Coordinate effects of human immunodeficiency virus type 1 protein Tat and cellular protein Purα on DNA replication initiated at the JC virus origin

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JC virus (JCV) causes progressive multifocal leukoencephalopathy, a demyelinating disease in brains of individuals with AIDS. Previous work has shown that the Tat protein, encoded by human immunodeficiency virus type 1 (HIV-1), can interact with cellular protein Purα to enhance both TAR-dependent HIV-1 transcription and JCV late gene transcription. Tat has been shown to activate JCV transcription through interaction with Purα, which binds to promoter sequence elements near the JCV origin of replication. DNA footprinting has shown that Purα and large T-antigen cooperatively interact at several binding sites in the origin and transcriptional control region. Overexpression of Purα inhibits replication initiated at the JCV origin by T-antigen. In transfected glial cells Tat reversed this inhibition and enhanced DNA replication. In an in vitro replication system maximal activation by Tat, more than sixfold the levels achieved with T-antigen alone, was achieved in the presence of Purα. Effects of mutant Tat proteins on both activation of replication and binding to Purα have revealed that Cys22 exerts a conformational effect that affects both activities. The origin of an archetypal strain of JCV was less susceptible to activation of replication by Tat relative to the rearranged Mad-1 strain. These results have revealed a previously undocumented role for Tat in DNA replication and have indicated a regulatory role for JCV origin auxiliary sequences in replication and activation by Tat.

Introduction

The circular DNA papovavirus JC (JCV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a demyelinating disease resulting from destruction of infected oligodendrocytes in the brain (for review see Berger & Major, 1999). Although more than 90% of adults demonstrate antibodies to JCV, only a small percentage of immunosuppressed people develop PML. With the onset of the AIDS epidemic PML has become prominent, and it is now found in approximately 4% of all AIDS cases (Berger & Major, 1999). Since the incidence of PML in AIDS is particularly high (Berger et al., 1987; Stoner et al., 1986), the hypothesis has been put forth that human immunodeficiency virus type 1 (HIV-1) may directly influence infection by JCV (Krachmarov et al., 1996; Tada et al., 1990). HIV-1 infects primarily microglial cells and astrocytes in the brain (Bagasra et al., 1996; Tornatore et al., 1994), while JCV infects primarily oligodendrocytes (ZüHBeathe & Chou, 1965). There is no evidence for coinfection of cells by the two viruses. Local, severe HIV encephalitis has previously been observed in PML lesions (Vazeux et al., 1990), highlighting the close proximity of cells infected by the two viruses. Although productive HIV infection is not always associated with PML lesions, it is known that HIV-1 can infect astrocytes in a limited fashion, and that early proteins, including Tat, may be produced in the absence of virus particles (Shahabuddin et al., 1996). The strains of JCV identified in the brains of individuals with PML are distinct from an archetypal strain frequently detected in the blood or urine of individuals without disease. While multiple JCV strains have now been detected in PML tissue, the patterns of difference from the archetypal strain are similar, all involving rearrangements in the late promoter side of the origin of...
replication. Little is known about the apparent reactivation of JCV in the brain in AIDS patients, but the observed sequence rearrangements may well play a prominent role. As does its kindred papovavirus, SV40, JCV relies on its virally encoded large T-antigen for initiation of replication. JCV T-antigen is highly homologous to the SV40 counterpart, and primary T-antigen binding sites in both viruses are also similar. Research has identified flanking sequences critical for initiation at the JCV origin. In particular a repeated pentanucleotide, AGGGA, at the late side of the origin of the Mad-1 strain of JCV is critical for maximal replication initiated by the JCV T-antigen (Chang et al., 1994; Lynch & Frisque, 1991). It has been reported that this element exists in a non-B DNA configuration (Amirhaeri et al., 1988). The pentanucleotide, with its G triplet repeats, is bound avidly by Purz (Chen et al., 1995). Both pentanucleotide repeats are adjacent to an A–T tract, also critical for initiation (Lynch & Frisque, 1990). This arrangement, while repeated twice at the Mad-1 origin, is absent from that of the archetypal strain.

