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Rapid Activation and Subsequent Down-Regulation of the Human Immunodeficiency Virus Type 1 Promoter in the Presence of Tat: Possible Mechanisms Contributing to Latency

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The mechanism of induction of gene expression of the human immunodeficiency virus type 1 long terminal repeat (LTR) by the Tat transactivator protein was studied in a cell fusion assay. Tat causes a rapid activation of both transcription from the LTR and accumulation of hybrid LTR-chloramphenicol acetyltransferase mRNAs. Approximately 4 h after induction by Tat, expression from the LTR promoter is down-regulated, resulting in a decrease in the accumulation of LTR mRNA. This down-regulation of expression occurs in the continued presence of Tat. Protein synthesis inhibitors can block this down-regulation; therefore, the postinduction repression of expression is dependent upon de novo protein synthesis. We propose that a labile cellular protein(s) is responsible for the low levels of human immunodeficiency virus type 1 expression, possibly contributing to the establishment of a latent state of viral expression.

The analysis of human immunodeficiency virus type 1 (HIV-1) gene expression during the course of infection and the resulting cellular responses is critical in the understanding of viral pathogenesis; these events may determine whether the progression of infection results in active viral replication and possibly cell death or the establishment of a latent state, characterized by little or no viral replication. We have used a cell fusion system to analyze the mechanism of both Tat-induced viral gene expression and the subsequent cellular responses.

The mechanism of the Tat-induced increase in HIV-1 gene expression has been controversial. Tat is an essential viral protein (8, 16) that increases expression from the long terminal repeat (LTR) promoter (1, 57; for recent reviews see references 22, 43, and 49), resulting in an increase in the accumulation of mRNA (6, 26, 27, 31, 40, 45, 47, 51, 62). Transcriptional activation has been reported to account for the Tat-induced increase in mRNA accumulation (3, 10, 24, 27, 31, 34, 47, 52). The mechanism of transcriptional activation has been proposed to involve a *cis*-acting RNA enhancer element present in the nascent transcript (3).

The Tat-responsive (TAR) element has been localized to a region 3′ to the transcriptional start site but 5′ to the first splice donor (6, 26, 40, 45, 51, 62); therefore, TAR is present as DNA sequences in proximity to the transcriptional initiation site and additionally is present in all viral mRNAs. The minimal TAR region includes sequences between nucleotides (nt) +19 and +44 from the start site of transcription (18, 23, 26, 56). In addition to transcriptional activation, Tat has been reported to increase the utilization of HIV-1 mRNAs in some (4, 6, 10, 40, 50, 62) but not all (45, 47) systems. Evidence presented here supports the role of Tat at both the transcriptional and posttranscriptional levels.

We have studied the kinetics of Tat induction of TAR-containing mRNAs. We show evidence that after a rapid Tat-induced increase in expression of the HIV-1 LTR, expression is down-regulated in the continued presence of Tat. We find that the down-regulation seen for LTR-driven mRNA is not seen for *neo* or actin control mRNAs in this system and requires de novo protein synthesis. We propose that a cellular factor(s) may be responsible for repression of gene expression from the LTR and that this repression may be relevant to mechanisms of latency associated with HIV-1 infection.

MATERIALS AND METHODS

Selection and maintenance of cell lines. HL3T1 cells are HeLa cells that contain the integrated plasmid pL3CAT (15, 62). This plasmid contains HIV-1 sequences from *Bam*HI to *Hin*dIII (+8021 to +9162), which include the entire U3 region and 80 bp of the R region of the LTR, linked to the chloramphenicol acetyltransferase (CAT) gene. Sequences 3′ to the CAT gene in this plasmid include splicing and polyadenylation sequences from simian virus 40 (SV40) (20, 21). CB2MX3 cells are mouse C127 cells that contain multiple copies of the plasmid pB2MX3 (62). This episomally maintained plasmid contains bovine papillomavirus sequences and expresses the Tat gene from the mouse metallothionein promoter. pB2MX3 contains HIV-1 sequences from +5332 to +8021. HIV-1 Rev sequences are present in this plasmid; however, pB2MX3 is functionally inactive for Rev in complementation experiments (55). In monolayer culture the CB2MX3 cell line produces high levels of Tat for more than 10 passages. Thereafter, a decrease in production of Tat has been observed. Therefore, the CB2MX3 cells used in these experiments were at passage 10 or less.

Fusion of stable cell lines. Separate tissue culture cell lines that stably express either *cis*-acting DNA sequences (LTR-CAT sequences) or *trans*-acting factors (Tat) were cocultivated and allowed to reach approximately 90% confluency. The medium was aspirated, and the cells were rinsed with phosphate-buffered saline (PBS). A solution of 50% (wt/vol) polyethylene glycol (PEG; Baker Analyzed PEG 1000) in Dulbecco's modified Eagle's medium was added to the cells. The PEG solution was left on the cells for 3 min and then aspirated. After three rinses with PBS, the medium was

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replaced and the cells were incubated at 37°C. Cells were harvested for analysis as described below.

