

Tat is required for efficient HIV-1 reverse transcription

David Harrich, Catherine Ulich,
León F.García-Martínez and
Richard B.Gaynor¹

Division of Molecular Virology, Departments of Internal Medicine and Microbiology, University of Texas Southwestern Medical Center, Dallas, TX 75235-8594, USA

¹Corresponding author

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 tRNA₃^{Lys} 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 Mizutani, 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 tRNA₃^{Lys} 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 tRNA₃^{Lys} (Barat *et al.*, 1993) can induce conformational changes in the heterodimeric reverse transcriptase (Robert *et al.*, 1990; Zakharova *et al.*, 1995). The combination of reverse transcriptase and tRNA₃^{Lys} 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 tRNA₃^{Lys} 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 Berkhout, 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; Berkhout *et al.*, 1989; Laspia *et al.*, 1989, 1990; Feinberg *et al.*, 1991; Marciniak 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; Laspia *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; Kliever *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 Δ *tat* 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, Δ *tat* or Δ *tat* 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 Δ *tat* 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 Δ *tat* 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

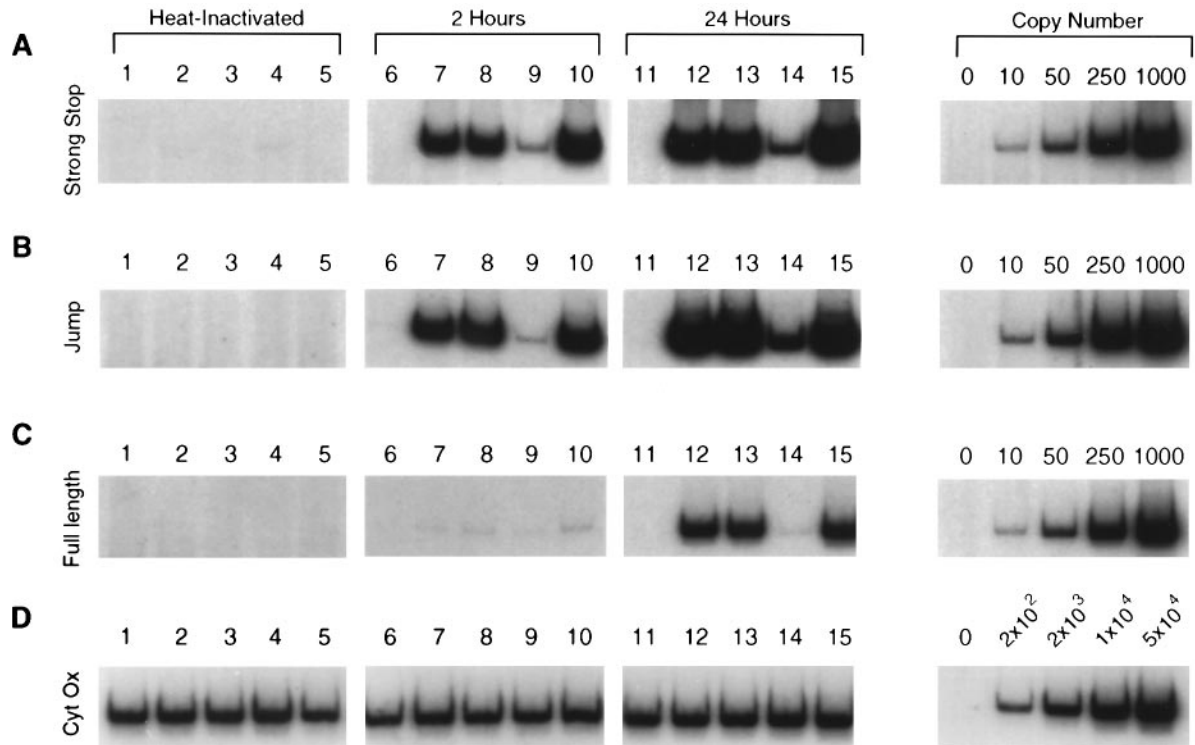


Fig. 1. HIV-1 virions lacking *tat* are defective in reverse transcription. Activated PBMCs were infected for 2 h with culture supernatant from 293 cells containing 6 ng of reverse transcriptase activity (5 U/ μ g) for either wild-type HIV-1 (A–D, lanes 7 and 12), wild-type HIV-1 produced from 293 cells following transfection with an RSV expression vector containing the 72 amino acid *tat* gene (A–D, lanes 8 and 13), Δ *tat* virus (A–D, lanes 9 and 14), Δ *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, Δ *tat* virus or Δ *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 (–) strong stop (A, lanes 1–15), the first strand jump (B, lanes 1–15) and full-length DNA (C, lanes 1–15). Quantitative PCR analysis of *cyt-ox 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, 2×10^2 , 2×10^3 , 1×10^4 and 5×10^4). 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 Δ *tat* virus produced following transfection with wild-type *tat* by analyzing jump DNA at 2 (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 Δ *tat* virus reverse transcription relative to wild-type HIV-1 was analyzed by Phosphorimager quantitation at 24 h post-infection and indicated that the Δ *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 Δ *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-ox 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, Δ *tat* and

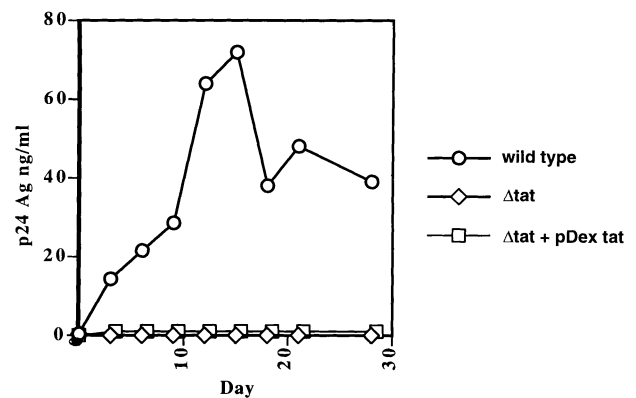


Fig. 2. Replication of HIV-1 *tat* mutant viruses in PBMCs. For each infection, 2×10^6 activated PBMCs were infected with filtered culture supernatant produced from 293 cells containing ~ 6 ng of reverse transcriptase activity for either wild-type HIV-1, Δ *tat* virus or Δ *tat* virus produced following transfection with a wild-type *tat* expression vector. The infected PBMCs were sampled every 3 days and assayed for p24 antigen by ELISA.