A variety of cellular proteins have been identified which bind to Tat (Desai et al., 1991; Jeang et al., 1993; Kashanchi et al., 1994; Ohana et al., 1993; Taylor et al., 1994; Yu et al., 1995), including cyclin T1, the activator of Cdk9, the PITALRE kinase capable of phosphorylating the C-terminal domain of RNA polymerase II (Mancebo et al., 1997; Wei et al., 1998). In addition, Tat binds the ubiquitous cellular single-stranded DNA- and RNA-binding protein Purz. Purz has been observed to bind the HIV-1 TAR RNA element, at a site distinct from that at which Tat binds the element, and to activate HIV-1 transcription in a TAR-dependent manner (Chepenik et al., 1998). Recently Tat has been colocalized with Purz in nuclei of cultured human glial cells constitutively producing both proteins (Wortman et al., 2000). Tat has been shown to activate transcription at the major late promoter of JCV through its interaction with Purz (Chen et al., 1995). Tat does not itself bind to JCV DNA, but Tat and Purz together bind to PUR elements and synergistically activate transcription (Krachmarov et al., 1996). These PUR elements are located in and near the JCV origin of DNA replication, where Purz and large T-antigen interact to influence binding of both proteins (Chen et al., 1995). Tat can freely traverse cell membranes and enter adjacent cells in a capacity to alter gene activity (Ensoli et al., 1990, 1993; Ezhevsky et al., 1997; Frankel & Pabo, 1988; Hofman et al., 1993; Schwarze et al., 1999). We have asked here whether the Tat–Purz–DNA interaction could affect not only gene transcription but DNA replication as well. Results have demonstrated that Tat stimulates replication initiated at the JCV origin both in vitro and in vivo, displaying a heretofore unknown activity of this pathogenic protein.

Methods

**JCV DNA replication in transfected human glial cells.** Plates of 1.5 × 10⁶ U-87MG human astrocytic glial cells were transfected with 3 µg of plasmid pBlCAT3-Mad₁, containing a 388 bp segment from the JCV Mad-1 strain comprising the origin of replication, as described previously (Chang et al., 1996). Alternatively, transfection was performed with a control plasmid, pBlCAT3, with no insert. The following additional plasmids were cotransfected as indicated: pCT, expressing JCV large T-antigen; pCMV-Purz, expressing the cellular protein Purz; and pTat, expressing the HIV Tat protein. All transfected plates were balanced to the same concentration of total plasmid DNA using respective empty vectors. At 72 h after transfection, plasmid DNA was recovered by the method of Hirt (1967) as previously modified (Johnson & Jelinek, 1986). This method involves extraction of total cellular DNA in SDS and selective removal of chromosomal DNA by precipitation with NaCl. Recovered DNA was treated with restriction endonucleases SacI and DpnI, and subjected to agarose gel electrophoresis as described previously (Chang et al., 1996). DpnI-resistant bands were detected in gel blots by hybridization to the origin insert labelled with [³²P]Phosphate. Intensities of fully DpnI-resistant SacI bands, representing replicated DNA, were obtained using Imagequant 1.2 and analysed using Photoshop 5.0.

**JCV DNA replication in vitro.** Plasmid DNA used for templates in the replication reaction were prepared without exposure to phenol, ethidium or UV light to minimize nicking and allow for high yields of highly supercoiled DNA. Plasmids were propagated in E. coli strain XL-1 Blue (Stratagene). After alkaline lysis, plasmid DNA was isolated using the Qiagen Maxi Prep kit. Plasmids used consisted of > 95 % supercoiled DNA. Plasmids, described in detail in the text, were pBlCAT3-Mad₁, containing the Mad-1 strain origin of replication, pCV archetyp, containing the archetypal origin in the same vector as that for the Mad-1 origin, and pBlCAT3 with no insert. Replication in vitro of plasmid DNA was carried out in 25 µl reaction vols containing, 30 µM HEPES buffer, pH 7.5, 7 mM MgCl₂, 4 mM ATP, 100 µM each of dATP, dGTP, dCTP, TTP, 50 µM each of GTP, CTP, UTP, 40 mM phosphocreatine, 0.625 units of creatine phosphokinase, 400 ng of plasmid DNA, 90 µl HeLa cell extract (CHIMERx, 240 µg protein, added last to begin the reaction) and 100 Ci of [³²P]dCTP (New England Nuclear; 3000 Ci/mmol). JCV T-antigen was prepared from extracts of SF9 cells infected with baculovirus vector bearing the T-antigen gene and purified by immunoaffinity chromatography. In certain experiments SV40 T-antigen (CHIMERx) was substituted with no noticeable effect. Glutathione S-transferase (GST)–Purz and GST–Tat were purified as previously described (Johnson et al., 1995). After 2.5 h at 37 °C, reactions were stopped by addition of 200 µl of 10 mM EDTA with 2 µg yeast tRNA. After three extractions with phenol–chloroform:isoamyl alcohol (50:49:1) and two extractions with ice-cold diethyl ether, DNA was recovered by the method of Hirt (1967) as previously modified (Johnson et al., 1995). Incorporation of [³²P] into a labelled reference band was determined by scintillation spectrometry. Comparison of band intensities with this reference band was used to calculate [³²P]dCMP incorporation.