Isolation of total RNA from tissue culture cells and Northern analysis. Total RNA was isolated by the hot phenol procedure (54) and analyzed by Northern (RNA blot) transfer as described previously (14, 62). Nick-translated CAT DNA and internal control *neo* DNA probes were used to hybridize to total RNA bound to nitrocellulose filters. All Northern blots were washed at 65°C in 0.1× SSC–0.1% sodium dodecyl sulfate (SDS) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The CAT probe contains sequences from *Hin*dIII to *Nco*I in pSV2CAT (21), which contains only CAT coding sequences. The *neo* probe contains only *neo* coding sequences from *Hin*dIII to *Sma*I of pSV2Neo (58). The 2-kb *Hin*dIII fragment of the actin plasmid pβ-2000 was used as the actin probe (5).

Transcription of LTR-CAT in stable cell lines. Transcription in permeabilized monolayer cells was measured essentially as described before (7). Medium was aspirated from the fused cells, and the cells were washed once with ice-cold PBS and once with ice-cold permeabilization buffer (150 mM sucrose, 30 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4], 33 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate). The tissue culture dishes were then placed on a bed of ice. The permeabilization buffer was aspirated and replaced with 300 μg of lysolecithin (L-α-lysophosphatidylcholine; Sigma L-4129) per ml in ice-cold permeabilization buffer in sufficient quantity to completely cover the monolayer. The optimal concentration of lysolecithin had been determined in a previous experiment by testing concentrations of lysolecithin ranging from 100 to 500 μg/ml. The optimal concentration was based on two criteria: maximum number of cells permeabilized and minimum amount of cell damage. Lysolecithin at 300 μg/ml was chosen because >95% of the cells were rendered permeable to 0.1% trypan blue with no obvious loss of cellular structure. Concentrations higher than 500 μg/ml resulted in loss of membrane integrity, as viewed under the light microscope. Incubation was for 1 min on ice, at which time the lysolecithin was aspirated. Transcription buffer (20 mM Tris-HCl [pH 8], 6 mM magnesium acetate, 84 mM KCl, 10 mM NH_4Cl, 10% glycerol, 30 mM EDTA, 1 mM dithiothreitol, 10 μg of creatine phosphokinase per ml, 10 mM creatine phosphate, 250 μM GTP, 250 μM CTP, 250 μM ATP, 500 μCi of [α-^{32}P]UTP [3,000 Ci/mmol] per ml) was added, and the cells were incubated for 15 min at 31°C. The transcription buffer was aspirated, 3 volumes of RNA extraction buffer (6 M guanidinium isothiocyanate, 0.5% Sarkosyl, 2% 2-mercaptoethanol, 50 mM Tris-HCl [pH 8], 5 mM EDTA, filter sterilized) were added, and the solution was vortexed vigorously. The solution was passed several times through a 25-gauge needle to shear the DNA. Cesium chloride was added to a final concentration of 0.4 g/ml, and the resulting solution was layered on a 1-ml cushion of 5.6 M CsCl–10 mM EDTA and centrifuged in an SW60.1 rotor at 35,000 rpm at 20°C for 18 h. The RNA pellet was suspended in 10 mM Tris-HCl (pH 8)–300 mM NaCl–5 mM EDTA–0.5% SDS, then ethanol precipitated, and suspended in 50 μl of H_2O. The radiolabeled RNA was used to probe nitrocellulose membranes to which 500 ng of the appropriate purified DNA fragments had been bound (36). The CAT and *neo* DNA fragments are the same as those described for Northern analysis above. Double-stranded M13 DNA was used as control. The nitrocellulose was prehybridized for 48 h in hybridization solution lacking labeled RNA. A 1.5-ml solution of the [α-^{32}P]UTP-labeled RNA was made to include 300 μl of 20× SSC, 60 μl of 50× Denhardt's solution (1× Denhardt's solution is 0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 30 μl of yeast tRNA (10 mg/ml), and 750 μl of deionized formamide. This solution was heated at 80°C for 10 min before hybridization. After hybridization, the membranes were washed at least 10 times in 0.1% SDS–0.1× SSC at 65°C for 10 min each. The membranes were air dried and subjected to autoradiography at −70°C.

Immunoprecipitations. The immunoprecipitations were performed as previously described (62) with nonimmune rabbit serum, anti-CAT rabbit serum (5'-3', Inc.), or anti-Tat rabbit serum. The Tat antibody was raised in rabbits against an N-terminal peptide of Tat (amino acids 1 to 62) (13).