Δ *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

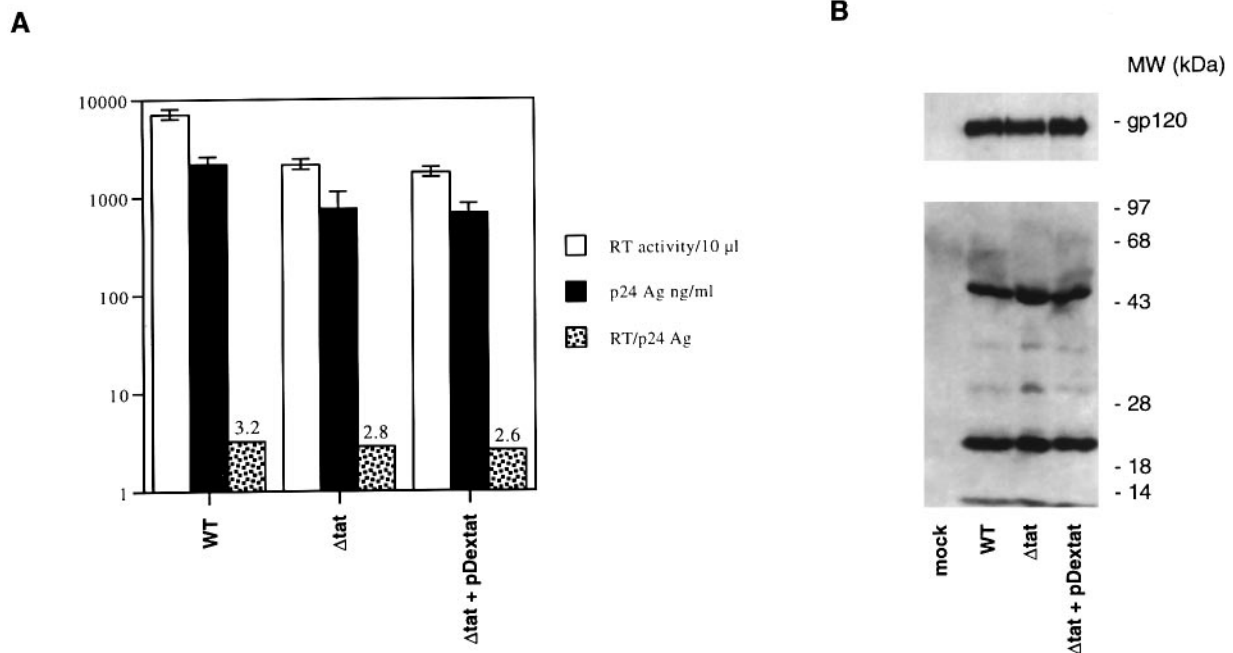


Fig. 3. Biochemical analysis of HIV-1 *tat* mutant viruses in PBMCs. (A) Filtered culture supernatants containing ~300 ng of HIV-1 p24 antigen were subjected to centrifugation and the pelleted virus was assayed for reverse transcriptase activity and p24 antigen content as described in Materials and methods. 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, Δ tat virus or Δ tat 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 (IIIB) (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 Δ tat virus produced in 293 cells resulted in the generation of virus that was able to replicate efficiently in PBMCs. Both Δ tat virus and Δ tat 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 Δ tat 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 Δ tat virus with wild-type *tat* did not result in the generation of wild-type HIV-1 recombinants.

HIV-1 wild-type and Δ tat virions have similar biochemical properties

It was important to characterize the biochemical properties of wild-type HIV-1 and Δ tat 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

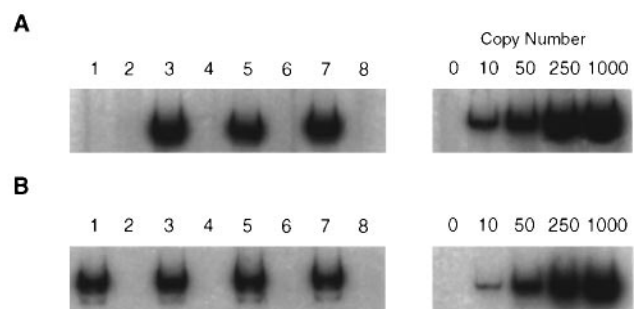


Fig. 4. Analysis of HIV-1 intravirion RNA in wild-type and *tat* mutant viruses. RNA was extracted from pelleted virus that contained 100 ng of p24 antigen and cDNA was synthesized in the presence (A and B, lanes 1, 3, 5 and 7) or absence (A and B, lanes 2, 4, 6 and 8) of M-MLV reverse transcriptase primed with an antisense oligonucleotide that hybridized to HIV-1 sequences extending from +242 to +219. (A) HIV-1 cDNA was detected by PCR with a nested antisense primer corresponding to HIV-1 sequences +236 to +214 and a sense primer corresponding to +96 to +118 for mock (lanes 1 and 2), HIV-1 wild-type (lanes 2 and 3), Δ tat virus (lanes 4 and 5) or Δ tat virus produced following transfection with a wild-type *tat* expression vector (lanes 7 and 8). (B) A synthetic RNA included in each sample during the RNA isolation and the M-MLV reverse transcription reaction was detected by PCR using a nested antisense primer made to HIV-1 sequences +62 to +42 and an oligonucleotide made to polylinker sequences present in the synthetic RNA (lanes 1–8).

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

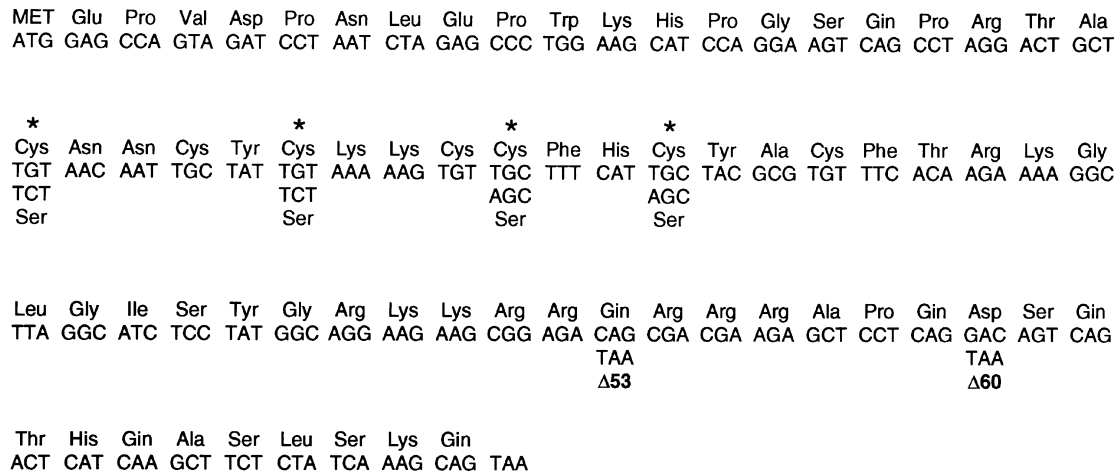


Fig. 5. Structure of the first coding exon of the *tat* gene. The sequence of the first 72 amino acids of the HIV-1 SF2 *tat* gene is shown. The position of the four cysteine residues that were mutated in the *cys* construct are indicated as are the positions of the truncations of the *tat* gene at amino acid position 53 ($\Delta 53$) and 60 ($\Delta 60$).

