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Interaction of HIV-1 Tat With Purα in Nuclei of Human Glial Cells: Characterization of RNA-Mediated Protein-Protein Binding

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Abstract A complex between the Tat protein, encoded by human immunodeficiency virus type 1 (HIV-1), and the cellular protein, Purα, has been implicated in activation of the late promoter of JC virus (JCV) and in enhancement of JCV DNA replication. JCV is the causative agent of progressive multifocal leukoencephalopathy (PML), an acquired immunodeficiency syndrome (AIDS) opportunistic infection of the brain. Purα also binds the HIV-1 *TAR* RNA element and activates HIV-1 transcription, suggesting a role for RNA binding in the action of this protein. Using immunoelectron microscopy, we find that in human glial cells expressing both proteins, Tat and Purα are colocalized in extranucleolar chromatin structural elements. The colocalized Purα and Tat are nearly exclusively nuclear, although individual proteins can be seen in both nucleus and cytoplasm, suggesting a preferential tropism of the complex for the nucleus. Analysis of the interaction between purified proteins indicates that the Tat-Purα interaction is strongly enhanced by the presence of RNA. Tat amino acids from 37–48 are essential for Tat binding. Residues 49–72, including the TAR RNA-binding domain, are critical for binding to Purα, while Cys_{22}, in the Tat transactivation domain, is responsible for an important global effect. Purα repeat II domains are involved in the interaction, and a polypeptide based on one such sequence inhibits binding. After RNase treatment of Purα enhancement of Tat binding can be partially restored by addition of a single-stranded JCV DNA *PUR* element, to which Tat does not bind. The results indicate that the Tat-Purα interaction is direct, rather than through an RNA link, and that RNA binding configures Purα for optimal interaction with Tat. J. Cell. Biochem. 77:65–74, 2000.

Key words: AIDS; JC virus; JCV; progressive multifocal leukoencephalopathy; PML

There is now extensive evidence that several pathological functions of the human immunodeficiency virus type 1 (HIV-1) Tat protein are mediated by the interaction of Tat with cellular protein Purα. In particular, Tat and Purα may mediate a direct effect of HIV-1 upon infection by JC virus (JCV) in the brains of individuals afflicted with acquired immunodeficiency syndrome (AIDS). Tat and Purα can be co-immunoprecipitated from extracts of cultured human glial cells in which both proteins are expressed [Krachmarov et al., 1996]. Tat and Purα synergistically activate transcription at the late promoter of JCV in transfected U87MG cells. In this case, Tat stimulates binding of Purα to a Tat-responsive Purα-binding element, *upTAR*, to which Tat alone does not bind [Chowdhury et al., 1993; Krachmarov et al., 1996]. Recent evidence indicates that Tat can enhance replication initiated by JCV T-antigen at the viral origin and that this effect in vitro is maximal in the presence of Purα (D.C. Daniel, K. Khalili, and E.M. Johnson, submitted for publication).

Tat is an RNA-binding protein that stimulates transcription of HIV-1 through a specific interaction with an element (transactivation

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region, or 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; Kamine et al., 1991; Luo et al., 1993]. While the precise mechanism of Tat activation is unknown, evidence suggests that the protein acts on transcription by introducing associated cellular factors into the vicinity of the transcriptional apparatus that enhance elongation [Graeble et al., 1993], possibly through phosphorylation of RNA polymerase II (RNAPII), increasing processivity [Parada and Roeder, 1996]. A variety of cellular proteins have been identified that bind to Tat [Jeang et al., 1993; Kashanchi et al., 1994; Ohana et al., 1993; Taylor et al., 1994; Yu et al., 1995], including Cdk7 [Cujec et al., 1997] and Cdk9, the PITALRE kinase capable of phosphorylating the C-terminal domain of RNA polymerase II [Mancebo et al., 1997; Wei et al., 1998]. It has recently been demonstrated that Purα binds the HIV-1 TAR RNA element, at a site distinct from that of Tat binding, and that Purα activates HIV-1 transcription in a TAR-dependent manner [Chepenik et al., 1998]. Although Purα also binds Tat, little is known about how RNA binding by Purα might influence its interaction with Tat.