**Binding of Tat or Tat mutant proteins to Purz.** HIV-1 Tat proteins were bacterially produced as GST fusion proteins and coupled to glutathione–agarose beads. Beads coupled to equimolar amounts of Tat or each of the Tat mutants were reacted with a 20-fold excess of Purz (2 × 10⁻⁷ M), derived by thrombin cleavage of GST–Purz. GST–Tat is not cleaved by thrombin in the binding buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% Nonidet P-40, 10 mM PMSE, 1 µg/ml aprotinin, 1 µg/ml leupeptin). After binding for 30 min and washing with binding buffer (Johnson et al., 1995), proteins
we sought to determine whether Tat modulates the replicative effect of Purα (Krachmarov et al., 1998, 1999)., 1998, 1999. Previous studies had demonstrated a pronounced inhibitory effect of Purα overexpression upon replication initiated at the JCV origin in U-87MG human astrocytic glial cells (Chang et al., 1996). Since Tat is known to bind to Purα (Chepenik et al., 1998; Gallia et al., 1998, 1999b; Krachmarov et al., 1996; Wortman et al., 2000), and to alter its in vivo functions (Krachmarov et al., 1996), we sought to determine whether Tat modulates the replicative effect of Purα. The effect of Tat upon replication initiated at the JCV origin in human cells was examined in vivo using human U-87MG glial cells transfected with plasmid pBLCAT3-Mad1L, bearing the JCV origin of replication (Fig. 1). The columns of the histogram were derived from densitometry of DpnI-resistant bands from a SacI digest of pBLCAT3-Mad1L, as revealed by hybridization. The first column, representing a relative density of zero, shows that replication did not occur in the absence of a plasmid, pJCT, expressing JCV large T-antigen. Overexpression of Purα in these cells inhibited plasmid replication (columns labelled Purα). Since high Purα levels in G0 have also been observed to block the CV-1 cell cycle at the G0–S boundary (Stacey et al., 1999), it is conceivable that inhibition of the onset of replication is a cellular function of Purα. However, U-87MG cells possess endogenous Purα, and it is thus also conceivable that the inhibition does not represent a replicative function of the protein. Instead, overexpressed Purα in the absence of partner proteins such as Tat, could exert a squelching effect by drawing associated proteins, such as cyclin A (Itoh et al., 1998), away from their site of action in replication. In any case, Tat expression reversed the inhibition by Purα to levels seen with T-antigen alone (columns labelled Tat + Purα). Expression of Tat in the glial cells yielded a dose-dependent activation of replication initiated at the JCV origin. The following additional plasmids were cotransfected as indicated: pJCT, expressing HIV-1 Tat in the presence of cellular Purα, known at precisely what levels Purα exerts its effects on replication, we have employed an in vitro DNA replication system. Slight incorporation into DNA. To more precisely dissect the mechanism by which Tat influences replication through an ability to enhance expression of T-antigen from the pJCT vector thereby eliciting the appearance of enhancing replication. We have previously ruled out this possibility by assaying levels of T-antigen in transfected U-87MG cells via immunoblotting. In that experiment Tat exerted no such effect on the T-antigen expression vector (Chang et al., 1996). It is likely that Tat functions indirectly in that Tat does not directly bind to JCV DNA. To more precisely dissect the mechanism by which Tat exerts its effects on replication, we have employed an in vitro JCV DNA replication system.

were extracted from beads in SDS sample buffer, subjected to SDS–PAGE on a 10% gel, blotted to an Immobilon P membrane and probed with anti-Purα monoclonal antibody 9C12. Detection was with the Pierce SuperSignal Enhancer system.

Results

Enhancement by Tat of replication initiated at the JCV origin in human glial cells and reversal of an inhibitory effect of Purα

Previous studies had demonstrated a pronounced inhibitory effect of Purα overexpression upon replication initiated at the JCV origin in U-87MG human astrocytic glial cells (Chang et al., 1996). Since Tat is known to bind to Purα (Chepenik et al., 1998; Gallia et al., 1998, 1999b; Krachmarov et al., 1996; Wortman et al., 2000), and to alter its in vivo functions (Krachmarov et al., 1996), we sought to determine whether Tat modulates the replicative effect of Purα. The effect of Tat upon replication initiated at the JCV origin in human cells was examined in vivo using human U-87MG glial cells transfected with plasmid pBLCAT3-Mad1L, bearing the JCV origin of replication (Fig. 1). The columns of the histogram were derived from densitometry of DpnI-resistant bands from a SacI digest of pBLCAT3-Mad1L, as revealed by hybridization. The first column, representing a relative density of zero, shows that replication did not occur in the absence of a plasmid, pJCT, expressing JCV large T-antigen. Overexpression of Purα in these cells inhibited plasmid replication (columns labelled Purα). Since high Purα levels in G0 have also been observed to block the CV-1 cell cycle at the G0–S boundary (Stacey et al., 1999), it is conceivable that inhibition of the onset of replication is a cellular function of Purα. However, U-87MG cells possess endogenous Purα, and it is thus also conceivable that the inhibition does not represent a replicative function of the protein. Instead, overexpressed Purα in the absence of partner proteins such as Tat, could exert a squelching effect by drawing associated proteins, such as cyclin A (Itoh et al., 1998), away from their site of action in replication. In any case, Tat expression reversed the inhibition by Purα to levels seen with T-antigen alone (columns labelled Tat + Purα). Expression of Tat in the glial cells yielded a dose-dependent activation of replication initiated at the JCV origin. The following additional plasmids were cotransfected as indicated: pJCT, expressing HIV-1 Tat in the presence of cellular Purα, known at precisely what levels Purα exerts its effects on replication, we have employed an in vitro JCV DNA replication system. Slight incorporation into DNA. To more precisely dissect the mechanism by which Tat influences replication through an ability to enhance expression of T-antigen from the pJCT vector thereby eliciting the appearance of enhancing replication. We have previously ruled out this possibility by assaying levels of T-antigen in transfected U-87MG cells via immunoblotting. In that experiment Tat exerted no such effect on the T-antigen expression vector (Chang et al., 1996). It is likely that Tat functions indirectly in that Tat does not directly bind to JCV DNA. To more precisely dissect the mechanism by which Tat exerts its effects on replication, we have employed an in vitro JCV DNA replication system.

