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Human Immunodeficiency Virus Tat Transactivation: Induction of a Tissue-Specific Enhancer in a Nonpermissive Cell Line

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The enhancer of the human neurotropic papovavirus JC virus (JCV) restricts viral transcription to glial cells. We utilized the tissue specificity of the JCV enhancer as a tool to investigate the function of human immunodeficiency virus (HIV) Tat in transcriptional activation. The reporter plasmid pJCTAR-CAT was constructed by inserting the HIV type 1 Tat-responsive element, TAR, between the JCV promoter and the chloramphenicol acetyltransferase (CAT) gene. Cotransfection of pJCTAR-CAT and pSV-Tat, an expression vector for Tat, resulted in a 50-fold increase in JCV promoter activity in cells nonpermissive for JCV expression. Both the 98-bp JCV enhancer and the HIV TAR sequences were required for transactivation of pJCTAR-CAT in nonpermissive cells. The transactivation by Tat occurred at the level of transcription, as the increase in CAT activity paralleled an increase in the steady-state levels of CAT mRNA in S1 nuclease and nuclear run-on analyses. In the presence of Tat, the JCV enhancer is functional in cells normally nonpermissive for JCV expression; therefore, our results provide unique evidence that HIV type 1 Tat may regulate the activity of specific transcription factors.

The enhancer of the human neurotropic papovavirus JC virus (JCV) is typical of an enhancer which has an intrinsically narrow range of tissue specificity (11, 12, 21–23, 26, 35, 45). In tissue culture, the JCV enhancer is active only in glial cells, not in a wide variety of other cells including HeLa and CV-1 (22). Although transcription factors ubiquitously expressed in several cell types bind to JCV enhancer sequences (2, 23), a 45-kDa glial cell-derived protein that is not present or is inactive in HeLa cells has been shown to bind directly to a specific domain within the JCV enhancer and to increase JCV promoter activity in in vitro transcription assays (1, 23). Thus, the interaction of transcription factors expressed specifically in glial cells with the JCV enhancer sequences likely contributes to the tissue specificity of JCV transcription.

The human immunodeficiency virus (HIV) genome encodes two regulatory proteins, Tat and Rev, that are essential for viral replication (8, 10, 39). Tat transactivates HIV long terminal repeat (LTR)-directed gene expression at both transcriptional and posttranscriptional levels (5, 7, 16, 24, 38, 41). Transactivation of the HIV LTR by Tat requires functional upstream regulatory elements and a Tat-responsive element (TAR) located between +19 and +42 in the R region of the LTR (4, 9, 13, 15, 17, 20, 25, 32, 33, 36, 37, 40, 42–44, 46). Whether Tat increases the activity of transcription factors which interact with these regulatory sequences is not evident from published studies.

We utilized the stringent tissue specificity of the JCV enhancer to analyze the transcriptional regulatory properties of the HIV Tat protein. Cotransfection of the HIV Tat gene and a reporter plasmid containing the JCV enhancer-promoter and TAR resulted in a 50-fold increase in JCV transcription in the nonpermissive cells. Transactivation of the JCV promoter by Tat occurred at the level of transcription and was dependent on the presence of the JCV enhancer and a functional TAR sequence downstream of the RNA initiation site. Our experiments suggest that Tat may have the capacity to regulate the activity of specific transcription factors in a cell.