proteins were similar for wild-type HIV-1, Δ *tat* virus and Δ *tat* 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 Δ *tat* 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), Δ *tat* virus (Figure 4A, lanes 5 and 6) and Δ *tat* 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 Δ *tat* 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 Δ *tat* virus. The 293 cells containing the Δ *tat* 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 ($\Delta 53$) or a *tat* deletion mutant that contained the first 60 amino acids of *tat* ($\Delta 60$) (Garcia *et al.*, 1988). The *tat/cys* and $\Delta 53$ *tat* mutants are very defective for *tat* activation of HIV-1 gene expression while the $\Delta 60$ *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

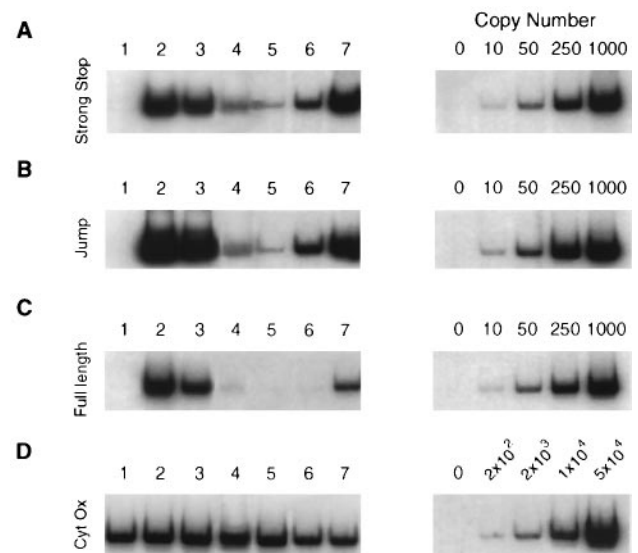


Fig. 6. Role of *tat* mutants on increasing Δ *tat* virus reverse transcription. Activated PBMCs were infected for 2 h with culture supernatant from 293 cells including mock supernatant (A–D, lane 1) or 6 ng of reverse transcriptase activity (5 U/ μ g) for either wild-type HIV-1 (A–D, lane 2), wild-type HIV-1 transfected with a wild-type *tat* expression vector (A–D, lane 3), Δ *tat* virus (A–D, lane 4) or Δ *tat* virus transfected with expression vectors containing either a *tat* cysteine mutant (*tat/cys*) (A–D, lane 5), a *tat* mutant truncated at amino acid 53 ($\Delta 53$) (A–D, lane 6) or a *tat* mutant truncated at amino acid 60 ($\Delta 60$) (A–D, lane 7). Hirt lysates were prepared from HIV-1-infected PBMCs at 24 h post-infection and the recovered cytoplasmic nucleic acids were assayed for HIV-1 DNA using PCR primers to detect (A) (–) strong stop (B) the first strand jump or (C) full-length DNA. (D) Total cytoplasmic nucleic acids from each infection were normalized to *cyt-ox II* content by PCR. 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, 2×10^2 , 2×10^3 , 1×10^4 and 5×10^4). Infections were performed using three independent stocks of each virus followed by PCR analysis.

Δ *tat* 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 Δ *tat* virions produced following transfection of

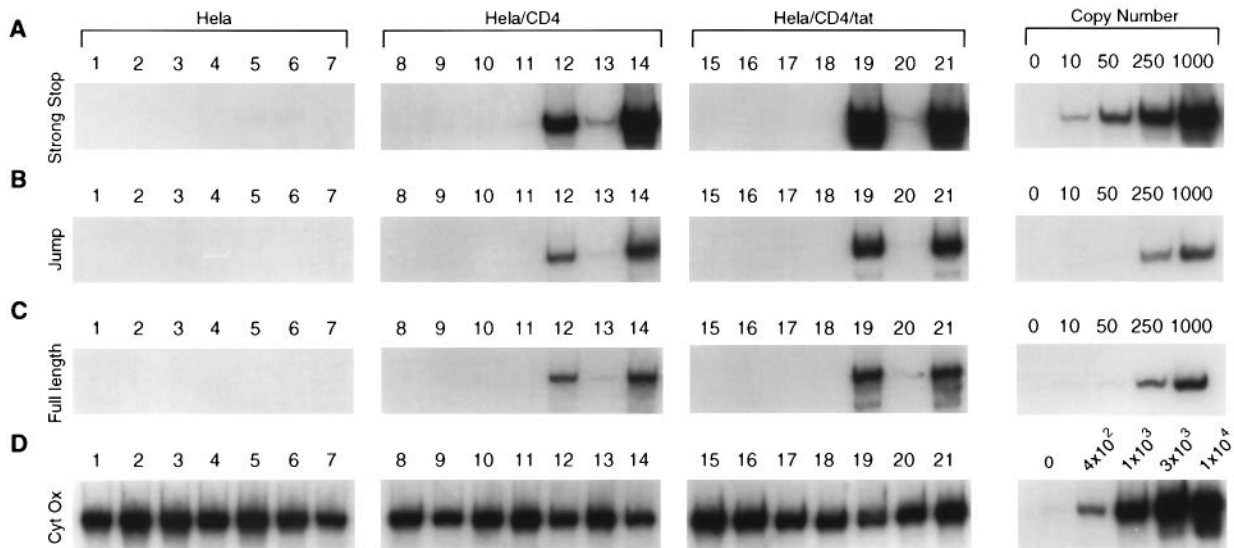


Fig. 7. Tat expressed in target cells does not increase Δ tat virus reverse transcription. Equivalent cell numbers of either HeLa, HeLa/CD4 or HeLa/CD4/tat were infected for 2 h with either mock supernatant (A–D, lanes 1, 8 and 15) or 15 ng of reverse transcriptase (5 U/ μ g) activity obtained from 293 cells for either wild-type HIV-1 (A–D, lanes 5, 12 and 19), HIV-1 Δ tat virus (A–D, lanes 6, 13 and 20) or HIV-1 Δ tat 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 Δ tat virus (A–D, lanes 3, 10 and 17) or Δ tat 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, 4×10^2 , 1×10^3 , 3×10^3 and 1×10^4).

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, Δ tat 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, Δ 53, when transfected into 293 cells containing the Δ tat 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 Δ 60 restored the ability of the Δ tat 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 Δ tat virus complemented with *tat/cys*; 5-, 6- and 50-fold for Δ tat virus complemented with Δ 53 and of 2-fold or less for Δ tat virus complemented with Δ 60. 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 Δ tat virus was dependent on a functional *tat* gene.