RESULTS

Fusion of stable cell lines containing *cis*- or *trans*-acting HIV-1 elements. We used the PEG-mediated fusion of two cell types to study Tat transactivation (44). One cell type is the HeLa-derived cell line HL3T1 (15, 62), containing the Tat-responsive HIV-1 LTR promoter linked to the bacterial CAT gene. In the absence of the Tat protein, this LTR-CAT construct is expressed at very low levels. The second cell type, CB2MX3, contains the HIV-1 Tat gene, constitutively expressed from the mouse metallothionein promoter (62). Brief treatments with PEG of cocultivated LTR-CAT-containing (HL3T1) and Tat-producing (CB2MX3) stable cell lines resulted in the loss of membrane integrity, the mixing of cytoplasmic compartments, and large-syncytium formation (Fig. 1A). Shortly after fusion, the Tat protein produced by the CB2MX3 cells stimulated a large increase in the accumulation of CAT protein. This is shown by both CAT immunofluorescence (Fig. 1A) and CAT enzymatic analysis (Fig. 1B). CAT assays were performed as described previously (20, 21, 62). By 24 h after fusion, the majority of the cellular membranes were reorganized (Fig. 1A); however, most cells were still multinucleate. Cocultivation of the two cell types in the absence of PEG-mediated fusion did not result in CAT activation.

The induction of the LTR-CAT gene by Tat is rapid in this system. CAT enzyme activity was elevated over basal level at 1 h after fusion (not shown) and continued to increase over the time course of this experiment. Relative CAT activity is shown at the top of Fig. 1B.

Kinetics of HIV-1 mRNA induction by Tat show a large increase in accumulation, followed by down-regulation. We analyzed the LTR-CAT mRNA by Northern analysis at different times after fusion. The Tat-induced accumulation of mRNA reached a peak at approximately 6 h and then declined to lower levels (Fig. 2A, top panel); longer exposure of the autoradiograph revealed that mRNA levels at 24 h were still higher than basal levels (Fig. 2B). Expression of the neomycin gene from the SV40 early promoter (58), which was integrated into the genome of the HL3T1 cells, remained relatively constant compared with LTR-CAT mRNA, indicating that the decline in LTR-CAT mRNA accumulation after 6 h is not a general property of mRNA in this system (Fig. 2A, bottom panel). Northern analysis with different nick-translated restriction fragments from the 3' end of the CAT gene indicated that the two forms of mRNA (Fig. 2 and 3) represent alternatively spliced forms of polyadenylated CAT mRNA (25). In addition to the quantitative decline of LTR-CAT mRNA after 6 h, the smaller of these transcripts becomes shorter over the time course of the experiment (Fig. 2). The reason for this is not known.