Maximal stimulation of JCV replication in vitro by HIV-1 Tat in the presence of cellular Purα

An in vitro system was employed to investigate whether Tat can directly influence JCV DNA replication. A system utilizing HeLa cell extracts has recently been shown to effectively replicate plasmids bearing a JCV origin (Nesper et al., 1997). Using a similar system it was affirmed in Fig. 2 that replication depends upon the presence of JCV T-antigen and upon the presence of a JCV origin of replication. The origin used for Fig. 2 was a 401 bp segment from the Mad-1 strain of JCV, a strain representative of rearranged strains frequently detected in brains of AIDS patients (Major et al., 1992; Newman & Frisque, 1997). In these experiments 32P-labelled plasmid DNA recovered from the replication reaction was linearized with HindIII and treated with DpnI. Resistance to restriction endonuclease DpnI is conferred upon plasmid DNA, propagated in dam+ strains of E. coli, upon replication in a mammalian system. Slight incorporation into DpnI-cleaved
bands in the absence of T-antigen was due to repair activities and artefactual nick translation. Only the topmost band, 4.3 kb, representing full-length DpnI-resistant DNA was taken as a measure of replication. There was no incorporation into this band in the absence of T-antigen. In the presence of T-antigen incorporation was seen only when the plasmid template contained a JCV origin of replication.

It has previously been reported that Tat effects on JCV late gene transcription are mediated through PUR elements in the promoter (Chowdhury et al., 1993; Krachmarov et al., 1996). Tat alters the ability of Purα to bind to these elements (Krachmarov et al., 1996), which are also part of the origin of replication. Footprinting has detailed the binding of Purα and T-antigen to these elements in the Mad-1 promoter (Chen et al., 1995), certain of which are reported to be critical for JCV DNA replication (Chang et al., 1994; Lynch & Frisque, 1990). Therefore, we have examined the effects of modulating either of these proteins upon replication initiated at the Mad-1 origin in vitro. Purα alone could not substitute for T-antigen in ability to initiate DNA replication. These data, constituting blank lanes for different doses of Purα, are not shown. This control was necessary since PUR elements overlap T-antigen binding sites in the JCV origin (Chang et al., 1996). In Fig. 3 the effects of Tat and Purα, either separately or together, upon replication initiated at the JCV origin are presented. Fig. 3(A) shows the effects of varying doses of GST–Tat upon replication with a constant level of GST–Purα. An incremental stimulation of replication by Tat can be seen, beginning at 4 × 10⁻¹⁰ M, peaking at 4 × 10⁻⁸ M and persisting to 10⁻⁷ M. At that point molar levels of Purα and Tat differ by less than an order of magnitude. Tat exerted maximal effects on replication at levels approximately five- to tenfold higher than that of plasmid DNA (10⁻⁸ M). At concentrations above 10⁻⁷ M effects of Tat in the presence of Purα were markedly inhibitory (Fig. 3A, two rightmost lanes). The maximal stimulation by Tat together with Purα vs T-antigen alone (lane 2) in this experiment was more than sixfold, as determined by densitometry of the 4.3 kb bands. Fig. 3(B) shows the effects of Tat and Purα separately upon replication initiated by T-antigen. The autoradiograph for Fig. 3(B) was exposed longer than that for Fig. 3(A). This is evident by comparing the two leftmost lanes in Fig. 3(A) with the two lanes labelled 0 in Fig. 3(B), which represent the effects of T-antigen alone. In both (A) and (B) there is a
Effects of Tat and Purz on JCV DNA replication in vitro