Tat expression in target cells does not increase the reverse transcription of Δ tat virions

A wild-type *tat* expression vector when transfected into 293 cells producing the Δ tat 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 Δ tat 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, Δ tat virus or Δ tat 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, Δ tat virus or the Δ tat virus complemented with wild-type *tat* were used to infect HeLa CD4 cells (Figures 7A–C, lanes 12–14). The Δ tat 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 Δ tat 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

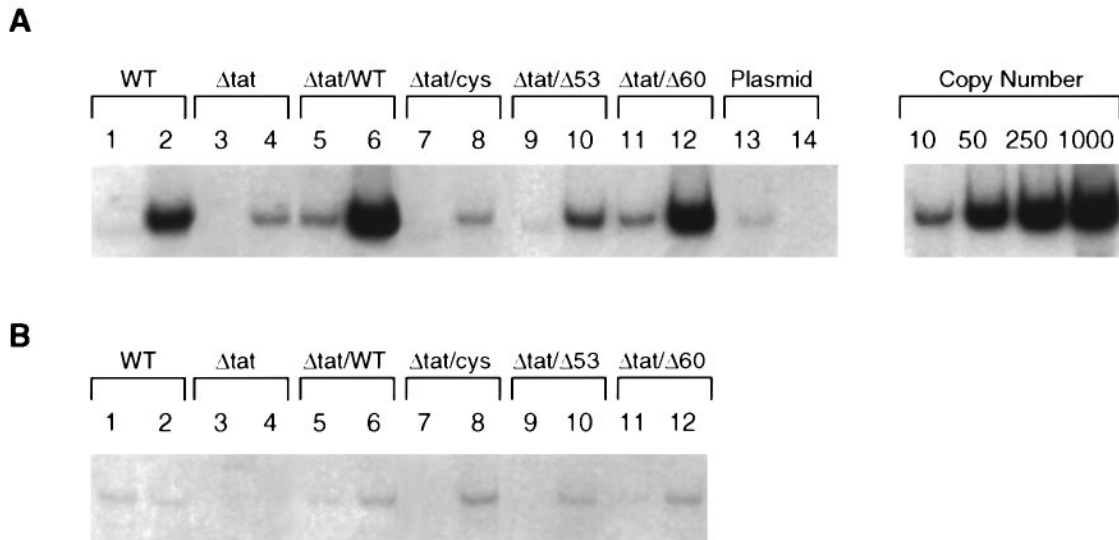


Fig. 8. Endogenous reverse transcription assays of HIV-1 wild-type and *tat* mutants. **(A)** and **(B)** Either wild-type HIV-1 (lanes 1 and 2), Δ tat virus (lanes 3 and 4) or Δ tat virus produced following transfection with either wild-type *tat* designated Δ tat/WT (lanes 5 and 6) or the *tat* mutants *tat/cys* designated Δ tat/cys (lanes 7 and 8), Δ 53 designated Δ tat/ Δ 53 (lanes 9 and 10) or Δ 60 designated Δ tat/ Δ 60 (lanes 11 and 12) were assayed in triplicate for reverse transcriptase activity. Culture supernatants (~150 μ l) 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 μ l 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 μ M dNTPs (A, lanes 2, 4, 6, 8, 10 and 12) or 12 μ M 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 μ l 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 HIV-1 (–) strong stop DNA as described in Materials and methods. All PCR reactions were performed within the linear range of the assay.

viruses produced in 293 cells was used to infect HeLa CD4 cells containing the *tat* gene, the Δ tat 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 Δ tat 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-ox*y 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 Δ tat 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, Δ tat virus or Δ tat 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 μ M

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 μ g/ml herring sperm DNA and 50 μ g/ml proteinase 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), Δ tat virus produced following transfection of 293 cells with wild-type *tat* (Figure 8A, lane 6) and Δ tat virus produced following transfection with the Δ 60 *tat* mutant (Figure 8A, lane 12). In contrast, there was a marked decrease in the amount of (–) strong stop DNA synthesized with the Δ tat virus (Figure 8A, lane 4) or the Δ tat virus produced following transfection of 293 cells with either the *tat/cys* mutant (Figure 8A, lane 8) or the *tat* mutant Δ 53 (Figure 8A, lane 10). Phosphorimager quantitation of the PCR assays indicated that Δ tat virus exhibited a 10-fold defect relative to wild-type HIV-1. The Δ tat virus produced following transfection of 293 cells with wild-type *tat* was 2-fold higher than wild-type virus, while the Δ tat virus produced following transfection of 293 cells with *tat/cys* exhibited a 16-fold defect, the Δ tat virus produced following transfection with Δ 53 *tat* exhibited a 5-fold defect and the Δ tat virus produced following transfection with Δ 60 *tat* exhibited no defect. DNase I treatment was sufficient to remove potential contaminating chromosomal DNA or cell nuclei as indicated by the near complete

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 (Kliwer *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 p24 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 tRNA₃^{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 tRNA₃^{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 tRNA₃^{Lys} 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 (–) 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; Weeks 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 tRNA₃^{Lys} 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 mechanism 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 pBH10 (Hahn *et al.*, 1984). The *tat* deletion construct pBRDH2puro- Δ *tat* was derived from pBRDH2puro that was linearized with the restriction enzyme *Ban*II 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 *tat* but 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 (LEASRKSA) 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 (Δ 60), 53 amino acids (Δ 53) or serine substitutions of cysteine at residues 22, 27, 31 and 34 (*tat/cys*) were placed downstream of the RSV promoter in a vector containing the SV40 splice acceptor and polyadenylation sequences (Garcia *et al.*, 1988).

Cells and cell lines

A 100 mm plate of 50% confluent 293 cells (Graham *et al.*, 1977) was transfected with 20 μ g of *Mro*I-linearized pBRDH2puro- Δ *tat* (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 μ g/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 ARR #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 μ g/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 Δ *tat* 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 5250 cells with either wild-type or mutant *tat* genes, 100 mm plates of 40% confluency were transfected using lipofectamine with 5 μ g of expression vectors for RSV-*tat* wild-type, RSV-*tat* Δ 60, RSV-*tat* Δ 53 or RSV-*tat* Δ /*cys* 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 μ m 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)₁₅ and digoxigenin–biotin-labeled dUTP, which was detected by an ELISA protocol (Boehringer Mannheim) when 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/ μ l in 4 \times 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 1:10 000 human anti-HIV-1 IgG

(NIH ARR #192) or 1:1000 dilution of IgG-purified rabbit anti-gp120 (Intracel). Secondary antibodies were horseradish peroxidase (HRP)-coupled sheep anti-human at 1:5000 or HRP-coupled goat anti-rabbit at 1:3000. Visualization of proteins was carried out using enhanced chemiluminescence (Amersham).