Purα is a highly conserved, eukaryotic sequence-specific single-stranded DNA-binding protein. Purα also binds to RNA, although with much lower affinity [Bergemann and Johnson, 1992; Bergemann et al., 1992]. Purα has been implicated in control of DNA replication [Bergemann and Johnson, 1992; Chang et al., 1996; Itoh et al., 1998; Ma et al., 1994] and in control of transcription of a variety of eukaryotic genes [Du et al., 1997; Herault et al., 1993; Kelm et al., 1997; Krachmarov et al., 1996; Osugi et al., 1996; Zambrano et al., 1997]. Purα and Tat cooperatively stimulate transcription of the JCV late promoter in cotransfected glial cells [Krachmarov et al., 1996]. In transfected U-87MG cells Purα is capable of stimulating transcription of the HIV-1 genome in a TAR-dependent manner [Chepenik et al., 1998]. Although the Tat-binding domains of Purα have been mapped [Krachmarov et al., 1996], the regions of Tat that interact with Purα have not yet been determined. Here we present such an analysis, performed using Tat deletion and point mutants and synthetic Purα peptides corresponding to Tat interaction domains. The results reveal a mechanism whereby binding of Purα to RNA helps configure Purα for maximal interaction with Tat.

MATERIALS AND METHODS

Immunogold Electron Microscopy of Tat-Purα Interaction In Vivo

The human astrocytoma cell line 5–10, constitutively expressing the HIV-1 protein, Tat, was grown in culture as previously described [Krachmarov et al., 1996]. Asynchronous cells in log phase were fixed with 3.7% paraformaldehyde, scraped from the culture dish, embedded in Unicryl, sectioned, and sequentially treated with antibodies or protein A as described previously for CV-1 cells [Itoh et al., 1998]. Controls in which various primary antibodies or secondary labeling agents were omitted are as described in the text. Antibodies and their titers were as follows: anti-Purα monoclonal antibody 9C12 (1:100), protein A coupled to 30-nm gold beads (1:40, Amersham), rabbit anti-Tat polyclonal antibody 705 (1:200, AIDS Research and Reference Reagent Program), goat anti-rabbit antibody coupled to 10-nm gold beads (1:100, Amersham). Sections were then fixed with glutaraldehyde, stained with uranyl acetate and lead citrate, and visualized with a Jeol JEM100CX electron microscope as previously described [Itoh et al., 1998].

Binding Studies Involving Purified Purα, Tat and Mutant Tat Proteins

HIV-1 Tat (from strain isolate HBX2) and Tat mutant proteins were bacterially produced as GST fusion proteins and coupled to glutathione-agarose beads [Krachmarov et al., 1996]. Purα was purified from GST-Purα cleaved with thrombin. In indicated experiments, GST-Purα was treated, before cleavage and purification, with a boiled mixture of RNases A and T1 (1.0 μg/ml each) in 10 mM EDTA and washed extensively with phosphate-buffered saline (PBS). Beads coupled to equimolar amounts of Tat or each of the Tat mutants were reacted with a 20-fold excess of purified Purα (2×10^{-7}M). After binding and washing in buffer containing protease inhibitors as described [Krachmarov et al., 1996], proteins were extracted from beads in sodium dodecyl sulfate (SDS) sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (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.