<table>
<thead>
<tr>
<th>Reaction additions*</th>
<th>T-Ag†</th>
<th>GST</th>
<th>GST–Tat</th>
<th>GST–Purz</th>
<th>[^{32}P]dCMP incorporated (pmol)‡</th>
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<tr>
<td>−</td>
<td>−</td>
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<td>3·2</td>
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<td>+</td>
<td>1·7 \times 10^{-8} M</td>
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<td>+</td>
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<td>−</td>
<td>3 \times 10^{-7} M</td>
<td>−</td>
<td>−</td>
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<tr>
<td>+</td>
<td>−</td>
<td>6 \times 10^{-7} M</td>
<td>−</td>
<td>−</td>
<td>3·3</td>
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<td>+</td>
<td>−</td>
<td>5 \times 10^{-8} M</td>
<td>−</td>
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<td>+</td>
<td>−</td>
<td>3 \times 10^{-7} M</td>
<td>−</td>
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<td>+</td>
<td>−</td>
<td>7·5 \times 10^{-7} M</td>
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<td>+</td>
<td>−</td>
<td>1·5 \times 10^{-6} M</td>
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* All reactions were performed with the Mad-1 origin of JCV DNA replication and HeLa cell extracts as described in Methods.
† When present, 1·0 μg of T-antigen was used per reaction.
‡ Incorporation was measured by quantitative densitometry of \[^{32}P\] incorporation into the 4·3 kb, fully replicated gel band.

stimulation of replication of more than twofold by 3 \times 10^{-7} M Purz in the absence of Tat. Stimulation persists to 7·5 \times 10^{-7} M, but at levels higher than 10^{-6} M, Purz is strikingly inhibitory. Given the enhanced exposure of the autoradiograph in Fig. 3(B), stimulation by Purz alone is much less than that by Tat and Purz together. This is further documented in Table 1, which provides quantitative data on effects of T-antigen, Tat and Purz on replication initiated at the JCV origin at a variety of protein concentrations. Table 1 also provides an important control for the in vitro replication reactions: it documents the lack of effect of GST alone on the replication reaction. The right lanes of Fig. 3(B) show the effects of Tat on replication in the absence of added Purz, and they reveal two important aspects of Tat activity. Firstly, there is only a slight effect of Tat on replication in the absence of added Purz. This may not be clearly evident in the published photograph, but band densitometry indicates a 1·2-fold stimulation by 10^{-7} M Tat. This is vastly less than the approximately sixfold stimulation by that concentration of Tat in the presence of Purz. The stimulation of replication by the two proteins is synergistic since the effects of 10^{-7} M Tat and 3 \times 10^{-7} M Purz together, nearly sixfold stimulation, are more than multiplicative of the effects of those concentrations of each protein alone, approximately 1·2-fold and twofold, respectively. Secondly, the effect of Tat alone at concentrations >10^{-7} M is not inhibitory to replication (Fig. 3B, two rightmost lanes), whereas those concentrations in the presence of Purz are markedly inhibitory (Fig. 3A, two rightmost lanes). The effect of high concentrations of Tat in the presence of Purz mimics the effect of higher concentrations of Purz alone (Fig. 3B, second and third lanes). This is further evidence for a cooperative interaction of Tat and Purz. In this and all other experiments purified bacterial GST was employed as a control. GST had only a slightly inhibitory effect upon labelling of the DNA in the presence of T-antigen (Table 1). The ability of Tat to affect replication at very low concentrations lends credence to the notion that Tat may have this effect in the brain. The effects of Tat and Purz in the in vitro system may well reflect the effects of the two proteins in transfected cells (Fig. 1). The effects of high concentrations of Purz are inhibitory to replication in vitro, as are the effects of overexpressed Purz in the U-87MG cells. It must be cautioned, however, that the precise concentration of a protein at a location of activity in a cell cannot be determined due to compartmentalization.

In the absence of Tat and Purz the level of incorporation of dCMP into DpnI-resistant plasmid DNA, 3·2 pmol, translates into a level of 32 pmol dNMP per μg of input plasmid DNA. This is very comparable to levels of incorporation reported by Nesper et al. (1997), who also employed a system using HeLa cell extracts and JCV T-antigen. Levels of incorporation reported here are also quite comparable to those reported for the original SV40 DNA in vitro replication system (Li & Kelly, 1984).

It is helpful to know the endogenous level of Purz in the HeLa cell extract since this would form a background for effects of added Purz. In HeLa cells the levels of Purz fluctuate dramatically during the cell cycle (Itoh et al., 1998). In an asynchronous culture, in which most cells would be in late G1, a time when Purz levels are relatively low, it can be estimated that the intracellular level of the protein is approximately 10^{-9} M. This previously published estimate is based on relative intensities of gel bands in immunoblots (Itoh et al., 1998), and it is undoubtedly crude. It is unlikely, however, to be in error by more than tenfold. Thus, the level of Purz in the HeLa cell extract employed for in vitro replication is likely to be low relative to the levels of GST–Purz and GST–Tat employed in the study.