HIV-1 infection

The HIV-1 wild-type and *tat* mutants were normalized for reverse transcriptase activity. Typically, HIV-1 supernatant containing reverse transcriptase activity equal to 6 ng (5 U/ μ g) of recombinant reverse transcriptase was supplemented with 10 mM MgCl₂ and 300 U of DNase I (Worthington Biochemical, New Jersey) and then incubated at 30°C for 30 min. DNase I-treated viral supernatant was heat inactivated at 60°C for 20 min for use in control infections. For each infection, 2 \times 10⁷ activated PBMCs were infected with the appropriate DNase I-treated virus stock 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 \times 10⁷ 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 \times 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 suspension was vortexed gently and lysis solution was added (10 mM Tris pH 7.0, 10 mM EDTA, 1.2% SDS and 2 mg/ml proteinase K), then incubated at 37°C for 2 h. Exactly 0.25 ml of 5.0 M NaCl was added to each lysate and left on ice overnight. Each lysate was subjected to centrifugation at 15 000 g for 1 h. The supernatant was decanted and phenol:chloroform:isoamyl alcohol (25:24:1) solution was added, vigorously vortexed, and centrifuged at 15 000 g for 15 min. The aqueous layer was transferred to a fresh tube and 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 at 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 50.0 ml of PCR quality TE (10 mM Tris pH 7.8, 0.1 mM EDTA). The remaining cells were cultured for an additional 22 h then processed by modified Hirt lysis.

To determine viral replication kinetics, 2 \times 10⁶ 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/ μ g) 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) and 30 U 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 10⁷ HeLa, 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, Δ tat or Δ tat complemented with wild-type *tat* viral-containing activity equal to 15 ng of reverse transcriptase (5 U/ μ g) 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 amounts were normalized using PCR with the *cyt-ox*y 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, Δ tat or Δ tat complemented with either wild-type *tat* or the *tat* mutants Δ cys, Δ 53 and Δ 60 were assayed for reverse transcriptase activity (Boehringer Mannheim). For each endogenous reverse transcription reaction, 150 μ l of viral supernatants containing reverse transcriptase activity equivalent to 20 pg of recombinant reverse transcriptase were supplemented with 10 mM MgCl₂ and 100 U of DNase I (Worthington Biochemical Corp.) then incubated at 30°C for 20 min. As a control for DNase I activity, 10⁶ copies of an HIV-1 LTR plasmid was added to the viral supernatant. Next, 50 μ l of the treated supernatants were added to 200 μ l of stop solution (10 mM Tris pH 8.0, 10 mM EDTA, 20 μ g/ml sheared herring sperm DNA, 50 μ g/ml proteinase K), then incubated at 50 and 95°C for 10 min at each temperature. The remaining supernatants were supplemented with either 12 μ M dNTP (3 μ M each dATP, dCTP, dGTP and dTTP) or 12 μ M dNTPs minus dTTP and incubated at 37°C for 60 min. The reactions was terminated with 400 μ l of stop solution then incubated at 50 and 95°C for 10 min at each temperature. Assays

of 10 μ l of each of the endogenous reverse transcription reactions were performed for (–) strong 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 (Hirt, 1967; Harrich *et al.*, 1996). The 5' and 3' LTRs and the *tat* gene from the 293 cell lines stably expressing the Δ tat mutant virus were obtained by PCR. Optimized PCR reagents were obtained from Invitrogen and *Taq* DNA polymerase was purchased from Boehringer Mannheim. Exactly 200 ng of purified chromosomal DNA and 50 ng of the oligonucleotide pairs (–436/–415) (5'-CCCAACAAGACAAGAGAT-TGA -3', sense) and (+242/+219) (5'-CCTGCGTCGAGAGAGCTCC-TCTGG-3', antisense) (5' LTR); (+8605/+8625) (5'-GCAGCTTTA-GATATTAGCCAC-3', sense) and (+9282/+9258) (5'-CTGCTAGA-GATTTTCCCACTGAC-3', antisense) (3' LTR); (+5417/+5446) (5'-GCCATAATAAGAATTCTGCAACAACCTGCTG-3', sense) and (+5653/+5634) (5'-GGTTGCATTACATGTACTAC-3', antisense) (*tat* gene) were subjected to 40 cycles of PCR at 95, 55 and 72°C for 1 min at each temperature using 1.25 U of *Taq* DNA polymerase (Zack *et al.*, 1990). The expected 678, 677 and 237 bp DNA products, respectively for the 5' LTR, 3' LTR and the *tat* gene were resolved and purified from a 6% polyacrylamide gel. The DNA fragments were ligated into TA vector (Invitrogen) and analyzed by DNA sequencing.

Nucleic acids concentrations of Hirt lysates were normalized by ‘hot’ PCR amplification targeted to the mitochondrial DNA-encoded *cyt-ox*y II gene. Hirt lysates were serially diluted in 5-fold increments and 5 μ l of each dilution was assayed by ‘hot’ PCR. *Taq* DNA polymerase was first inactivated with Taqstart Ab (Clontech) for 5 min at room temperature, then added to a master PCR mix that contained 1 \times optimized buffer D (Invitrogen), 0.1 mM dNTPs, 50 ng of an unlabeled oligonucleotide (5'-CACATGCAGCGCAAGTAGGT-3', sense) and 25 ng of a ³²P end-labeled oligonucleotide (>5 \times 10⁸ c.p.m./ μ g) (5'-GGAAATGATTATG-AGGGCGTG-3', antisense) which hybridized to the human *cyt-ox*y II gene. After a 95°C denaturation for 5 min, 20–23 cycles of amplification were performed at 93°C for 1 min and 65°C for 2 min. The PCR products were separated by electrophoresis on a 6% polyacrylamide gel. The dried gels were quantified on a Molecular Dynamics PhosphorImager and visualized by autoradiography. Generally, the PCR of the *cyt-ox*y II mitochondrial gene produced linear amplification in the 10²–10⁴ dilution of the original Hirt lysates. The concentrations of the Hirt lysate nucleic acids were normalized for *cyt-ox*y II content.

Exactly 5 μ l of normalized Hirt lysate or 10 μ l of endogenous reverse transcription reactions were assayed directly for HIV-1 DNA by 30–35 cycles of ‘hot’ PCR as previously described. Oligonucleotides were used to detect SF2 LTR DNA that amplified (i) an 87 bp DNA fragment that contained R \geq U5 sequence between +96 and +118 (5'-CAAGTAGTGTGTGCGCTCTGTT-3', sense) and +182 and +158 (5'-CTGCTAGAGATTTTCCCACTGAC-3', antisense); (ii) a 111 bp DNA fragment that contained R \geq U3 sequences between –49 and –30 (5'-TGGCTGCCCTCAGATGCTG-3', sense) and between +62 and +42 (5'-AAGCAGTGGGTCCCTAGTTAG-3', antisense) and (iii) a 147 bp DNA fragment that contained the R \geq 5'-untranslated *gag* sequences between +96 and +118 (5'-CAAGTAGTGTG-TGCCCGT-CTGT-3', sense) and between +242 and +219 (5'-CCTGCGTCGAG-AGAGCTCCTCTGG-3', antisense). All PCR reactions were subject to electrophoresis in polyacrylamide gels as described.

