Trans-activation of the JC virus late promoter by the tat protein of type 1 human immunodeficiency virus in glial cells

HIROOMI TADA*, JAY RAPPAPORT†, MONIR LASHGARI*, SHOHREH AMINI*, FLOSSIE WONG-STAAI†, AND KAMEL KHALILI*‡

*Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107; and †Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system caused by the JC virus (JCV), a human papovavirus. PML is a relatively rare disease seen predominantly in immunocompromised individuals and is a frequent complication observed in AIDS patients. The significantly higher incidence of PML in AIDS patients than in other immunosuppressive disorders has suggested that the presence of human immunodeficiency virus type 1 (HIV-1) in the brain may directly or indirectly contribute to the pathogenesis of this disease. In the present study we have examined the expression of the JCV genome in both glial and non-glial cells in the presence of HIV-1 regulatory proteins. We find that the HIV-1-encoded trans-regulatory protein tat increases the basal activity of the JCV late promoter, JCV_L, in glial cells. In a reciprocal experiment, the JCV early protein, the large tumor antigen, stimulates expression from JCV_L and HIV-1 long terminal repeat promoter in both glial and non-glial cells. This trans-activation occurs at the level of RNA synthesis, as measured by the rate of transcription, stability of the message, and translation. We conclude that the presence of the HIV-1-encoded tat protein may positively affect the JCV lytic cycle in glial cells by stimulating JCV gene expression. Our results suggest a mechanism for the relatively high incidence of PML in AIDS patients than in other immunosuppressive disorders. Furthermore, our findings indicate that the HIV-1 regulatory protein tat may stimulate other viral and perhaps cellular promoters, in addition to its own.

AIDS is associated with a variety of neurologic disorders (1–5). Opportunistic infection of the central nervous system (CNS) and primary CNS lymphoma are often found in the later stages of this disease (3, 5, 6). In addition to inducing these secondary manifestations of immune suppression, human immunodeficiency virus (HIV) is thought to play a direct role in neuropathogenesis (7). HIV has been detected in brain tissue and cerebrospinal fluid by a variety of procedures (8–12). Furthermore, recent studies have clearly demonstrated the presence of HIV-1 virus in oligodendroglial and astroglial cells of patients with AIDS (13). The presence of HIV in brain appears to be associated with white matter changes, which include vacuolar degeneration, enlarged astrocytes, and demyelination. Similar histopathology is also observed in patients with the demyelinating disease progressive multifocal leukoencephalopathy (PML) (14–16).

Demyelination in brains of patients with PML is caused by the destruction of oligodendrocytes, the myelin-producing cells of the CNS. The JC virus (JCV), a human papovavirus, has been repeatedly isolated from brain plaques of PML patients and is thought to be the etiologic agent of this disease (14–19). This virus preferentially infects oligodendroglial cells of the CNS and propagates only in glial cells in tissue

culture (18). It has been shown that the highly restricted host range/tissue specificity of JCV to glial cells rests in the expression of its viral genome (20–22). It would appear that the JCV control region contains a regulatory element(s) that is recognized by trans-acting factors present predominantly in glial cells.

Although PML is a relatively infrequent disorder, latent infection with JCV appears to be fairly common (15). Virus reactivation and resulting neuropathology appears to be a consequence of the suppression of cell-mediated immunity. The striking similarity between PML and AIDS leukoencephalopathy suggests that JCV reactivation is a consequence of HIV infection, either directly by HIV-encoded trans-acting factors or secondarily through T4 cell depletion. Since glial cells are productively infected by both JCV and HIV-1, JCV reactivation through superinfection could be an *in vivo* mechanism of pathogenesis.

In addition to the standard retroviral genes *gag*, *pol*, and *env*, the HIV-1 genome encodes several accessory proteins (*vif*, *vpr*, *tat*, *rev*, and *nef*) (23). The best studied of these proteins, *tat*, *rev*, and *nef*, have regulatory roles in viral gene expression. We examined the relationship between JCV and HIV-1 gene expression by testing the ability of HIV-1-encoded trans-regulatory proteins to activate the JCV promoter. Our results indicate that the HIV-1-encoded protein *tat* stimulates expression of the JCV late (JCV_L) promoter predominantly in cells of human glial origin. This stimulatory effect occurs primarily at the level of RNA synthesis. The potential of the JCV-encoded trans-activator, the large tumor antigen (T antigen), to affect HIV long terminal repeat (LTR)-directed gene expression was also examined.