Tat transactivation of the JCV enhancer in the presence of the HIV response element. The plasmid pJCTAR-CAT was constructed to determine the effect of Tat on a tissue-specific enhancer. To create the pJCTAR-CAT plasmid, the parental plasmid pJC-CAT was cleaved at the unique *Hin*dIII restriction site. The *Hin*dIII site (JCV map positions 5112 to 5117) is located 5 nucleotides downstream of the major JCV early mRNA cap site at map position 5122. The HIV +1 to +57 TAR regulatory sequence was chemically synthesized with *Hin*dIII linkers and inserted into pJC-CAT at the *Hin*dIII site (Fig. 1). Since the level of Tat-dependent activity is inversely proportional to the distance between the promoter and TAR sequences (42), the position of TAR sequences with respect to the JCV cap site was designed to provide maximum TAR activity. In initial experiments, pJC-CAT and pJCTAR-CAT were transfected into HeLa cells, which are nonpermissive for JCV transcription, in the presence or absence of Tat. Following a 48-h incubation, cell extracts were prepared and the level of chloramphenicol acetyltransferase (CAT) enzyme activity was determined. The JCV enhancer exhibited no activity in HeLa cells (0.5 to 1.0% conversion; Fig. 2A, lanes 1 and 2). Similarly, extracts from HeLa cells transfected with pJCTAR-CAT (Fig. 2A, lanes 3 and 4) contained no CAT enzyme activity (0.4 to 0.5%). However, when pJCTAR-CAT was cotransfected with pSV-Tat into HeLa cells, a 50- to 100-fold increase in the level of CAT activity was observed with the pJCTAR-CAT but not with the parental pJC-CAT (Fig. 2A, lanes 5 to 8). For comparison, the cells were also transfected with the HIV LTR CAT plasmid, pBennCAT, in the presence or absence of the Tat expression plasmid (Fig. 2A, lanes 9 and 10, respectively). In the presence of Tat, approximately a 50- to 55-fold increase in pBennCAT activity was observed.

To determine the tissue specificity of pJC-CAT and pJCTAR-CAT transcriptional activity, the plasmids were transfected into HJC cells. These cells were derived by the transformation of hamster fetal glial cells with JCV (26). It has been shown previously that these transformed glial cells

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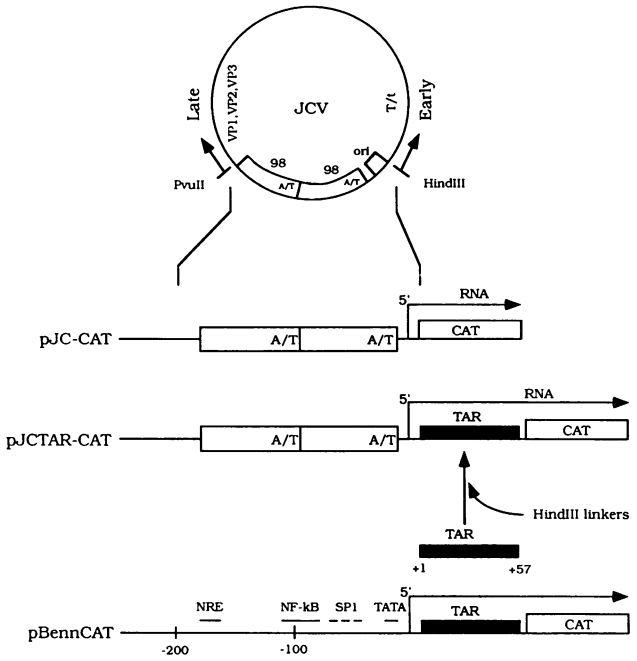

FIG. 1. Construction of pJCTAR-CAT plasmid. The organization JCV genome and the coding strands for early and late transcription are indicated. The JCV transcriptional regulatory region contains two 98-bp repeats, which control the tissue-specific transcription from the viral genome. The CAT vector containing the entire JCV enhancer (map position 5112 to 268) (pJC-CAT) has been described previously (22). pJCTAR-CAT was constructed by inserting an oligonucleotide containing the HIV TAR +1 to +57 transcriptional control sequence into the unique *Hin*dIII site of the pJC-CAT plasmid. The orientation of the TAR insert was determined by restriction enzyme digestion and DNA sequence analysis.