Quantitative RT–PCR was performed using HIV-1 virion-associated RNA isolated from pelleted virus particles. DNase I-treated virus stocks for either the wild-type or Δ tat mutant viruses were subjected to centrifugation at 22 000 g for 90 min and resuspended in 1 \times PBS/BSA. The viral suspensions were assayed for p24 antigen and reverse transcriptase activity. Exactly 100 ng of p24 antigen were suspended in Trizol reagent (Gibco-BRL) according to the manufacturer's recommendations. 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 μ l of PCR TE (10 mM Tris pH 7.8, 0.1 mM EDTA). Duplicate reactions that contained 5 μ l of each viral RNA, 50 ng of the oligonucleotide primer (+242/+219) (5'-CCTGCGTCGAGAGAGCTCCTCTGG-3', antisense) and 5 ml 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

provided by the manufacturer plus 0.1 mM dNTPs at 37°C for 30 min. First, each reverse transcription reaction was serially diluted in 5-fold increments and assayed for the internal control RNA (I.C. RNA) by 'hot' PCR for 30 cycles as previously described, using oligonucleotide primers made to the polylinker region of the I.C. RNA (5'-CTTGCATGCCTGCAGGTCGACT-3', forward) and HIV-1 sequences from +62 to +42 (5'-AAGCAGTGGGTTCCCTAGTTAG-3', antisense) (Harrich et al., 1990). The reverse transcription reactions were normalized to I.C. RNA concentrations and amplified by 'hot' PCR for 30 cycles as described using the HIV-1-specific oligonucleotide primers (+96/+118) (5'-CAAGTAGTGTGTGCCGTCGTGTT-3', sense) and a primer nested to the cDNA first strand primer (+236/+214) (5'-CGAGAGAGCTCTCTGGTTCTAC-3'-antisense). The PCR products were separated on a 6% polyacrylamide gel system in 1× TBE, dried and quantified on a Molecular Dynamics PhosphorImager.

Acknowledgements

We thank Sharon Johnson for the preparation of this manuscript, Anthony Cancel for preparation of the figures and Anne Snider-Mette for expert technical assistance. This work was supported by grants from the NIH and Veterans Administration.