MATERIALS AND METHODS

Cells and Transfection Procedure. U-87MG (HTB-14) is an established human glioblastoma cell line, which was obtained from American Type Culture Collection. H9 is a continuous line of human T4 lymphocyte cells. The HeLa cell line was derived from a cervical carcinoma as described (24). All cell types except H9 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum and plated in 60-mm dishes for 24 hr prior to transfection. Cells were transfected by the calcium phosphate/DNA coprecipitation method (25). Transfected plasmid DNA was kept constant at 15 µg per dish by adding pUC19 plasmid DNA with the test plasmids and was coprecipitated with calcium phosphate in a final volume of 1.5 ml. H9 cells were transfected by using the DEAE-dextran method (26).

Abbreviations: CNS, central nervous system; HIV-1, human immunodeficiency virus type 1; PML, progressive multifocal leukoencephalopathy; JCV, JC virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; T antigen, large tumor antigen; JCV_L promoter, JCV late promoter.

‡To whom reprint requests should be addressed.

Plasmids. pJC_L-CAT (22) was previously constructed by cloning the 286-base-pair (bp) *Pvu* II–*Hind*III fragment (map units 0.67–0.72) of JCV, containing the late gene control region, into the *Bgl* II site of pCAT3M (27). pCV-1 contains the *tat*, *rev*, and *nef* (28) open reading frames of HIV-1. *ptat* is a recombinant plasmid expressing only the *tat* protein (29). pBJC-T plasmid was constructed by placing the JCV DNA fragment that codes for viral early region under the control of the herpes simplex virus ICP4 promoter (kindly provided by J. Remenick, National Cancer Institute).

Chloramphenicol Acetyltransferase (CAT) Assay. All extracts were made 48 hr posttransfection, and CAT enzyme assays were performed as described (30).

S1 Nuclease Analysis. Total cellular RNA was prepared by the hot acid phenol procedure 48 hr posttransfection (31). Input DNA was removed by treatment with DNase I (10 μ g/ml) in the presence of RNasin (Promega Biotec). RNA (50 μ g) was probed for CAT mRNA with a single-stranded DNA probe uniformly labeled with [³²P]dCTP (400 Ci/mmol; 1 Ci = 37 GBq) during primer extension synthesis from an M13 phage vector. RNA was analyzed by S1 nuclease protection as described (32).

Nuclear Run-On Assay. Nuclei were prepared 48 hr posttransfection from HTB-14 cells in two 100-mm plates containing 30 μ g of plasmid DNA and resuspended in hypotonic buffer [10 mM KCl, 1.5 mM MgCl₂, 10 mM Hepes (pH 7.9), and 5 mM dithiothreitol] for 10 min at 0°C. Cells were disrupted with a Dounce homogenizer. Nuclei were isolated from the cytosol by centrifugation at 1500 rpm with a Sorvall H1000B rotor for 5 min and resuspended in the same buffer for further purification by glycerol gradient centrifugation. The isolated nuclei were incubated in transcription buffer containing 50 mM KCl, 10 mM Hepes (pH 7.9), 5 mM MgCl₂, and [α -³²P]UTP plus other unlabeled nucleotides (33). Hybridization of the newly synthesized ³²P-labeled RNAs to the

DNA spotted on the nitrocellulose filter was performed by a standard procedure using dot-blot techniques (34).