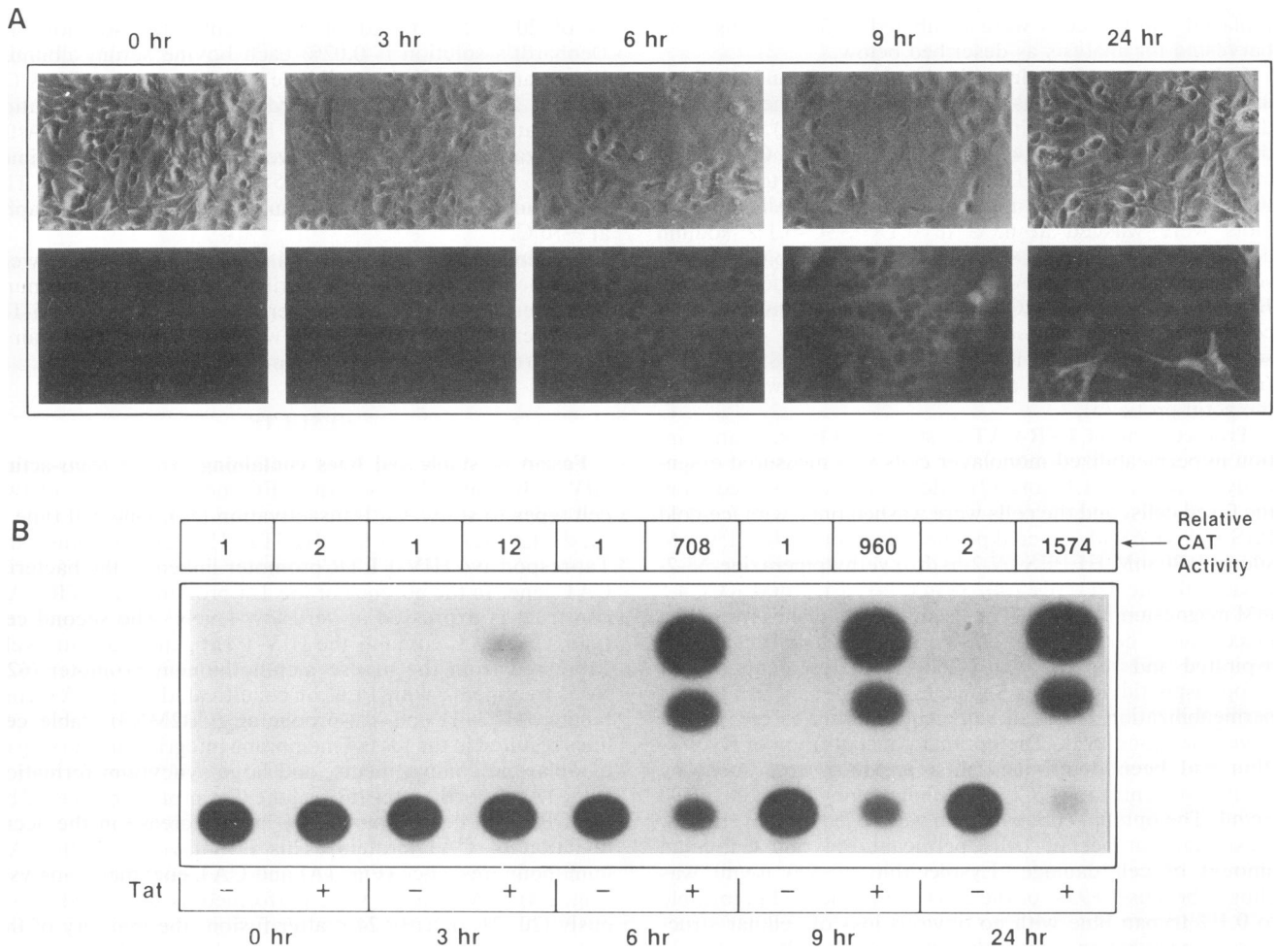

FIG. 1. Kinetics of expression of LTR-CAT after cell fusion. The stable cell lines HL3T1, containing the HIV-1 LTR-CAT gene, and CB2MX3, expressing the Tat protein, were fused and harvested for analysis at the indicated times after fusion. The fixed cells (A), the CAT assays (B), the Northern analysis in Fig. 2A, and the immunoprecipitations in Fig. 5 represent the analysis of matching sets of fused cells from one representative experiment. (A) Bright-field (top panel) and indirect immunofluorescence (bottom panel) of matching fields of fused LTR-CAT-containing and Tat-producing cells. After fusion, fixed cells were incubated with anti-CAT antibody and fluorescein isothiocyanate-labeled second antibody to identify the CAT protein. Fusion of LTR-CAT-containing cells to non-Tat-producing control cell lines was consistently negative for CAT protein. (B) CAT assays of fused cells. The unacetylated substrate (bottom species) and two acetylated products (top species) were separated by thin-layer chromatography. The CAT assays for the 6-, 9-, and 24-h time points shown, in the presence of Tat, were outside the linear range of activity; therefore, the activities of 1:4 and 1:20 dilutions of cellular extracts were used to calculate the relative activities. Relative CAT activity is shown above the individual CAT assays. Symbols: +, fusion of LTR-CAT-containing cells to Tat-producing cells; −, fusion of LTR-CAT-containing cells to non-Tat-producing cells.

A cell line containing a different indicator gene, that for human placental alkaline phosphatase (2), linked to the HIV-1 LTR (LTR-SEAP) was also analyzed in fusion experiments as described above. The LTR-SEAP mRNA showed induction kinetics similar to those of LTR-CAT mRNA after fusion to Tat-producing cells (data not shown). Therefore, the Tat-induced increase in LTR mRNA accumulation and subsequent decline to lower levels were HIV-1 LTR specific and not dependent on the reporter gene. LTR-CAT mRNA transcribed from deleted HIV-1 promoters retaining only sequences from nt −120 to +80 showed mRNA accumulation kinetics similar to those for the promoter shown here, which contains HIV-1 sequences from nt −453 to +80 (data not shown). Therefore, sequences between nt −120 and +80 in the LTR are sufficient *cis*-acting elements for this Tat-induced increase and subsequent decline in mRNA accumulation.

We have shown previously that the Tat-induced activation of the LTR is more pronounced in human cells than in mouse cell lines (44, 62). Since the Tat-producing cells (CB2MX3) in the fusion experiments were mouse cells and the LTR-CAT-containing cells (HL3T1) were human cells, it was important to determine whether the LTR mRNA expression was also down-regulated in a fusion system containing only human cells. Fusion of the human HL3T1 cells to HeLa cell line HLTat (55), which stably expresses the HIV-1 Tat gene, showed kinetics similar to those in mixed human and mouse cells (Fig. 2B), while internal control actin and *neo* mRNA levels remained relatively constant. Therefore, the observed kinetics of LTR mRNA accumulation were not due to mouse-specific factors.

The decline in LTR mRNA levels after 6 h of fusion was prevented by the inhibition of protein synthesis (Fig. 3). The steady-state levels of many mRNAs are affected by protein