References

- Aboul-ela,F., Karn,J. and Varani,G. (1995) The structure of the human immunodeficiency virus type-1 TAR RNA reveals principles of RNA recognition by Tat protein. *J. Mol. Biol.*, **253**, 313–332.
- Aiyar,A., Cobrinik,D., Ge,Z., Kung,H.J. and Leis,J. (1992) Interaction between retroviral U5 RNA and the psi C loop of the tRNA (Trp) primer is required for efficient initiation of reverse transcription. *J. Virol.*, **66**, 2464–2472.
- Aiyar,A., Ge,Z. and Leis,J. (1994) A specific orientation of RNA secondary structures is required for initiation of reverse transcription. *J. Virol.*, **68**, 611–618.
- Allain,B., Lapadat-Tapolsky,M., Berlioz,C. and Darlix,J.-L. (1994) Transactivation of the minus-strand DNA transfer by nucleocapsid protein during reverse transcription of the retroviral genome. *EMBO J.*, **13**, 973–981.
- Arts,E.J., Li,X., Gu,Z., Kleiman,L., Parniak,M.A. and Wainberg,M.A. (1994) Comparison of deoxyoligonucleotide and tRNA (Lys-3) as primers in an endogenous human immunodeficiency virus type-1 *in vitro* reverse transcription/template-switching reaction. *J. Biol. Chem.*, **269**, 14672–14680.
- Arts,E.J., Ghosh,M., Jacques,P.S., Ehresmann,B. and Le Grice,S.F.J. (1996a) Restoration of tRNA^{Lys}-primed (–)-strand DNA synthesis to an HIV-1 reverse transcriptase mutant with extended tRNAs. *J. Biol. Chem.*, **271**, 9054–9061.
- Arts,E.J. et al. (1996b) Initiation of (–) strand DNA synthesis from tRNA^{Lys} on lentiviral RNAs: implications of specific HIV-1 RNA–tRNA^{Lys} interactions inhibiting primer utilization by retroviral reverse transcriptases. *Proc. Natl Acad. Sci. USA*, **93**, 10063–10068.
- Baltimore,D. (1970) RNA-dependent DNA polymerization in virions of RNA tumor viruses. *Nature*, **226**, 1209–1211.
- Barat,C., Schatz,O., Le Grice,S. and Darlix,J.L. (1993) Analysis of the interactions of HIV-1 replication primer tRNA(Lys3) with nucleocapsid protein and reverse transcriptase. *J. Mol. Biol.*, **231**, 185–190.
- Berkhout,B., Silverman,R.H. and Jeang,K.T. (1989) Tat trans-activates the human immunodeficiency virus through a nascent RNA target. *Cell*, **59**, 273–282.
- Braaten,D., Franke,E.K. and Luban,J. (1996) Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 before the initiation of reverse transcription. *J. Virol.*, **70**, 3551–3560.
- Calnan,B.J., Tidor,B., Biancalana,S., Hudson,D. and Frankel,A.D. (1991) Arginine-mediated RNA recognition: the arginine fork. *Science*, **252**, 1167–1171.
- Cobrinik,D., Soskey,L. and Leis,J. (1988) A retroviral RNA secondary structure required for efficient initiation of reverse transcription. *J. Virol.*, **62**, 3622–3630.
- Cobrinik,D., Aiyar,A., Ge,Z., Katzman,M., Huang,H. and Leis,J. (1991) Overlapping retrovirus U5 sequence elements are required for efficient integration and initiation of reverse transcription. *J. Virol.*, **65**, 3864–3872.
- Das,A.T., Klaver,B. and Berkhout,B. (1995) Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNA^{Lys3}. *J. Virol.*, **69**, 3090–3097.
- Dayton,A.I., Sodroski,J.G., Rosen,C.A., Goh,W.C. and Haseltine,W.A. (1986) The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell*, **44**, 941–947.
- Dingwall,C., Ernberg,I., Gait,M.J., Green,S.M., Heaphy,S., Karn,J., Lowe,A.D., Singh,M. and Skinner,M.A. (1990) HIV-1 Tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. *EMBO J.*, **9**, 4145–4153.
- Feinberg,M.B., Baltimore,D. and Frankel,A.D. (1991) The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc. Natl Acad. Sci. USA*, **88**, 4045–4049.
- Feng,S. and Holland,E.C. (1988) HIV-1 Tat *trans*-activation requires the loop sequence within TAR. *Nature*, **334**, 165–167.
- Fisher,A.G. et al. (1986) The trans-activator gene of HTLV-III is essential for virus replication. *Nature*, **320**, 367–371.
- Franke,E.K., Yuan,H.E.H. and Luban,J. (1994) Specific incorporation of cyclophilin A into HIV-1 virions. *Nature*, **372**, 359–362.
- Garcia,J.A., Harrich,D., Pearson,L., Mitsuyasu,R. and Gaynor,R.B. (1988) Functional domains required for *tat*-induced transcriptional activation of the HIV-1 long terminal repeat. *EMBO J.*, **7**, 3143–3147.
- Garcia,J.A., Harrich,D., Soutanakis,E., Wu,F., Mitsuyasu,R. and Gaynor,R.B. (1989) Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation. *EMBO J.*, **8**, 765–778.
- Goff,S.P. (1990) Retroviral reverse transcriptase: synthesis, structure, and function. *J. AIDS*, **3**, 817–831.
- Graham,F.L., Smiley,J., Russell,W.C. and Nairn,R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.*, **36**, 59–72.
- Hahn,B.H., Shaw,G.M., Arya,S.K., Popovic,M., Gallo,R.C. and Wong,S.F. (1984) Molecular cloning and characterization of the HTLV-III virus associated with AIDS. *Nature*, **312**, 166–169.
- Harrich,D., Hsu,C., Race,E. and Gaynor,R.B. (1994) Differential growth kinetics are exhibited by human immunodeficiency virus type 1 TAR mutants. *J. Virol.*, **68**, 5899–5910.
- Harrich,D., Mavankal,G., Mette-Snider,A. and Gaynor,R.B. (1995) Human immunodeficiency virus type 1 TAR element revertant viruses define RNA structures required for efficient viral gene expression and replication. *J. Virol.*, **69**, 4906–4913.
- Harrich,D., Ulich,C. and Gaynor,R.B. (1996) A critical role for the TAR element in promoting efficient human immunodeficiency virus type 1 reverse transcription. *J. Virol.*, **70**, 4017–4027.
- Hirt,B. (1967) Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.*, **26**, 365–369.
- Hu,W.S. and Temin,H.M. (1990a) Retroviral recombination and reverse transcription. *Science*, **250**, 1227–1233.
- Hu,W.S. and Temin,H.M. (1990b) Genetic consequence of packaging two RNA genomes in one retroviral particle: pseudodiploidy and high rate of genomic recombination. *Proc. Natl Acad. Sci. USA*, **87**, 1556–1560.
- Hu,Y.F., Luscher,B., Admon,A., Mermod,N. and Tjian,R. (1990) Transcription factor AP-4 contains multiple dimerization domains that regulate dimer specificity. *Genes Dev.*, **4**, 1741–1752.
- Huang,L., Joshi,A., Willey,R., Orenstein,J. and Jeang,K.T. (1994) Human immunodeficiency viruses regulated by alternative *trans*-activators: genetic evidence for a novel non-transcriptional function of Tat in virion infectivity. *EMBO J.*, **13**, 2886–2896.
- Isel,C., Marquet,R., Keith,G., Ehresmann,C. and Ehresmann,B. (1993) Modified nucleotides of tRNA^{Lys} modulate primer/template loop–loop interaction in the initiation complex of HIV-1 reverse transcriptase. *J. Biol. Chem.*, **268**, 25269–25272.
- Isel,C., Ehresmann,C., Keith,G., Ehresmann,B. and Marquet,R. (1995) Initiation of reverse transcription of HIV-1: secondary structure of the HIV-1 RNA/tRNA(Lys3) (template/primer) complex. *J. Mol. Biol.*, **247**, 236–250.
- Isel,C., Lanchy,J.-M., Grice,S.F.J.L., Ehresmann,B. and Marquet,R. (1996) Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcription require the post-transcriptional modifications of primer tRNA^{Lys}. *EMBO J.*, **15**, 917–924.
- Jardine,D., Tachedjian,G., Locarni,S. and Birch,C. (1993) Cellular topoisomerase I activity associated with HIV-1. *AIDS Res. Hum. Retroviruses*, **9**, 1245–1250.