RESULTS

Transcriptional Activities of the JCV_L and HIV-1 Promoters in Glial and Non-Glial-Origin Cell Lines. The activity of the JCV_L promoter and the HIV-1 LTR was first compared in the presence and absence of the JCV T antigen and HIV-1 trans-regulatory proteins. Indicator plasmids containing the bacterial CAT gene, under the control of the HIV-1 LTR (pHIV-CAT) or the JCV_L enhancer/promoter (pJC_L-CAT), were transfected into glial (HTB-14) and non-glial (HeLa and H9) cells alone or with plasmids encoding HIV-1 *tat* (pCV-1) or JCV T antigen (pBJC-T). Fig. 1 illustrates the organization of the JCV and HIV-1 genomes and the plasmids that were used in this study. As shown in Fig. 1B, pCV-1 contains a cDNA fragment from the HIV-1 genome harboring three overlapping open reading frames, those for *tat*, *rev*, and *nef*, which are expressed from the adenovirus major late promoter (28). In the pBJC-T construct, the JCV T antigen is constitutively expressed by the herpes simplex virus immediate early promoter, ICP4 (Fig. 1). In extracts examined 48 hr after transfection with pJC_L-CAT, virtually no CAT activity was detected in glial and non-glial (H9 and HeLa) cells after a 120-min enzyme reaction (Fig. 2A–C, lane 4). Except in glial cells (Fig. 2A, lane 1), transfection of pHIV-CAT alone indicated no detectable CAT activity (Fig. 2B and C, lane 1). Cotransfection of pHIV-CAT with pCV-1, as expected (28, 36, 37), significantly enhanced the basal CAT levels in all three cell types examined (Fig. 2A–C, compare lanes 1 and 2). When pJC_L-CAT was cotransfected with the pCV-1 plasmid, an increased level of CAT enzyme activity was observed in glial cells (Fig. 2A, compare lanes 4 and 5). In HeLa cells low, but detectable, levels of CAT enzyme activ-

FIG. 1. Schematic representation of the HIV-1 and JCV genomes and the related HIV-1 and JCV plasmids. (A) Locations of the HIV-1-encoded structural and regulatory proteins in the HIV-1 genome. The current nomenclature for HIV-1 genes is as follows: *tat* (*tat*3, TAT), *rev* (*art*, *trs*), *Vif* (*sor*, A, P', a), *VPr*(R) and *nef*(3'orf, B, E, F). pCV-1 contains a 1.8-kilobase cDNA fragment corresponding to mRNAs whose synthesis involved splicing events at nucleotides 287–5356 and 5625–7956. The 1.8-kilobase DNA fragment in pCV-1 contains three overlapping open reading frames, those for *tat*, *rev*, and *nef*, which are under adenovirus type 2 major late promoter. *ptat* is a derivative of the pCV-1 cDNA clone that contains the entire *tat* coding regions subcloned into pUC19 (16). (B) The genomic map and control region of JCV. The diagram presents the control region for expression of the JCV early gene (large and small tumor antigens), late genes (VP1, -2, -3), and late leader protein. The origin for viral DNA replication is indicated by "OR." To the late side of the origin are the transcriptional control sequences for the early and the late genes containing the tandem 98-bp enhancer/promoter elements with A+T-rich sequence. The late viral transcripts have heterologous 5' ends (35), indicated by an arrow. Agno, small open reading frame present in the leader sequences of the late RNAs. (C) pHIV-CAT was made by replacing the simian virus 40 regulatory region with the HIV-1 LTR (nucleotide +258) in the pSV2-CAT expression vector (28). pJC_L-CAT plasmid contains a 286-bp fragment, from 0.67 to 0.72 map units, of the JCV genome that spans the two 98-bp repeats in front of the CAT gene is the pCAT₃m expression vector (27). The JCV fragment was placed in the antisense orientation (relative to its positions in the JCV genome); therefore, CAT gene expression is under the control of the JCV_L promoter (22). The pBJC-T plasmid was constructed by placing the JCV DNA fragment containing the early coding region in front of the ICP4 promoter.