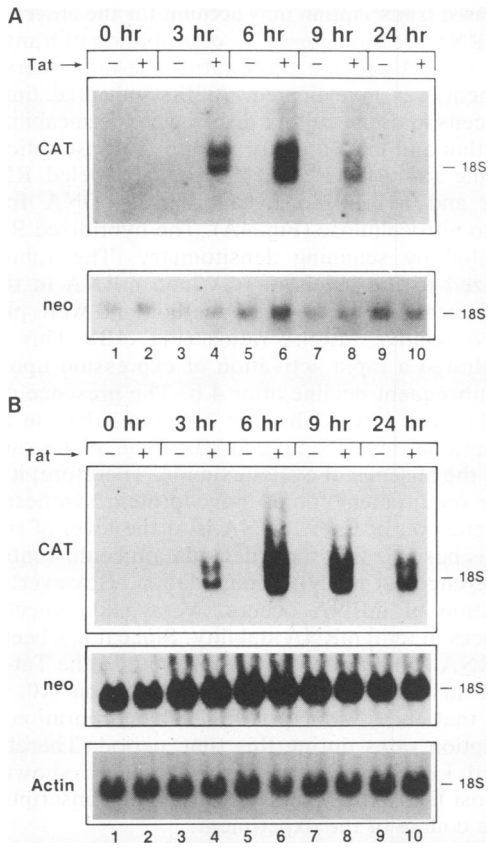

FIG. 2. Northern analysis of LTR-CAT mRNA accumulation after Tat induction. The indicated times are the times of RNA isolation after fusion. (A) Fusion of human to mouse cells. Fusion of HL3T1 cells to the non-Tat-producing 7-4 cell line (−) and to the Tat-producing CB2MX3 cell line (+) is shown. Nick-translated CAT DNA (top panel) and internal control *neo* DNA (bottom panel) probes were used to hybridize to 20 μg of total RNA bound to nitrocellulose filters. The CAT probe was a *Hin*dIII to *Nco*I fragment of pSV2CAT, which contains only CAT-coding sequences. The *neo* probe was a *Hin*dIII to *Sma*I fragment from pSV2Neo, which contains only *neo*-coding sequences. The position of 18S RNA is indicated at the right. (B) Fusion with two human cell lines. Experimental conditions were the same as for panel A, except that the HL3T1 cells were fused to either non-Tat-producing HeLa cells (−) or Tat-producing HLTat cells (+). Northern analysis of the *neo* and actin mRNAs from the same experiment is shown at the bottom. The CAT and *neo* probes are the same as in panel A.

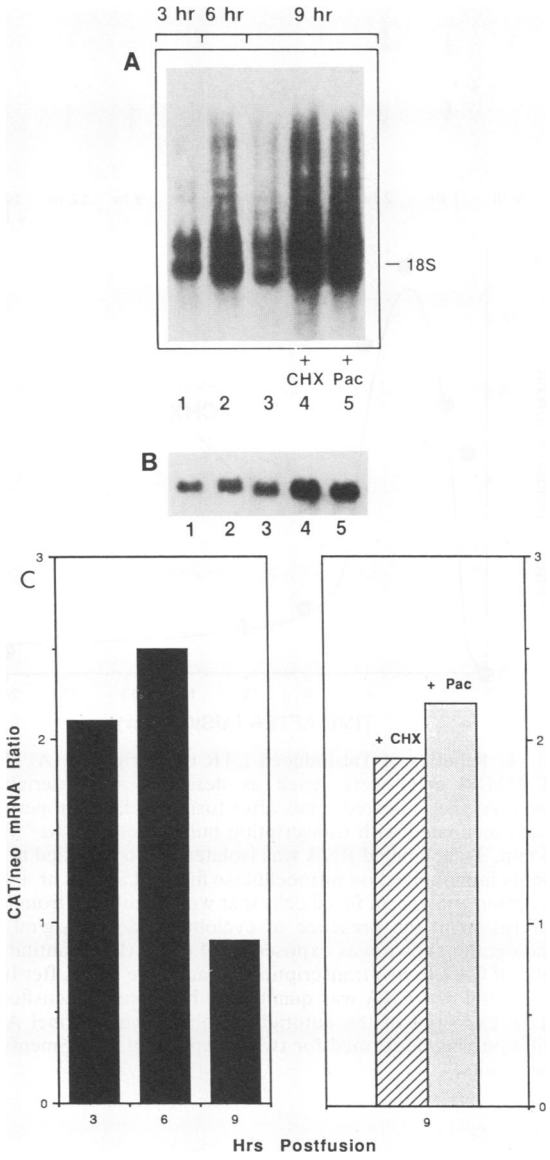

FIG. 3. Protein synthesis inhibitors (cycloheximide and pactamycin) prevent the down-regulation of LTR mRNA in fused HL3T1 and CB2MX3 cells. RNA was isolated at the indicated times after fusion. (A) RNA from fused cells that were treated with cycloheximide (CHX, 10 μg/ml) from 6 to 9 h after fusion (lane 4) and RNA from fused cells that were treated with pactamycin (Pac, 2 μg/ml; a gift from Upjohn Chemical Co.) from 6 to 9 h after fusion (lane 5). Only the analysis of mRNA from fusions to Tat-producing cell lines is shown, as fusion to the non-Tat-producing cell lines was routinely negative for CAT mRNA. The position of 18S RNA is shown at the right. (B) Rehybridization of nitrocellulose shown in panel A with the nick-translated *neo* probe, shown in Fig. 2. (C) Quantitation by densitometry of the mRNA shown in panels A and B. Quantitation was performed on a shorter exposure than that shown here and included only the two major CAT mRNA species. Two independent experiments were quantitated by densitometry with the same results. Values at each time point are expressed as the CAT/*neo* mRNA ratio.