- Kao,S.Y., Calman,A.F., Luciw,P.A. and Peterlin,B.M. (1987) Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature*, **330**, 489–493.
- Kato,H., Sumimoto,H., Pognonec,P., Chen,C.H., Rosen,C.A. and Roeder,R.G. (1992) HIV-1 Tat acts as a processivity factor *in vitro* in conjunction with cellular elongation factors. *Genes Dev.*, **6**, 655–666.
- Keen,N.J., Gait,M.J. and Karn,J. (1996) Human immunodeficiency virus type-1 Tat is an integral component of the activated transcription-elongation complex. *Proc. Natl Acad. Sci. USA*, **93**, 2505–2510.
- Klaver,B. and Berkhout,B. (1994) Evolution of a disrupted TAR RNA hairpin structure in the HIV-1 virus. *EMBO J.*, **13**, 2650–2659.
- Kliwer,S., Garcia,J., Pearson,L., Soultanakis,E., Dasgupta,A. and Gaynor,R. (1989) Multiple transcriptional regulatory domains in the human immunodeficiency virus type 1 long terminal repeat are involved in basal and E1A/E1B-induced promoter activity. *J. Virol.*, **63**, 4616–4625.
- Lapadat-Topolsky,M., De Rocoquigny,H., Van Gent,D., Roques,B., Plasterk,R. and Darlix,J.L. (1993) Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle. *Nucleic Acids Res.*, **21**, 831–839.
- Lapadat-Topolsky,M., Pernelle,C., Borie,C. and Darlix,J.-L. (1995) Analysis of the nucleic acid annealing activities of nucleocapsid protein from HIV-1. *Nucleic Acids Res.*, **23**, 2434–2441.
- Laschia,M.F., Rice,A.P. and Mathews,M.B. (1989) HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell*, **59**, 283–292.
- Laschia,M., Rice,A. and Mathews,M.B. (1990) Synergy between HIV-1 Tat and adenovirus E1A is principally due to stabilization of transcriptional elongation. *Genes Dev.*, **4**, 2397–2408.
- Li,X., Mak,J., Arts,E.J., Gu,Z., Kleiman,L., Wainberg,M.A. and Parniak,M.A. (1994) Effects of alterations of the primer-binding site sequences on human immunodeficiency virus type 1 replication. *J. Virol.*, **68**, 6198–6206.
- Maddon,P.J., Dalgleish,A.G., McDougal,J.S., Clapham,P.R., Weiss,R.A. and Axel,R. (1986) The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell*, **47**, 333–348.
- Marciniak,R.A. and Sharp,P.A. (1991) HIV-1 Tat protein promotes formation of more-processive elongation complexes. *EMBO J.*, **10**, 4189–4196.
- Masuda,T., Planelles,V., Krogstad,P. and Chen,I.S.Y. (1995) Genetic analysis of human immunodeficiency virus type 1 integrase and the U3 *att* site: unusual phenotype of mutants in the zinc finger-like domain. *J. Virol.*, **69**, 6687–6696.
- Mavankal,G., Du,S.I., Oliver,N., Sigman,D. and Gaynor,R.B. (1996) HIV-1 and HIV-2 Tat proteins specifically interact with RNA polymerase II. *Proc. Natl Acad. Sci. USA*, **93**, 2089–2094.
- Morgenstern,J.P. and Land,H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.*, **18**, 3587–3596.
- Muesing,M.A., Smith,D.H. and Capon,D.J. (1987) Regulation of mRNA accumulation by a human immunodeficiency virus trans-activator protein. *Cell*, **48**, 691–701.
- O'Brian,W.A., Namazi,A., Hamidreza,K., Mao,S.H., Zack,J.A. and Chen,I.S.Y. (1994) Kinetics of human immunodeficiency virus type 1 reverse transcription in blood mononuclear phagocytes are slowed by limitations of nucleotide precursors. *J. Virol.*, **68**, 1258–1263.
- Panet,A.C., Haseltine,W.A., Baltimore,D., Peters,G., Harada,F. and Dahlberg,J. (1975) Specific binding of tRNA^{trp} to AMV reverse transcriptase. *Proc. Natl Acad. Sci. USA*, **72**, 2535–2539.
- Peliska,J.A., Balasubramanian,S., Giedroc,D.P. and Benovic,S.J. (1994) Recombinant HIV-1 nucleocapsid protein accelerates HIV-1 reverse transcriptase catalyzed DNA strand transfer reactions and modulates RNase H activity. *Biochemistry*, **33**, 13817–13823.
- Priel,E., Showalter,S.D., Roberts,M., Oroszlan,S., Segal,S., Aboud,M. and Blair,D.G. (1990) Topoisomerase I activity associated with human immunodeficiency virus (HIV) particles and equine infectious anemia virus core. *EMBO J.*, **9**, 4167–4172.
- Rhim,H., Park,J. and Morrow,C.D. (1991) Deletions in the tRNA(Lys) primer-binding site of the human immunodeficiency virus type 1 identify essential regions for reverse transcription. *J. Virol.*, **65**, 4555–4564.
- Robert,D., Sallafranque-Andreola,M.L., Bordier,B., Sarih-Cottin,L., Tarrago-Litvak,L., Graves,P.V., Barr,P.J., Fournier,M. and Litvak,S. (1990) Interactions with tRNA^{Lys} induce important structural changes in human immunodeficiency virus reverse transcriptase. *FEBS Lett.*, **277**, 239–242.
- Rosen,C.A., Sodoroski,J.G. and Haseltine,W.A. (1985) The location of *cis*-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. *Cell*, **41**, 813–823.
- Schwartz,O., Marechal,V., Danos,O. and Heard,J.M. (1995) Human immunodeficiency virus type 1 *nef* increases the efficiency of reverse transcription in the infected cell. *J. Virol.*, **69**, 4053–4059.
- Selby,M.J., Bain,E.S., Luciw,P.A. and Peterlin,B.M. (1989) Structure, sequence, and position of the stem-loop in TAR determine transcriptional elongation by Tat through the HIV-1 long terminal repeat. *Genes Dev.*, **3**, 547–558.
- Sherman,P.A. and Fyfe,J.A. (1989) Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. *Anal. Biochem.*, **180**, 222–226.
- Takahashi,H., Matsuda,M., Kojima,A., Sata,T., Andoh,T., Kurata,T., Nagashima,K. and Hall,W.W. (1995) Human immunodeficiency virus type 1 reverse transcriptase: enhancement of activity by interaction with cellular topoisomerase I. *Proc. Natl Acad. Sci. USA*, **92**, 5694–5698.
- Temin,H.M. and Mizutani,S. (1970) RNA-directed DNA polymerase in virions of Rous sarcoma virus. *Nature*, **226**, 1211–1213.
- Thali,M., Bukovsky,A.A., Kondo,E., Rosenwirth,B., Walsh,C.T., Sodoroski,J. and Gottlinger,H.G. (1994) Specific association of cyclophilin A with human immunodeficiency virus type 1 virions. *Nature*, **372**, 363–365.
- Varmus,H. and Brown,P. (1989). Retroviruses. In Berg,D.E. and Howe,M.M. (eds), *Mobile DNA*. American Society for Microbiology, Washington, DC, pp. 53–108.
- von Schwedler,U., Song,J., Aiken,C. and Trono,D. (1993) *Vif* is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *J. Virol.*, **67**, 5067–5074.
- Wakefield,J.K., Rhim,H. and Morrow,C.D. (1994) Minimal sequence requirements of a functional immunodeficiency virus type 1 primer binding site. *J. Virol.*, **68**, 1605–1614.
- Wakefield,J.K., Wolf,A.G. and Morrow,C.D. (1995) Human immunodeficiency virus type 1 can use different tRNA as primers for reverse transcription but selectively maintains a primer binding site complementary to tRNA(Lys3). *J. Virol.*, **69**, 6021–6029.
- Weeks,K.M. and Crothers,D.M. (1991) RNA recognition by Tat-derived peptides: interaction in the major groove? *Cell*, **66**, 577–588.
- Whitcomb,J.M. and Hughes,S.H. (1992) Retroviral reverse transcription and integration: progress and problems. *Annu. Rev. Cell Biol.*, **8**, 275–306.
- Zack,J.A., Arrigo,S.J., Weitsman,S.R., Go,A.S., Haislip,A.M. and Chen,I.S. (1990) HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell*, **61**, 213–222.
- Zack,J.A., Haislip,A.M., Krogstad,P. and Chen,I.S. (1992) Incomplete reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. *J. Virol.*, **66**, 1717–1725.
- Zakharova,O.D., Tarrago-Litvak,L., Fournier,M., Andreola,M.L., Repkova,M.N., Venyaminova,A.G., Litvak,S. and Nevinsky,G.A. (1995) Interaction of primer tRNA (Lys3) with p51 subunit of human immunodeficiency virus type 1 reverse transcriptase: a possible role in enzyme activation. *FEBS Lett.*, **361**, 287–290.

Received on October 2, 1996; revised on November 28, 1996