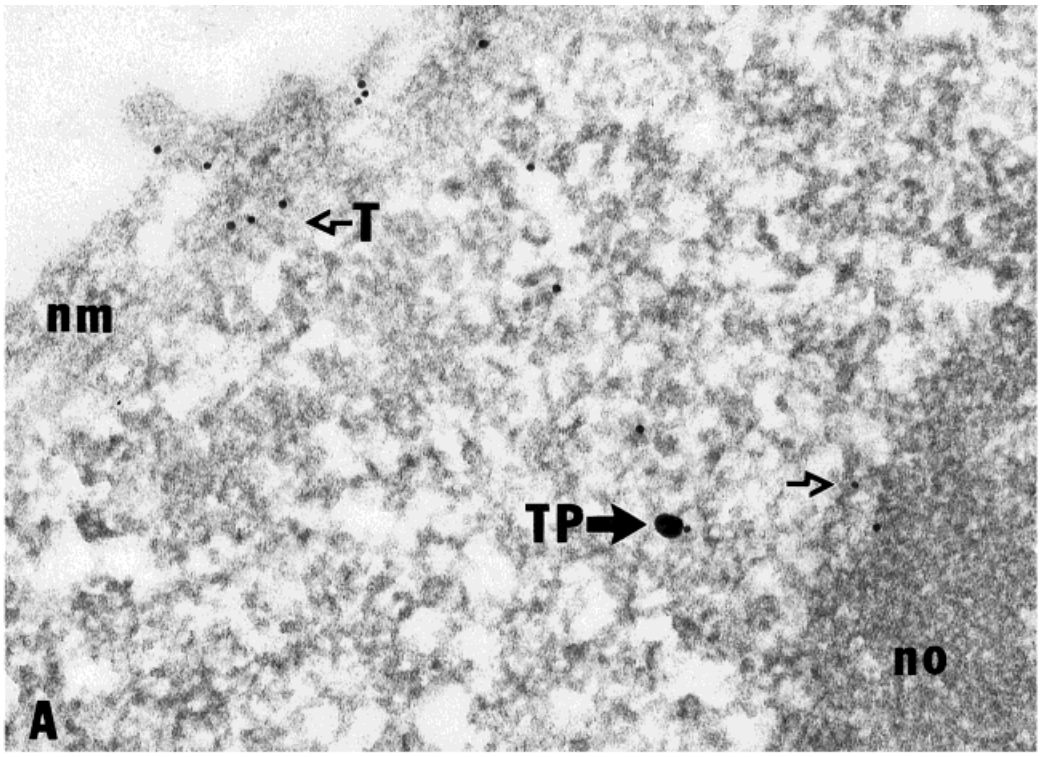
RESULTS

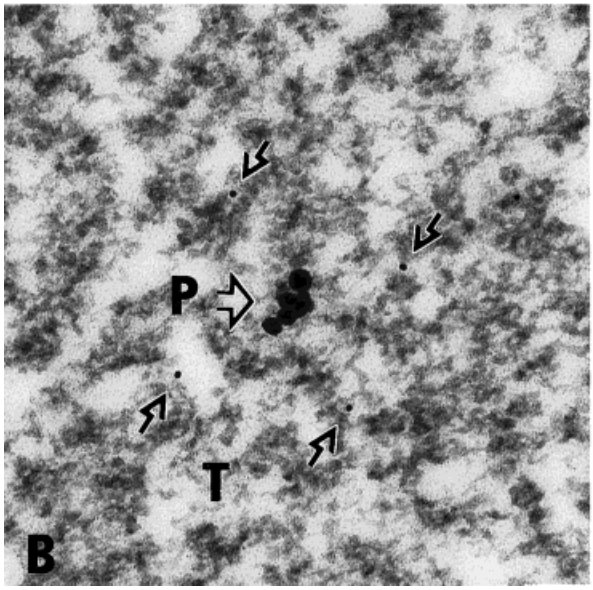
Colocalization of Tat and Purα by Immunogold Electron Microscopy in Nuclei of Human Glial Cells Constitutively Expressing Tat

Although Tat and Purα have been co-immunoprecipitated from extracts of glial cells [Krachmarov et al., 1996], no data exist regarding the intracellular location of such complexes. We examined whether Tat and Purα are colocalized in specific subcellular structures in cells in which Tat is expressed. Cells of the human astrocytoma line 5–10 constitutively express the HIV-1 Tat protein under control of a simian virus 40 promoter [Taylor et al., 1992]. Cells were fixed and treated with rabbit anti-Tat antibody 705 and with mouse monoclonal anti-Purα antibody 9C12. Secondary antibody or protein A, employed as described under Materials and Methods, were labeled with either 10 nm (Tat) or 30 nm (Purα) gold beads, respectively. Controls included cells treated with either primary antibody together with both secondary antibodies. There was virtually no colocalization of beads in these controls. It is not feasible to present all these negative control results in this report, but a detailed description of how the controls are performed has been published [Krachmarov et al., 1995]. Additional controls included cells treated with either primary antibody together with appropriate secondary antibody. In these cases, both proteins were detected primarily in the nucleus although significant cytoplasmic levels were also seen. Tat and Purα were frequently colocalized. As a result of Tat overexpression, Tat beads outnumber Purα beads by nearly 10:1; most of the Tat beads in the nucleus are seen in discrete foci. As shown in Figure 1, Tat and Purα beads are seen juxtaposed primarily in dense chromatin structures surrounding the nucleoli. Quantitation of Tat and Purα localization can be summarized as follows. Of beads visualized in cells, 74% of Tat beads were in the nucleus, while 56% of Purα beads were in the nucleus. Approximately 25% of all Purα beads were recorded as juxtaposed to (<10 nm from) Tat. Four hundred colocalized beads were recorded. Of these colocalized beads, 94.5% were in the nucleus. The nearly exclusive detection of Tat and Purα together in the nucleus, although both proteins are also cytoplasmic, suggests that the Tat-Purα complex has properties that preferentially dispose it toward the nucleus.