FIG. 2. HIV-1 and JCV_L promoter-dependent CAT protein expression in glial and non-glial cells. Plasmids containing the CAT reporter gene under the control of either the HIV-1 LTR (pHIV-CAT) or the JCV_L promoter (pJCV_L-CAT) were transfected into human glioblastoma U-87MG cells (A), human T4 lymphocytes (H9) (B), or human cervical carcinoma (HeLa) cells (C) by the DEAE dextran method for H9 cells (26) and by calcium phosphate coprecipitation (25) for the remaining cells. pHIV-CAT or pJCV_L-CAT DNAs were introduced alone or with pCV-1 or pBJC-T into cells. Three micrograms of pHIV-CAT or pJCV_L-CAT and 12 μ g of pCV-1 or pBJC-T DNAs were mixed in the transfection assays. The final DNA concentration was adjusted to 15 μ g with pUC19 DNA in all transfections. Cell extracts were prepared 48 hr posttransfection and CAT enzymatic activity was determined (30). Cell extracts were incubated with 0.5 μ Ci of [¹⁴C]chloramphenicol and 0.5 mM acetyl-CoA for 2 hr, and the acetylated forms were separated from the nonacetylated forms by thin-layer chromatography. Lanes: 1, pHIV-CAT; 2, pHIV-CAT plus pCV-1; 3, pHIV-CAT plus pBJC-T; 4, pJCV_L-CAT; 5, pJCV_L-CAT plus pCV-1; 6, pJCV_L-CAT plus pBJC-T.

ity were observed (Fig. 2C, lane 5). In contrast, transfected H9 cells showed virtually no detectable CAT activity (Fig. 2B, lane 5). Consistent with our previous observations (38), the JCV early protein, T antigen, increased viral late promoter activity, JCV_L, in HTB14 and HeLa cells (Fig. 2A and C, compare lanes 4 and 6). In the presence of T antigen, moderate enhancement in the HIV-1 LTR basal activity was observed in glial and non-glial HeLa cells (Fig. 2A and C, compare lanes 1 and 3). It should be noted that the trans-activation by T antigen is independent of DNA replication and occurs at the transcriptional level (38, 39).

HIV-1 tat Protein Trans-Activates Expression of the JCV_L Promoter in a Glial Origin Cell Line. To identify the genetic component within pCV-1 responsible for JCV_L promoter activation, we used a plasmid (ptat) expressing only tat protein in our studies. As shown in Fig. 3, in the presence of ptat, an even greater enhancement of basal CAT levels expressed by the JCV_L promoter was obtained (Fig. 3 *Inset*, compare lanes 1–3). The variation in the extent of trans-activation derived by pCV-1 and ptat expressor plasmids may be reflected by the differences in the levels of tat protein produced by these plasmids in the transfected cells. Recently, we have found that trans-activation by tat is exquisitely sensitive to the amount of ptat plasmid in the cotransfection experiments (M. Chowdhury, J. P. Taylor, H.T., J.R., F.W.-S., S.A., and K.K., unpublished results). Thus, it is likely that similar to HTLV-1 Tax protein (40), the concentration of tat protein in the cells is critical for induction of the responsive promoters. Alternatively, it is possible that the other open reading frames present in pCV-1, by expressing their corresponding proteins (i.e., rev and nef) down-modulate the trans-activation phenomenon. Preliminary results of the effect of nef protein on the basal and induced

FIG. 3. CAT activity directed by JCV late promoter is induced by HIV-1 tat protein in glial cells. pJCV_L-CAT (3 μ g) was introduced alone (lane 1) or with 12 μ g of pCV-1 (lane 2), 12 μ g of ptat (lane 3), 12 μ g of pBJC-T (lane 4), or 15 μ g of pCV-1 plus pBJC-T (7.5 μ g of each) (lane 5) by calcium phosphate procedures. The final DNA concentration was adjusted to 30 μ g with plasmid pUC18 DNA. Extracts were prepared 48 hr posttransfection and analyzed for CAT activity as described (30). (*Inset*) Conversion of chloramphenicol to its acetylated forms was determined by thin-layer chromatography. Relative stimulations by the cotransfected plasmids are graphed.

levels of JCV_L activity have indicated that nef has no effect, positive or negative, on JCV_L promoter function (M. Chowdhury, J. P. Taylor, H.T., J.R., F.W.-S., S.A., and K.K., unpublished results).

HIV-1 tat and JCV T antigen appear to activate JCV_L promoter gene expression through different mechanisms. Whereas trans-activation by T antigen is not cell type-specific, trans-activation by tat is more significant in glial cells. Furthermore, the effects of these proteins on promoter activity appear to be synergistic. Cotransfection of pJCV_L-CAT with pCV-1 and pBJC-T results in an 85-fold increase in CAT activity (Fig. 3, lane 5) as compared to their individual trans-activation potential of 6-fold and 10-fold, respectively (Fig. 3, lanes 2 and 4). This is not likely to be due to increased concentrations of tat or T antigen as a result of activation by one viral protein or the production of the other. Thus, the observed CAT level in the triple-transfection experiment described above predominantly reflects the effects of the JCV T antigen and HIV-1 tat on the JCV_L promoter.