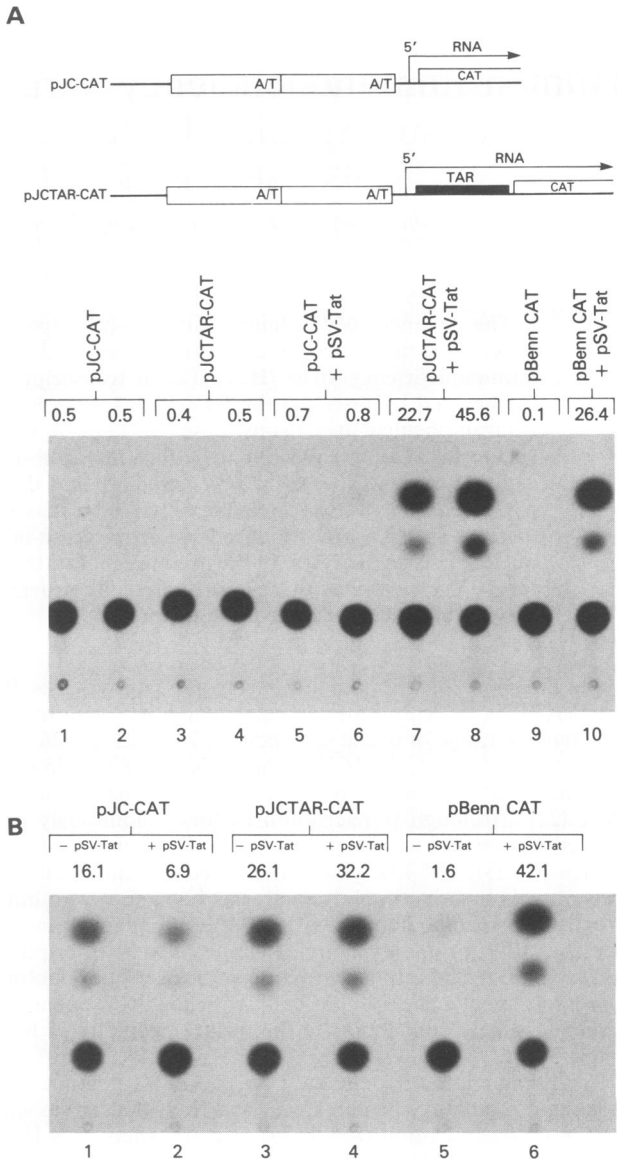

FIG. 2. CAT assay of extracts from HeLa and HJC cells transfected with pJC-CAT and pJCTAR-CAT. Plasmid DNAs were purified and transfected by the calcium phosphate precipitation technique as described previously (6, 14). Briefly, 100-mm-diameter plates were seeded at a density of 10^6 cells per plate and transfected with 4 μg of the CAT reporter plasmid and 1 μg of pSV-Tat as indicated. All transfection samples were brought to a total of 20 μg of DNA by the addition of calf thymus DNA as carrier. The cells were harvested at 48 h posttransfection. (A) HeLa cells; (B) HJC cells. Contents of lanes are as indicated. The number above each lane denotes the percent conversion of the ^{14}C-chloramphenicol substrate.

are fully permissive for JCV early gene expression. Consistent with these previous observations, the basal level of pJC-CAT (16%) was elevated in these cells compared with that in the nonpermissive HeLa cells (0.5%) (Fig. 2A, lanes 1 and 2, and Fig. 2B, lane 1). Similarly, the basal level of pJCTAR-CAT (26%) was elevated in the HJC glial cells compared with that in HeLa cells (0.5%) (Fig. 2A, lanes 3 and 4, and Fig. 2B, lane 3). The increase in JCV promoter activity was not due to increased transfection efficiency in the HJC cells, since the control pBennCAT plasmid basal activity remained low (Fig. 2B, lane 5). When the plasmids were cotransfected with the Tat-coding plasmid, a stimulation of pBennCAT (1.6 versus 42%) (Fig. 2B, lanes 5 and 6) but not of pJCTAR-CAT (26 versus 32%) (Fig. 2B, lanes 3 and 4) or pJC-CAT (16 versus 7%) (Fig. 2B, lanes 1 and 2) activity was observed. These results suggested that Tat was able to stimulate JCV promoter activity in nonpermissive HeLa cells.

Tat transactivation is dependent on JCV enhancer sequences. To determine whether JCV enhancer sequences were required for Tat responsiveness in HeLa cells, deletion mutants in the JCV enhancer were prepared and analyzed for their responsiveness to Tat transactivation (Fig. 3). Consistent with the results presented above, transfection of the pJCTAR-CAT plasmid with pSV-Tat into HeLa cells resulted in increased CAT activity (>100-fold). In HJC cells, the basal activity of the pJCTAR-CAT promoter was high and did not change significantly in the presence of Tat (less than 2-fold). Deletion of one JCV 98-bp repeat (pJC TAR Δ98-CAT) resulted in a 17-fold decrease in Tat responsiveness in HeLa cells (13.6 versus 0.8 pg of CAT). Deletion of one 98-bp repeat also diminished the activity of the JCV promoter 27-fold in HJC cells (21.6 versus 0.8 pg of CAT). Deletion of both the 98-bp repeat and upstream JCV regulatory sequences (pJCTAR 4D-CAT) abolished the responsiveness of the JCV promoter to the Tat gene product (0.1