Effects of mutant Tat proteins on JCV replication in vitro and on binding to Purz

A series of deletion and point mutations of Tat were used to examine the contributions of different Tat domains to both Purz binding and Tat’s replicative effects. The method of standardizing molar concentrations of the mutant Tat proteins has been described in two previous publications, which are in good agreement regarding effects of the mutations on Tat binding to Purz (Gallia et al., 1999a; Wortman et al., 2000).
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**Fig. 4.** Effects of Tat mutations on the ability of Tat to bind to Purα and to enhance replication initiated at the JCV origin. Top, mutational analysis of Tat protein domains involved in activation of replication initiated at the JCV origin. Replication reactions were performed as described for Fig. 2 in the presence of 1 μg of JCV T-antigen and 150 ng of Purα (10^{-7} M). No Tat was added in lane 1. In the remaining lanes GST–Tat (Tat86) or GST–Tat mutants were present at the same molar concentration of 1.3 × 10^{-7} M, corresponding to 127 ng of wt Tat. Bottom, mutational analysis of Tat protein domains involved in binding to Purα. Binding of Purα to Tat or its mutant proteins fused to GST and immobilized on glutathione–agarose beads was performed as described in Methods. Shown is an immunoblot showing mutant Tat protein effects on replication are positioned directly above respective lanes showing effects on binding to Purα.

The bottom panel of Fig. 4 presents Purα binding to different GST–Tat mutants immobilized on glutathione–agarose beads, and the top panel presents effects of purified GST–Tat and its mutants on replication initiated in vitro at the JCV origin. Since wild-type Tat with a deletion of aa 2–36, Tat86A (2–36), bound Purα, as did Tat48, albeit weakly, Tat amino acids from 37–48 were critical for binding to Purα. There was no Tat mutation that restricted Purα binding while still allowing enhanced replication of the JCV origin-bearing plasmid. As seen in the top panel of Fig. 4, and consistent with Fig. 3(A), full-length, wild-type Tat86 produced a dramatic stimulation of replication of the JCV origin-bearing plasmid. Intriguingly, all of the examined Tat mutants inhibited overall replication, but to different degrees. The reason for such inhibition is not known, but it reflects the ability of different domains in the Tat protein to affect the replicative process. Both replicative effects of Tat and Tat ability to bind Purα were influenced by a global, conformational effect of Tat aa Cα22. When Cα22 was mutated to G in either Tat72Cα22 → G or Tat48Cα22 → G, the resulting protein was especially inhibitory to replication. Consistent with mediation of Tat replicative effects through a Tat interaction with Purα is the observation that when Cα22 was mutated in Tat48 it was more detrimental to both Purα binding and JCV DNA replication than when Cα22 was mutated in Tat72. It is likely that presence of Cα22 induced a global change in Tat that helped configure it for Purα binding. This configuration could also be induced when the entire amino terminal region was deleted, as in Tat86Δ(2–36). This mutation still allowed Purα binding although it diminished Tat replicative effects. This may indicate that amino acids in the region 2–36 promoted effects on replication complementary to effects requiring Purα binding. Furthermore, Cα22 may have, through a conformational effect, influenced Purα binding without actually contacting Purα. While a global effect of Cα22 is important for both Purα binding and replicative effects of Tat, it is clear that Tat domains other than those involved in Purα binding are also critical for these effects. Tat72 bound Purα very well whereas this deletion was detrimental to DNA replication. It is reasonable that certain domains of Tat could interact with Purα while other domains would remain accessible to interact with additional proteins or RNA that could influence the replication apparatus. It has been reported that Tat binding to transcription factor TFIID is dependent upon Tat residues 36–50 (Kashanchi et al., 1994). The critical role of Cα22 in influencing Purα binding may play a role not only in effects of Purα on JCV DNA replication, but also in observed effects of Purα on HIV-1 transcription (Chepenik et al., 1998). It is notable that while Tat72 was capable of HIV-1 transcriptional activation, Tat72Cα22 → G was not (Rhim et al., 1994). The specific importance of Cα22 is further emphasized by the observation that whereas Tat48 bound transcription factor TFIH in vitro, Tat48Cα22 → G did not (Parada & Roeder, 1996).