synthesis inhibitors (11, 17, 33, 46). Therefore, we compared the ratio of LTR-CAT mRNA (Fig. 3A) and *neo* mRNA (Fig. 3B) in the same cells and showed that the presence of cycloheximide or pactamycin from 6 to 9 h postfusion prevented the decline of the LTR mRNA normally seen at 9 h (Fig. 3C). Because cycloheximide causes an arrest of translational elongation, it could be argued that the transcripts were protected from degradation by the polysomes. However, pactamycin treatment gave results similar to cycloheximide. Pactamycin inhibits translation at initiation, while elongating transcripts continue translation until termination and release (60). Therefore, protein synthesis inhibition per se prevents the decline of Tat-induced LTR mRNA. We conclude that de novo protein synthesis is required for the down-regulation of LTR mRNA at 6 to 9 h postfusion, suggesting the involvement of labile factors in this process.

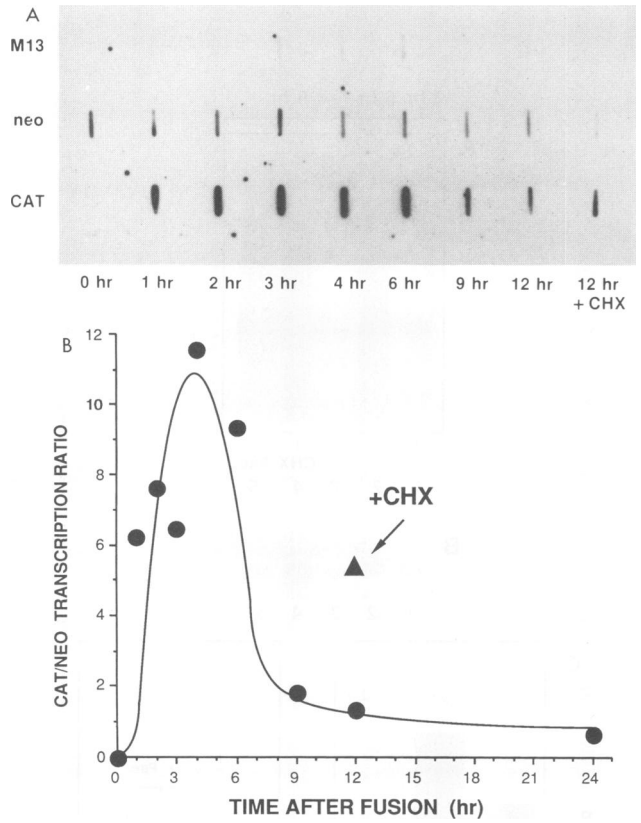

FIG. 4. Kinetics of Tat-induced LTR transcription. (A) HL3T1 and CB2MX3 cells were fused as described in Materials and Methods. At the indicated times after fusion, cells were permeabilized and incubated with transcription buffer including [α-^{32}P]UTP for 15 min. Total labeled RNA was isolated and hybridized to DNA fragments immobilized on nitrocellulose filters. Lane 12 hr + CHX, transcription analysis of fused cells that were incubated from 6 to 12 h after fusion in the presence of cycloheximide (10 μg/ml). The autoradiograph shown was exposed for 4 days. (B) Quantitation of the ratio of CAT to *neo* transcription at each time point after fusion. The CAT and *neo* RNA was quantitated by scanning densitometry of a 12-h exposure of the autoradiograph shown in panel A. The quantitation was performed for two independent experiments with similar results.

Increased transcription may account for the observed kinetics of mRNA accumulation. The contribution of transcription rates to mRNA accumulation during the time course of the experiment was investigated. At the indicated times after fusion, cells in tissue culture dishes were permeabilized with lysolecithin and incubated for 15 min in transcription buffer containing [α-^{32}P]UTP, and then total labeled RNA was purified and hybridized to appropriate DNA fragments bound to nitrocellulose (Fig. 4A). The hybridized RNA was quantitated by scanning densitometry. The values were normalized to the levels of pSV2neo mRNA in the same nuclei determined by the same method and were plotted as CAT/*neo* relative mRNA ratios (Fig. 4B). This analysis demonstrated a rapid activation of expression upon fusion and a subsequent decline after 4 h. The presence of cycloheximide from 6 to 12 h after fusion resulted in a higher transcriptional level compared with that at the same time point in the absence of cycloheximide. Therefore, it appears that the requirement for de novo protein synthesis in the downregulation of CAT mRNA is at the level of transcription. It is possible that rapid degradation could contribute to the differences in mRNA accumulation. However, if rapid degradation of mRNA occurs, we would expect to see differences in total mRNA stability. Since it has been shown that mRNA stability does not contribute to the Tat-induced increase in LTR-CAT mRNA accumulation (10, 24), we believe that the 15-min [α-^{32}P]UTP incorporation reflects transcription rates during this time period. Therefore, the observed kinetics of mRNA accumulation shown above were most likely the result of changes in transcription over the time course of the experiment.