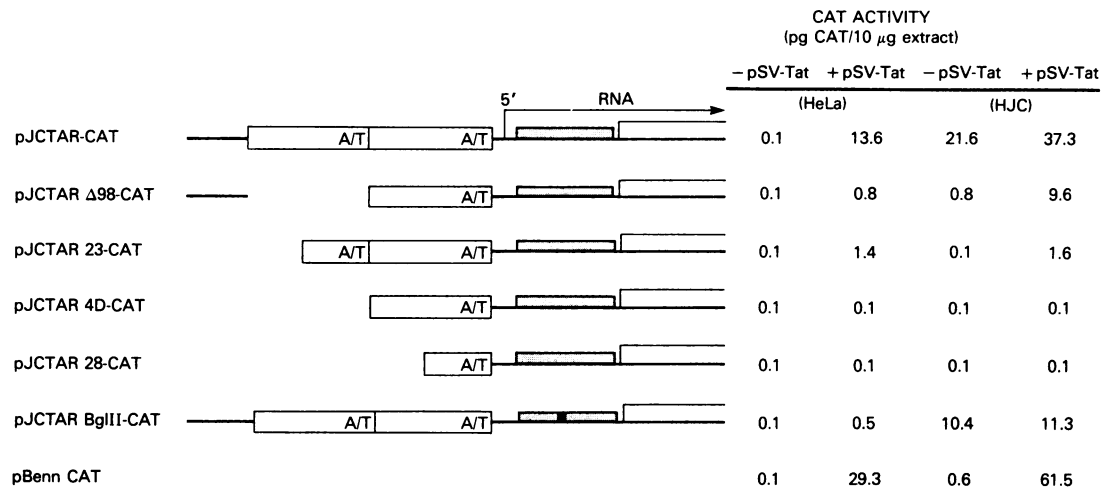

FIG. 3. Transfection of JCTAR enhancer deletion mutants in HeLa and HJC cells. The transfection of pJCTAR-CAT plasmid DNAs was performed in the presence or absence of pSV-Tat as indicated. pJCTAR Δ98-CAT was derived from pJCTAR-CAT by partial digestion with *Sac*I and ligation to remove one of the 98-bp repeats. pJCTAR 4D-CAT and pJCTAR 28-CAT were derived from pJCTAR-CAT by partial digestion with *Sac*I, digestion with *Nde*I, and ligation. pJCTAR Bgl-CAT was derived from pJCTAR-CAT by digestion with *Bgl*II, filling in with Klenow enzyme, and ligation. This construct resulted in a duplication of bases 20 to 23 in the TAR sequence. The plasmid constructs were verified with restriction enzyme mapping and DNA sequence analysis. The CAT enzyme activity (in picograms of CAT per 10 μg of extract) is designated in the columns to the right of the figure.

versus 0.1 pg of CAT) in both cell lines. The presence of the proximal portion of the second 98-bp repeat (JCV map positions 113 to 164) (pJCTAR 23-CAT) did not significantly increase the response to Tat (1.4 pg of CAT), suggesting that the distal enhancer sequences between map positions 165 and 213 were critical for Tat responsiveness. That the same nucleotide sequence was present between map positions 63 and 111 in the first 98-bp repeat suggests that duplication of the sequence was necessary for activity. These results demonstrated that JCV enhancer sequences were necessary for Tat transactivation of pJCTAR-CAT.

We also analyzed the effect of a site-specific mutation in the HIV TAR sequence (pJCTAR Bgl-CAT), a 4-bp insertion at nucleotide +20 in the TAR regulatory sequence. Mutation of this TAR regulatory sequence destroyed Tat transactivation of the JCV enhancer in HeLa cells (0.1 versus 0.5 pg of CAT) (Fig. 3). When the pJCTAR Bgl-CAT plasmid was transfected into HJC cells, the enhancer was active and maintained its tissue specificity but was not transactivated by Tat (10.4 versus 11.3 pg of CAT). These results demonstrated that TAR regulatory elements were also required for Tat transactivation of pJCTAR-CAT.