**Effects of rearrangements in JCV origin sequences upon enhancement of replication by Tat**

Tat exerted a differential effect upon replication of Mad-1 vs archetype strains of JCV. Due to the nature of sequence differences between strains, it is generally believed that Mad-1 and other strains found in PML have arisen from the archetype through rearrangement. Detection of both archetype and several rearranged variants of JCV in multiple tissues from a paediatric PML patient have raised the question of whether archetype and rearranged strains of JCV have different replication capabilities in different tissues (Newman & Frisque, 1997). In Fig. 5(A) a comparison is presented of the abilities of Tat and Purα to enhance replication initiated at either the Mad-1 or archetype origin in the in vitro system. For this figure a low concentration of T-antigen was used to ensure that both plasmids would be in the linear response range. The two origins have previously been shown to sustain T-antigen-dependent replication in glial cells (Ault, 1997). It can be seen in Fig. 5(A) that both origins initiated full-length plasmid replication in the presence of T-antigen. Replication initiated at both origins was enhanced by Tat and Purα. However, in the presence of Purα the Mad-1 origin responded dramatically to the presence of Tat (7-2-fold stimulation vs Purα alone), while the archetype origin responded weakly (1-6-fold stimulation vs...
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Fig. 5. Differential effects of Tat and Purα on DNA replication initiated at origins of Mad-1 vs archetype JCV strains. The in vitro DNA replication system was employed as described in Methods. Labelled plasmids were linearized with HindIII and treated with DpnI as described for Fig. 2 to assess replication. (A) Comparison of plasmid replication initiated at Mad-1 or archetype origins. The plasmids subjected to replication were pBLCAT3-Mad1L and pJCV archetype, containing the Mad-1 and archetype replication origins, respectively. For these plasmids each origin insert was cloned into the BamHI site of pBLCAT3. The origin inserts were generated by PCR using the same primer set: forward, 5'-CATTTTTGCTTTTTGTAGC and reverse, 5'-CCAAAACAGCTCTGGCTCGC, both coupled to BamHI linkers. The BamHI Mad-1 insert was thus 401 bp, and the archetype insert was 391 bp. In the replication system SV40 T-antigen was supplied at 0–25 µg; GST–Purα at 150 or 450 ng; GST–Tat at 75 or 150 ng. A DpnI-resistant band at 4–3 kb represents full-length replication for each plasmid. (B) Comparison of sequences in the late-promoter sides of Mad-1 and archetypal origins. Numbering is that of Frisque et al. (1984). While there is considerable sequence variation among both strain types, the Mad-1 insert used here can be considered to be derived from the archetype strain by deletion of 23 and 66 bp segments (shaded boxes) and duplication of a resulting 98 bp segment, as indicated by dashed lines. The original sequences at break points are shown above the archetype map. The asterisk denotes common sequence variants at one break point. New sequences created by the deletions and repetition are shown below the Mad-1 map. The L and arrow indicate the direction of late gene transcription.

450 ng of Purα alone) as revealed by densitometry of the bands at 4:3 kb in Fig. 5(A). This experiment indicates that Tat and Purα affect initiation, rather than some aspect of elongation, since the two plasmids are of nearly the same size and since vector sequences outside the origins are identical. Nonetheless, further experiments will be necessary to determine whether effects are on initial unwinding or on entry of proteins comprising the replication apparatus. This result highlights the importance of JCV origin auxiliary sequences to overall replication.

The sequences of the two origins are schematized in Fig. 5(B), which specifies new sequences created in the Mad-1 variant by deletion of 23 and 66 bp regions from the archetype and by repetition of a resulting 98 bp segment. The greater response of the Mad-1 origin to Tat and Purα may reflect the fact that the A–T tract of the first 98 bp Mad-1 repeat is between two sets of Purα-binding elements: the GAGGC T-antigen binding repeat, located at −13 to +12 on the Mad-1 map at bottom of Fig. 5, and an AGGGA repeat created by the rearrangement, symbolized by the line at +37. It has previously been shown that Purα binds to both of these elements (Chang et al., 1996). The repeated AGGGA pentamer is critical for JCV DNA replication (Chang et al., 1994; Lynch & Frisque, 1990). Previous footprinting studies have docu-
mented a cooperative effect of Purz and T-antigen on binding to DNA between the AGGGGA and GAGGC repeats (Chang et al., 1996). New studies, to be presented elsewhere, reveal effects of Purz and Tat on local DNA unwinding at the A–T tract in the different JCV origins. While there may be conditions, other than those used here, under which Tat can more strongly alter archetype DNA replication, the present results highlight the different replicative capacities of the two origins in a given system.

Discussion

The present results indicate that, in addition to the demonstrated effects of Tat on JCV gene transcription (Chowdhury et al., 1993; Krachmarov et al., 1996), Tat may act to directly stimulate JCV DNA replication. Aside from the possibility of cell co-infection with HIV-1 and JCV, which has not been demonstrated, it is conceivable that Tat, secreted from an HIV-1-infected cell, could enter a neighbouring cell harbouring JCV. In fact, fusion with a Tat domain has been used as a method of transducing cells directly with protein (Schwarze et al., 1999). Since the mechanism by which Tat is secreted and taken up by cells is essentially that of diffusion, concentrations of the protein in cells adjacent to those producing Tat could be effectively high, although that remains to be experimentally demonstrated for cells in the brain. Tat, in conjunction with Purz, can activate the late promoter of JCV (Chen et al., 1995; Chowdhury et al., 1993; Krachmarov et al., 1996). Finally, Tat can enhance JCV DNA replication, activated by T-antigen. While such a mechanism is demonstrably applicable to brain, it may also be applicable to other tissues infected by HIV-1 since both Purz and DNA or RNA PUR elements are ubiquitously present in human tissues. It should be noted that potential exposure to Tat would not fully explain the activation of JCV infection in humans. Many rearrangements of JCV regulatory sequences have been observed in PML, and we have here examined only one of them. It remains to be determined whether any pattern of rearrangements is specifically associated with AIDS. Clearly, rearrangement of JCV from the archetypal strain is an important aspect of activation in AIDS, and presently little is known about where this rearrangement occurs or what induces it (Major et al., 1992). To help assess relevance of the present results to PML, future experiments should involve transfection of human oligodendrocyte cultures with origin-containing clones from different JCV strains followed by a detailed analysis of response to Tat.

