverse transcriptase (Gibco-BRL) with buffers.

References


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References


HIV-1 reverse transcription requires Tat


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