S1 nuclease analysis of RNAs. To determine whether the level of CAT expression accurately reflected a change in the steady-state levels of CAT mRNA, cytoplasmic RNA was isolated 48 h posttransfection and quantitated by S1 nuclease analysis (Fig. 4). A 31-base oligonucleotide complimentary to the CAT RNA coding sequence, 5′-GCCATTGGGATA TATCAACGGTGGTATATCC-3′ (pSV2CAT map positions 4921 to 4951), was extended to the terminus of the *Nde*I-linearized pJCTAR-CAT plasmid in the presence of [α^{32}P]dCTP. The 438-base polymerase chain reaction fragment was then purified by electrophoresis on a 6% acrylamide-urea gel. Protected DNA bands of 145 and 160 nucleotides were expected for HIV-CAT and JCTAR-CAT RNAs, respectively. Consistent with the CAT analysis presented above, the basal RNA abundance from the pJCTAR-CAT plasmid was extremely low in HeLa cells (Fig. 4, lane 7). The level of the 160-nucleotide protected fragment was increased significantly in HeLa cells transfected with pJCTAR-CAT and pSV-Tat (Fig. 4, lane 8). The tissue specificity of the JCV enhancer was demonstrated by the elevation of the basal level of JCTAR-CAT RNA in the permissive HJC cells (Fig. 4, lane 3). The relatively high basal level of JCTAR RNA was not increased significantly by Tat in HJC cells (Fig. 4, lanes 3 and 4). The inability of Tat to activate JCTAR transcription in the HJC cell line was not due to inactivity of the Tat protein. Cotransfection of pBenn-CAT and pSV-Tat resulted in an increase in CAT RNA in both HJC (Fig. 4, lanes 1 and 2) and HeLa cells (Fig. 4, lanes 5 and 6).

Analysis of JCV transcription by nuclear run-on assay. The results of the S1 nuclease analysis demonstrated that Tat increased the steady-state level of JCTAR-CAT RNA. In order to determine whether this increase was due to a change in the stability of the RNA or to an increased rate of RNA synthesis, nuclear run-on assays were performed on cells transfected with pJCTAR-CAT or pJCTAR-CAT and pSV-Tat. At 24 h posttransfection, nuclei were isolated and run-on assays were performed in the presence of [α-^{32}P] UTP. Subsequently, the labeled RNA was purified by guanidinium extraction and CsCl centrifugation. The ^{32}P-labeled RNA was then hybridized to nitrocellulose membranes containing the pJCTAR-CAT or control β-actin plasmid DNA at 45°C for 72 h. Following extensive washing, the filters were exposed to X-ray film. The blots from the run-on assays presented in Fig. 5 showed an increase in the level of newly synthesized JCTAR-CAT RNA in the presence of Tat in HeLa cells (Fig. 5A). Direct radioactivity measurements of the hybridized ^{32}P-RNA indicated that JCTAR-CAT RNA synthesis was increased approximately 50-fold in the presence of Tat. As a control for these studies, nuclear run-on assays were also performed with mock-transfected cells or ^{32}P-RNA was hybridized to control β-actin DNA (Fig. 5A and B). ^{32}P-RNA isolated from mock-transfected cells did not hybridize to pJCTAR-CAT DNA, nor was nonspecific