Transcription from the LTR and LTR mRNA levels decline in the presence of continuous Tat synthesis. The decline in the transcription of the LTR-CAT gene described above could be explained by a decrease in the levels of Tat protein after fusion. To determine the levels of Tat protein synthesis, we analyzed the rate of protein synthesis over the time course of the experiment (Fig. 5). Fused cells were labeled for 30 min with [^{35}S]cysteine at the times indicated and immediately thereafter harvested for analysis. This experiment demonstrated that the synthesis of Tat protein continued throughout the course of the experiment. Approximately the same levels of Tat protein were being synthesized at 3 and 24 h after fusion, yet LTR-CAT mRNA levels (Fig. 2A) and transcription from the LTR (Fig. 4) were very different at early (3 h) and late (12 or 24 h) times, being higher at 3 h and

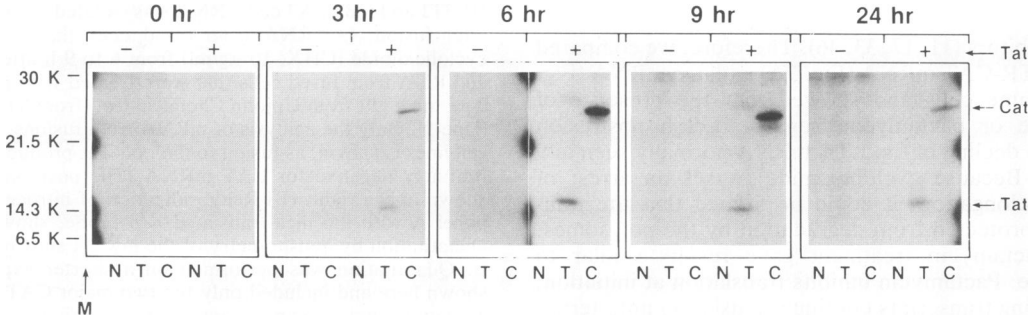

FIG. 5. Rates of synthesis of CAT and Tat proteins after fusion. After fusion at time zero, 30-min labeling with [^{35}S]cysteine was performed at the times indicated at the top of the figure, and cell lysates were harvested immediately for immunoprecipitation as described before (62) with normal rabbit (N), rabbit anti-CAT (C), or rabbit anti-Tat (T) antibodies, under conditions of antibody excess. The positions of CAT and Tat protein are indicated at the right. Fusion of HL3T1 cells to non-Tat-producing cell line 7-4 (−) or to the Tat-producing CB2MX3 cell line (+) is indicated. Lane M, ^{14}C-labeled protein markers (10^3 M_r).

lower at 12 or 24 h. Therefore, the continual synthesis of Tat is not sufficient for the maintenance of high levels of transcription from the LTR or for LTR mRNA accumulation.

Posttranscriptional effects of Tat. It has been reported that the increase in LTR mRNA accumulation in the presence of Tat cannot account for the increase in CAT enzyme (6, 10, 40, 62). In transient-transfection experiments with HeLa cells, the Tat-induced increase in mRNA accumulation was 20-fold, while CAT activity increased by several hundredfold (62). CAT activity over the time course of the experiment shown here increased steadily (Fig. 1B), even as the mRNA was declining (Fig. 2A). This could be the result of an increase in utilization of the LTR-CAT mRNA at later time points after fusion, a reflection of the accumulation of an enzyme with a greater stability than its mRNA, or both. To determine whether the levels of LTR mRNA correlated with the levels of translation of those messages, we compared CAT protein synthesis rates (Fig. 5) and mRNA accumulation (Fig. 2A) in duplicate samples in the same experiment. Figure 5 shows the immunoprecipitation of cell lysates with anti-CAT antibody labeled for 30 min with [^{35}S]cysteine at the indicated time points after fusion and immediately harvested for analysis. The rate of CAT synthesis increased greatly after Tat induction and then decreased to lower levels, as did the level of LTR-CAT mRNA (Fig. 2A). However, the levels of LTR-CAT mRNA at a given time point do not correlate well with the rate of CAT protein synthesis. For example, the rate of CAT synthesis at 9 h after induction was relatively high compared with that at 3 h (Fig. 5), yet the mRNA was less abundant at 9 h (Fig. 2A). Similarly, the levels of LTR-CAT mRNA at 24 h were very low compared with the levels at 3 h (Fig. 2A), yet CAT protein synthesis was approximately the same (Fig. 5). Therefore, the mRNA is utilized more efficiently at later times after Tat induction than at earlier times. Possible mechanisms for this apparent increase in utilization include delayed transport of mRNA out of the nucleus and changes in polysomal loading.

DISCUSSION

Analysis of Tat transactivation by the fusion method described here has several advantages over methods involving transiently transfected or virally infected cells. Analysis after transfection or infection involves a mixed population of kinetic events, and as such reflects an averaging of effects. The fusion method applied here provides a rapid switch from Tat-minus to Tat-plus conditions; the effects of Tat on transcription can be detected as early as 1 h after fusion. In addition, fusion is very efficient; virtually all of the cells in the culture are fused. For these reasons, the responses are highly coordinate and the signals are strong. By comparison with results obtained from other systems, it is reasonable to assume that the effects we have analyzed by the fusion technique are an accurate representation of events occurring on an individual cellular basis upon activation of the LTR by Tat.