HIV-1 tat Protein Stimulates Transcription of JCV_L Promoter. Although there are a number of studies suggesting a post transcriptional component of tat-induced trans-activation of HIV-1 LTR (36, 41–43), it appears that the initial effect of the viral trans-activator is to increase the rate of transcription initiators from HIV-1 LTR (44, 45). To better understand the mechanism by which HIV-1 tat protein modulates JCV gene expression in glial cells, we measured steady-state RNA levels in transient transfection experiments by the S1 nuclease protection assay. A uniformly labeled, single-stranded probe containing a sequence complementary to the CAT coding sequence was hybridized to total cellular RNA extracted 48 hr after transfection. In this assay we specifically

measured the levels of CAT RNA but not the start sites of RNA synthesis. The experiment illustrated in Fig. 4A demonstrates the protection of an appropriately cleaved 256-nucleotide DNA fragment, corresponding to protection by CAT mRNA transcribed from the JCV_L promoter in glial cells. The S1-protected fragment was observed with RNA samples from cells cotransfected with pJCV_L-CAT plus either pCV-1 (Fig. 4A, lane 2), ptat (Fig. 4A, lane 3), or pBJC-T (Fig. 4A, lane 4). Stable CAT mRNA was not detected in cells transfected with pJCV_L-CAT alone. The most substantial RNA increases were obtained by cotransfection with ptat. To

FIG. 4. CAT RNA analysis by S1 protection and nuclear run-on assays. (A) S1 nuclease analysis of CAT RNA obtained from transfected glial cells. At 48 hr posttransfection, total RNAs were isolated from the cells by the hot phenol method as described by Queen and Baltimore (31) and hybridized to a single-stranded uniformly labeled CAT DNA probe (32). Hybridization was done according to the procedure described previously (34). After hybridization, 9 volumes of buffer [0.25 M NaCl, 0.03 M sodium acetate (pH 4.6), 1 mM ZnSO₄], which had been preequilibrated to 4°C, was added. After addition of S1 nuclease to a final concentration of 800 units/ml, the reaction mixture was incubated for 90 min at 37°C. Protected DNA fragments were then purified and analyzed by electrophoresis in a denaturing acrylamide/urea gel (46). Lanes: 1, pJCV_L-CAT; 2, pJCV_L-CAT plus pCV-1; 3, pJCV_L-CAT plus ptat; 4, pJCV_L-CAT plus pJC-T; lane 6, control S1 probe. The positions of the 458-nucleotide probe and the 256-nucleotide S1-resistant fragment are shown on the left side of the gel. (B) Nuclear run-on assay using nuclei prepared from the transfected cells. Briefly, 48 hr after transfection, cells were washed with phosphate-buffered saline, resuspended in hypotonic buffer containing 5 mM dithiothreitol for 10 min at 0°C, and then disrupted with a Dounce homogenizer. Nuclei were collected by centrifugation at 1500 rpm (in a Sorvall H1000B rotor) for 5 min, and the pellet was resuspended in the same buffer and underlaid with the buffer containing 10% (vol/vol) glycerol. After centrifugation, the collected nuclei were resuspended in the same buffer and used for *in vitro* transcription. Nuclear transcription was carried out by the methods of Groudine *et al.* (33). Hybridization of the *in vitro*-labeled RNAs to filter-bound DNAs (10, 5, and 0.5 µg; lanes 1, 2, and 3, respectively) were performed by the method described previously (34) using a dot-blot apparatus.

determine whether tat exerts its effects on pJCV_L-CAT RNA at the level of transcription or RNA stabilization, nuclear run-on transcription analysis was performed. The ³²P-labeled RNAs synthesized in isolated nuclei from transfected cells were hybridized to a 250-bp HindIII-EcoRI DNA fragment containing the CAT coding sequence bound to a nitrocellulose filter. Fig. 4B demonstrates the rate of CAT RNA transcription. No CAT RNA was detected in cells transfected with pJCV_L-CAT alone. Similar to results obtained from stable mRNA measurements, high levels of pulse-labeled CAT RNA were synthesized in nuclei from cells cotransfected with pCV-1 or ptat. The level of actin RNA synthesis was low but remained unaffected by cotransfection with trans-activator plasmids (data not shown).