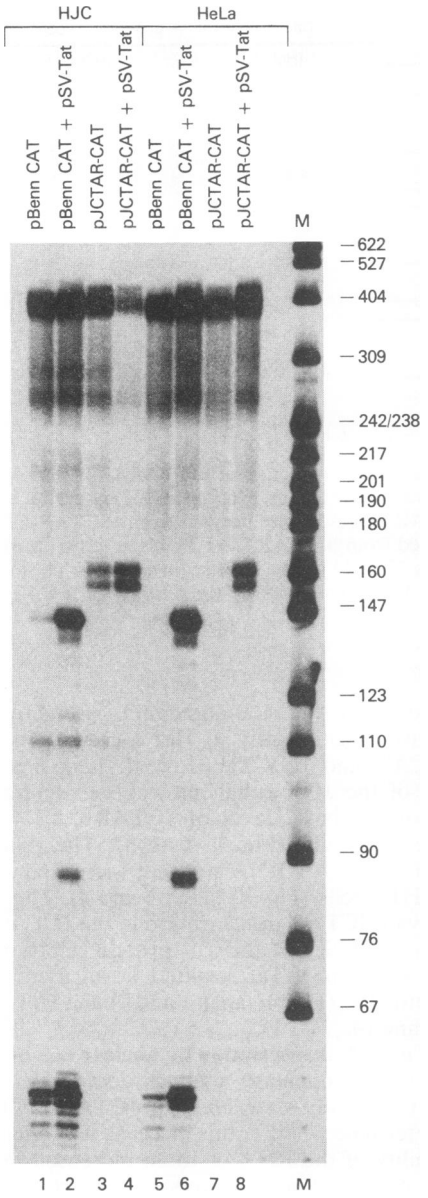

FIG. 4. S1 nuclease analysis of JCTAR- and HIV-CAT RNAs following transfection in HeLa and HJC cells. Transfection of pJCTAR-CAT and pBennCAT plasmids in the presence or absence of pSV-Tat was performed as indicated above each lane. DNA probes were made by polymerase chain reaction amplification as described in the text. RNA was isolated from cells 24 h after transfection by the Nonidet P-40 lysis method (3). Total RNA (30 μg) was hybridized with the DNA probe in hybridization buffer (80% formamide, 0.4 M NaCl, 0.04 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES, pH 6.4], 1 mM EDTA) for 14 h at 50°C. The mixture was then digested with 200 U of S1 nuclease in digestion buffer (0.25 M NaCl, 0.03 M sodium acetate [pH 4.6], 1 mM zinc acetate) for 60 min at 30°C. Following ethanol precipitation, the samples were analyzed on an 8% denaturing polyacrylamide-urea gel. Protected bands of 145 and 160 nucleotides are expected for the HIV- and JCTAR-CAT RNAs, respectively. The plasmids that were transfected for each assay are indicated above each lane. M, molecular markers. Molecular sizes (in nucleotides) are indicated to the right.

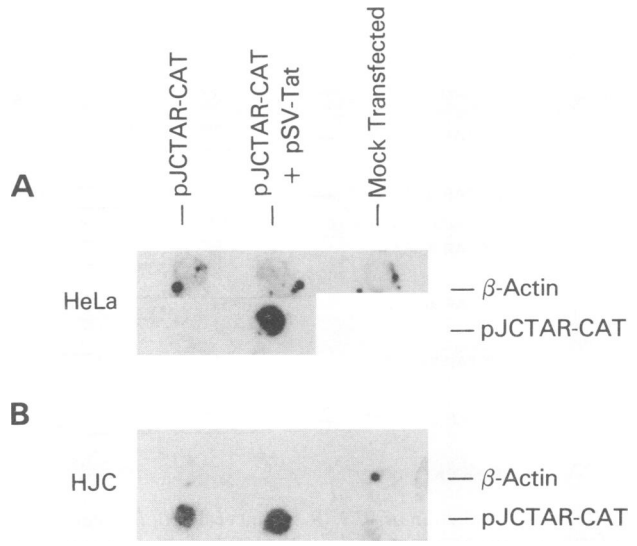

FIG. 5. Analysis of JCTAR transcription by the nuclear run-off assay. HeLa cells were transfected with either pJCTAR-CAT alone or pJCTAR-CAT and pSV-Tat, or they were mock transfected, as indicated. Nuclei were isolated from the transfected cells; washed two times and resuspended in buffer containing 20 mM Tris (pH 8), 20% glycerol, 150 mM KCl, 10 mM $MgCl_2$, 1 mM MnCl, 14 mM β-mercaptoethanol, 5 μl of [^{32}P]UTP (400 Ci/mmol), and 5 mM (each) ATP, GTP, and CTP; and incubated 30 min at 30°C (3). The RNA was purified and hybridized to membranes which contained 1 μg of denatured pJCTAR-CAT or pActin plasmid DNA. Hybridization was performed at 45°C for 72 h. The hybridized membranes were washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at room temperature until background contamination was removed.

hybridization of the ^{32}P-RNA from transfected cells to the β-actin DNA observed. The results of these controls demonstrated the specificity of the hybridization conditions used in these experiments. In another series of experiments, nuclear run-on assays were performed on HJC cells transfected with either pJCTAR-CAT or pJCTAR-CAT and pSV-Tat (Fig. 5B). Consistent with the results of the CAT assay, we found that the basal level of JCV transcription was elevated in HJC cells, and cotransfection of pSV-Tat did not increase significantly the level of pJCTAR-CAT RNA synthesis.