The interaction between Tat and Purz is remarkable in that the action of Purz alone in JCV replication appeared inhibitory whereas the action of Tat and Purz together was stimulatory. Overexpression of Purz upon transfection inhibited replication initiated at the JCV origin in Fig. 1, confirming earlier observation of that effect (Chang et al., 1996). In the earlier study anti-sense expression of Purz cDNA stimulated replication of the JCV-origin-containing plasmid, strongly suggesting that the effect of endogenous Purz was also inhibitory. Any inhibitory effect of Purz is likely to be at the level of initiation rather than elongation. In CV-1 cells microinjection of Purz in S phase had no effect on ongoing cellular DNA synthesis although injected cells completing replication were blocked from entering mitosis (Stacey et al., 1999). Tat clearly reversed the inhibitory effect of over-expressed Purz in vivo (Fig. 1). The stimulation observed with Tat alone on JCV-initiated replication in the U-87MG cells is likely to be due to the same type of interaction of Tat with endogenous Purz. The effect of Purz alone in the in vitro JCV replication system was stimulatory at low concentrations (Fig. 3B, Table 1). This may reflect the known ability of Purz to bind a variety of cell cycle regulatory proteins that it may not necessarily have access to in vivo. The inhibitory effect of Purz at high concentrations may reflect the cellular function of Purz, but it may also be due to an indiscriminate binding of Purz to single-stranded DNA at replication bubbles or to competition with the essential single-stranded DNA-binding protein RPA. In contrast, Tat and Purz acted synergistically to activate JCV replication. This suggests that the effect of Tat is not simply one of titrating away inhibitory Purz. Rather, it is likely that Tat changes the configuration of Purz to generate an altered activity of that protein. Such a change has previously been documented. In the presence of Tat the affinity of Purz for its specific PUR element is strongly enhanced (Krachmarov et al., 1996). Work is currently in progress to obtain genetically deficient Purz cell lines, which will aid in dissecting the synergistic nature of interaction of Tat and Purz.

Results from the in vivo and in vitro JCV DNA replication systems are in reasonably good agreement. In both cases Tat stimulated replication initiated at the JCV origin. In both cases Purz exhibited an inhibitory effect, either when overexpressed in vivo or at higher concentrations in vitro. Future experiments using cells with genetically inactivated PURA genes may provide insight into the stimulatory effect of Purz seen at low concentrations in vitro. Note that one might not necessarily expect any in vivo or in vitro replication systems to be in complete agreement due primarily to issues of compartmentalization. The access of many known regulatory proteins to the replication apparatus is controlled in vivo by nuclear import or exclusion and by post-synthetic modifications, processes difficult to duplicate in vitro. Recent studies indicate that Purz nuclear localization is highly regulated by cell cycle-dependent signals (Barr & Johnson, 2001).

Aside from any potential relevance to PML, the interactions of Tat and Purz with JCV regulatory sequences provide a very useful model system for dissecting molecular pathways of Tat pathogenicity. Purz is expressed in every human cell type thus far examined. PUR elements, such as the Tat-responsive element in the JCV origin/promoter region, are present in many cellular gene promoters, in origins of replication and in human telomeric repeats. In addition, both Tat and Purz are
known to interact with specific RNA sequences (Chepenik et al., 1998; Herault et al., 1995; Kobayashi et al., 2000; Tretiakova et al., 1998). Characterization of the Tat and Purz interaction with PUR elements may help elucidate mechanisms of HIV-1 pathogenicity in AIDS as well as principles of normal cellular regulation.

Results from the in vitro replication system illuminate aspects of initiation of JCV DNA replication. The importance of auxiliary sequences adjacent to virus origins has previously been noted (Gutierrez et al., 1990; He et al., 1993; Li & Botchan, 1993). Auxiliary sequences near the SV40 origin strongly facilitate DNA unwinding by T-antigen while only weakly influencing the binding of that protein to the origin (Gutierrez et al., 1990). While effects of Tat and Purz on JCV replication are clearly dependent on T-antigen, it remains to be determined whether they act at the step of initial DNA unwinding or on the DNA synthetic apparatus. In contemplating how Tat and Purz stimulate JCV replication, parallels may be found in Tat effects on transcription. Tat stimulates transcription of HIV-1 through interaction with an RNA element, TAR, present within the 5′ untranslated leader of HIV-1 transcripts (Berkhout et al., 1989; Churcher et al., 1993; Dingwall et al., 1990; Hamy et al., 1993; Kamne et al., 1991; Luo et al., 1993). Tat enhances HIV-1 transcription, at least in part, by binding to TAR and introducing protein kinases that phosphorylate and enhance processivity of RNA polymerase II (Parada & Roeder, 1996; Inamoto et al., 1993). Tat activates the human immunodeficiency virus through a nascent RNA target. Cell 59, 273–282.


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