DISCUSSION

The HIV-1 *tat* gene product stimulates gene expression from the viral LTR and is an essential component for the establishment of a productive viral infection (23, 47). The results of the experiments presented here establish that tat protein is able to induce a heterologous viral promoter such as JCV_L as well as its own promoter. An intriguing feature of this finding is that the trans-activation of JCV_L by tat occurs in a cell type-specific manner; i.e., it is more significant in glial cells. A number of studies have suggested that tat exerts its trans-activation function on HIV-1 LTR by increasing steady-state levels of viral mRNAs (37, 42, 48). It appears that these increases are the result of transcriptional activation (44, 45, 49), but evidence indicating the involvement of tat on transcription elongation (48) and mRNA utilization (41, 42) have also been reported. We have shown by nuclear run-on and S1 nuclease experiments that the primary effect of tat on JCV_L expression is to enhance the rate of transcription. Whether or not tat directly influences initiation of JCV_L gene transcription or functions as an anti-terminator of transcription is presently unknown.

Deletion analysis within the HIV-1 LTR has suggested that induction of HIV LTR is mediated by a short DNA segment located within the region from +18 to +44 relative to the transcription initiation site (49, 50). Nucleotide sequence comparison of the JCV_L control region with the HIV-1 LTR showed 63% sequence similarity between the JCV 98-bp enhancer/promoter at nucleotides 58–87 (51) and the HIV-1 LTR at nucleotides +20 to +48. The sequence CTGGGA, which is found at the tip of the predicted stem-loop structure of HIV-1 transcripts, has been demonstrated to be required for tat trans-activation (52). The sequence CTAGGGA found within the homologous region of the JCV enhancer is almost identical to this motif, although it contains one additional nucleotide. Whether the LTR-homologous sequence located within the JCV genome serves as a target for the observed trans-activation of the JCV_L promoter by tat in glial cells remains to be determined. It is tempting to speculate that a similar mechanism may contribute to the observed effect, since some late viral transcripts contain the trans-activation responsive homologous sequences in their 5' leader regions (35).

Cross-communication between HIV-1 and several DNA viruses has previously been investigated (53). Results from those studies suggest that a number of trans-regulatory proteins from the papovavirus, adenovirus, and herpesvirus families elevate HIV-1 gene expression in various host cells. In this communication, we demonstrate that the HIV-1-encoded regulatory protein tat could alter the regulatory pathway of a eukaryotic transcription unit in the proper cell type. We believe that a number of other viral and perhaps cellular genes may contribute, through activation and/or suppression by HIV-1-encoded regulatory proteins, to the wide range of clinical diseases prevalent in AIDS patients.

Indirect evidence that HIV-1-encoded proteins may stimulate cellular gene expression comes from several sources. Vogel *et al.* (54) have shown that transgenic mice expressing the HIV-1 *tat* gene develop dermal lesions similar to Kaposi sarcoma. Nakamura *et al.* (55) have found that T lymphocytes infected with some human retroviruses, including HIV-1, produce several growth factors *in vivo* that are required for the growth of Kaposi sarcoma cells *in vitro*. Whether these observations are the direct result of HIV-1-encoded proteins or cellular responses to viral gene expression is presently unknown.

The molecular mechanism involved in the neuropathogenesis of AIDS patients is not well understood. Our results suggest that interaction between HIV-1 and JCV in infected cells may facilitate JCV replication by stimulation of viral late gene expression. Studies on the JCV lytic cycle in a tissue culture system should determine whether or not coinfections of HIV-1 and JCV increase replication of the JCV in glial cells. These findings present a possible *in vivo* mechanism for the high prevalence of PML in AIDS patients and provide some clues to understanding the mechanisms involved in the development of AIDS dementia and other neurologic disorders seen in AIDS patients.

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