HIV Tat activated the level of RNA transcription from the tissue-specific JCV enhancer in a nonpermissive cell. The transactivation by Tat occurred at the level of transcription, since the increase in CAT activity was paralleled by an increase in the level of CAT mRNA abundance and synthesis in S1 nuclease and nuclear run-on analyses, respectively. Both the JCV enhancer and the HIV TAR sequences were required for transactivation of pJCTAR-CAT in nonpermissive HeLa cells.

Enhancer activity is normally regulated by the relative abundance and specific activity of transcription factors that interact with *cis*-acting DNA sequences upstream of that promoter (19, 27, 29–31). The JCV enhancer is tissue specific and is normally expressed only in fetal glial cells. Binding sites for cellular proteins are present within the JCV enhancer (1, 2, 23). By using gel shift and UV-cross-linking assays, experiments demonstrate that proteins of 82 and 78 to 80 kDa which bound to the 5′ and 3′ regions of the enhancer were present both in fetal glial and HeLa cells. In

contrast, regulatory proteins which interact with the central region of the enhancer are distinct in HeLa (85 kDa) and fetal brain (45 kDa) extracts. The 45-kDa protein, purified from calf brain, stimulates in vitro transcription of the JCV enhancer (1). Tat may regulate the functional levels of cellular transcription factors which interact with the JCV enhancer.

Insertion of the TAR regulatory sequence downstream of promoters such as the simian virus 40 promoter results in these promoters becoming Tat responsive. It could be argued, therefore, that the activity of Tat on the JCTAR template may be regulated through any number of transcription factors which are capable of supplying basal transcription activity to the JCV promoter in the nonpermissive cell. Certainly, this is consistent with the observation that Tat-TAR works poorly 3′ to active promoters, such as the Rous sarcoma virus LTR or JCV promoter in permissive cells, but works well next to promoters that display a low basal activity, such as the HIV type 1 LTR or JCV promoter in nonpermissive cells. This would argue against regulation of specific JCV transcription factors in the nonpermissive cell. This interpretation is possible but unlikely, since basal activity from the JCV promoter-enhancer in a nonpermissive cell is undetectable.

Tat transcriptional activation of the HIV LTR is the result of increased rates of transcription initiation and elongation (24). The S1 nuclease and nuclear run-on assays utilized in our analysis do not distinguish between the activity of Tat function on JCTAR transcription at the level of initiation or elongation. The ability of Tat to increase transcriptional elongation is not incompatible with the results presented in this article. One could envision a scenario in which the initiation of transcription of pJCTAR-CAT was occurring in the "nonpermissive" cell, possibly mediated by some non-neuronal DNA-binding proteins, with premature termination. Similar to the mechanism proposed for HIV LTR transcription, Tat could override the termination effect by increasing transcriptional elongation. Nuclear run-on analysis of RNA synthesis, utilizing probes covering various regions downstream of the JCTAR promoter, should help to clarify the function of Tat.

Development of in vitro transcription systems which are responsive to Tat is critical to understanding and dissecting the role of Tat in transcriptional regulation. Okamoto et al. (34) have reported that extracts from HIV-infected cells activate transcription from the HIV LTR. In addition, Jeyapaul et al. (18) and Marciniak et al. (28) recently reported that Tat increased transcription from the HIV LTR in vitro. By using a recombinant Tat protein which is biologically active in in vivo assays, our laboratory has demonstrated that Tat activates HIV LTR transcription in vitro by increasing the assembly of initiation complexes (submitted for publication). At present, we are analyzing the effect of Tat on the assembly of the transcriptional initiation complexes on the pJCTAR-CAT template in the presence and absence of tissue-specific enhancer proteins.

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