In the fusion system used here, Tat causes an increase in transcription from the viral LTR, leading to an increase in mRNA accumulation, consistent with the results of other studies with different systems (3, 24, 26, 27, 31, 34, 47, 52). This report presents evidence that the initial high level of expression of the HIV-1 LTR by Tat is transient in nature and that de novo protein synthesis is required for subsequent down-regulation. This increase and subsequent decrease in mRNA is not seen in the control mRNAs examined (*neo* and actin).

The region of the promoter between −120 and +80 contains multiple protein-binding domains involved in both basal and induced levels of transcription, including two NF-κB- and three SP1-binding sites (9, 28–30, 32, 41, 43, 63). Tat$^-$ viral mutants are not viable (8, 16). The low activity of the HIV-1 promoter in the absence of Tat raises the possibility of a repression mechanism, which nullifies the effects of NF-κB and SP1 factors, and/or additional positive factors (Tat or Tat associated) for activation. This mechanism may be at the level of transcription initiation or elongation or may involve rapid degradation of nascent transcripts in the nucleus. Mosca et al. (39) have shown that inactive integrated HIV-1 LTR-CAT genes can be reactivated in the absence of Tat with cycloheximide and that the reactivation is at the level of transcription. These results indicate that one mechanism of Tat activation is at the transcriptional level, possibly by overcoming a transcriptional block.

The regulation of HIV-1 transcriptional activation may reflect complex strategies involved in the propagation of the virus in human cells, and its elucidation may aid in the understanding of possible mechanisms of latency (12, 64). The molecular mechanism of latency has not been determined. Recently, a human gene has been cloned by virtue of its ability to bind Tat protein (42). When cotransfected into human cells with an HIV-1 LTR-CAT plasmid and a Tat-producing plasmid, this gene suppressed expression from the LTR. Whether this human gene is involved in the suppression of expression that we describe here for the HIV-1 LTR is not known.

Cellular genes such as c-*myc* c-*fos*, interferon beta (IFN-β), and several IFN-β-inducible promoters show rapid, transient induction kinetics similar to those shown here for the HIV-1 LTR. The downregulation of c-*myc* transcription that occurs after induction can be reversed by protein synthesis inhibitors (38). The c-*fos* promoter contains a 14-bp serum response element core, which is sufficient to mediate both the serum induction and subsequent down-regulation of transcription (48). Transcription from the IFN-β promoter is transiently induced by viral infection, and the subsequent down-regulation is dependent upon de novo protein synthesis (61). Several IFN-β-responsive genes (35) are transiently induced at the transcriptional level, and protein synthesis inhibitors reverse the subsequent down-regulation of transcription. The promoters of these genes contain homologous sequence elements that may bind both positive- and negative-acting factors (35, 37). The levels of expression of the HIV-1 promoter may also be determined by a balance between the positive- and negative-acting factors, which may vary according to cell type and specific stimuli. The data presented here support a model in which Tat, a positive activator, can shift the balance in favor of high levels of expression. However, the equilibrium favoring Tat over negative-acting factors may be subsequently reversed, resulting in a reduced level of expression of the LTR. In our system, after the initial Tat-mediated induction and subsequent down-regulation of the LTR, the levels of expression remain higher than those seen prior to the introduction of Tat. It is conceivable that the levels of expression of the LTR vary in different cells infected by HIV-1, leading to either rapid viral replication or complete shutoff of viral expression. Further experiments are required to elucidate the mechanisms of LTR activation and down-regulation. The system described in this report may provide a useful assay

for the identification of both positive and negative factors involved in HIV-1 expression.

We have previously addressed the discrepancy between activation of HIV-1 at the mRNA and protein levels (6, 40, 62). On the basis of such measurements, it was suggested that Tat acts both at the transcriptional level to increase viral mRNA and at the posttranscriptional level to increase the utilization of mRNA. These experiments were done by transient transfections, and results were analyzed 1 to 3 days after transfection. In the present work we have compared activation at the mRNA and protein levels over time, since it was difficult to define a steady state for this promoter. We show that the translational efficiency of the mRNA continued to increase for 24 h after Tat activation, even as the levels of mRNA were declining. We have previously shown that one aspect of the posttranscriptional activation of TAR-containing mRNAs by Tat is the increased association of those mRNAs with polysomes (10). This may explain the large protein/mRNA ratios seen in the presence of Tat (6, 40, 62).

In conclusion, our results suggest that, when stably integrated in the genome, the HIV-1 promoter is maintained in an inactive state, presumably by cellular factors, and that regions of the promoter between −120 and +80 are sufficient for this regulation. Tat and other stimulatory agents (19, 39, 53, 59) perturb this repressed state. A balance between cellular inhibitory factors and viral or cellular stimulatory factors may determine the outcome of HIV-1 expression, resulting in either progression to mature virus production and possible cell death or continued maintenance of a repressed state.

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