Tat Domains Involved in Binding to Purα

We sought to determine the amino acid domains in the HIV-1 Tat protein responsible for binding to cellular Purα . Full-length Tat (Tat86), a Tat deletion mutant with 35 aa near the N-terminus removed (Tat86Δ2/36), Tat protein encoded by *tat* exon I (Tat72), a Tat deletion mutant lacking the C-terminal 38 aa (Tat48) and two mutants which include a point mutation at aa22 ($Tat72C_{22}\rightarrow G$ and $Tat48C_{22}\rightarrow G$) were immobilized on glutathione-agarose beads. These beads were then employed in binding reactions with purified Purα, which had been generated by cleavage of bacterially produced GST-Purα with thrombin. The binding buffer employed, previously described [Krachmarov et al., 1996], included several protease inhibitors as well as excess bovine serum albumin (BSA); it was determined that >99% of immobilized Tat remained on the beads under binding conditions. In all cases, Purα was in vast excess over the GST-Tat proteins. It can be seen in Figure 2 that Tat72 has an even greater capacity to bind Purα than does Tat86, indicating that the ^{14}C-terminal aa are not involved in binding. Removal of 35 aa near the N-terminus also does not affect binding (cf. Tat86 with Tat86Δ2/36). However, point mutation of C_{22} to G strongly reduces binding to Purα, whether on a deletion background of Tat72 or Tat48. Deletion from Tat72 to Tat48 very strongly reduces binding, indicating that residues from 49–72 are important but not essential. Although Tat 48 binds weakly, it does bind Purα, indicating that residues from 37–48 are essential for Purα binding. Comparison of Tat72 and $Tat72C_{22}\rightarrow G$ with Tat48 and $Tat48C_{22}\rightarrow G$ reveals that when Tat aa 49–72 are removed, mutation of C_{22} virtually eliminates binding. The fact C_{22} that is important in this context, whereas when aa 2–36 are deleted it is not, suggests that C_{22} exerts a global effect on Tat-Purα interaction, most likely through an effect on Tat conformation. Deletion of the large segment near the Tat N-terminus may obviate the need for that effect. Tat residues 36–50 are important for binding of Tat to the TBP subunit of transcription factor TFIID [Kashanchi et al., 1994]. It has previously been reported that whereas Tat72 is capable of HIV-1 transcriptional activation in vivo, $Tat72C_{22}\rightarrow G$ is negative for activation





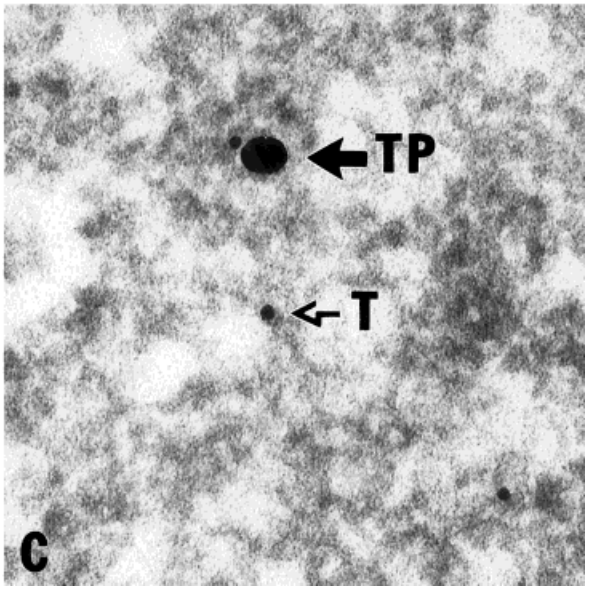


Fig. 1. Colocalization of the human immunodeficiency virus type 1 (HIV-1) Tat protein with cellular Purα in nuclei of cultured human glial cells constitutively expressing Tat using immunogold electron microscopy. Growth and preparation of 5–10 cells for electron microscopy is as described under Materials and Methods. Cell sections were treated with anti-Tat and anti-Purα antibodies and gold-labeled secondary antibody or protein A as described. The smaller beads, 10 nm in diameter, represent Tat. The larger beads, 30 nm, represent Purα. **A:** Tat and Purα colocalized in an extranucleolar chromatin structure. Note high levels of Tat in cytoplasm and near nuclear membrane, as well as lack of extracellular staining. **B:** Focus of Tat and Purα in a roundly symmetrical nuclear structure with four peripheral Tat beads. **C:** High-magnification micrograph of Tat and Purα colocalized with fibrous chromatin material. T, Tat; P, Purα; TP, Tat, and Purα; nm, nuclear membrane; no, nucleolus.

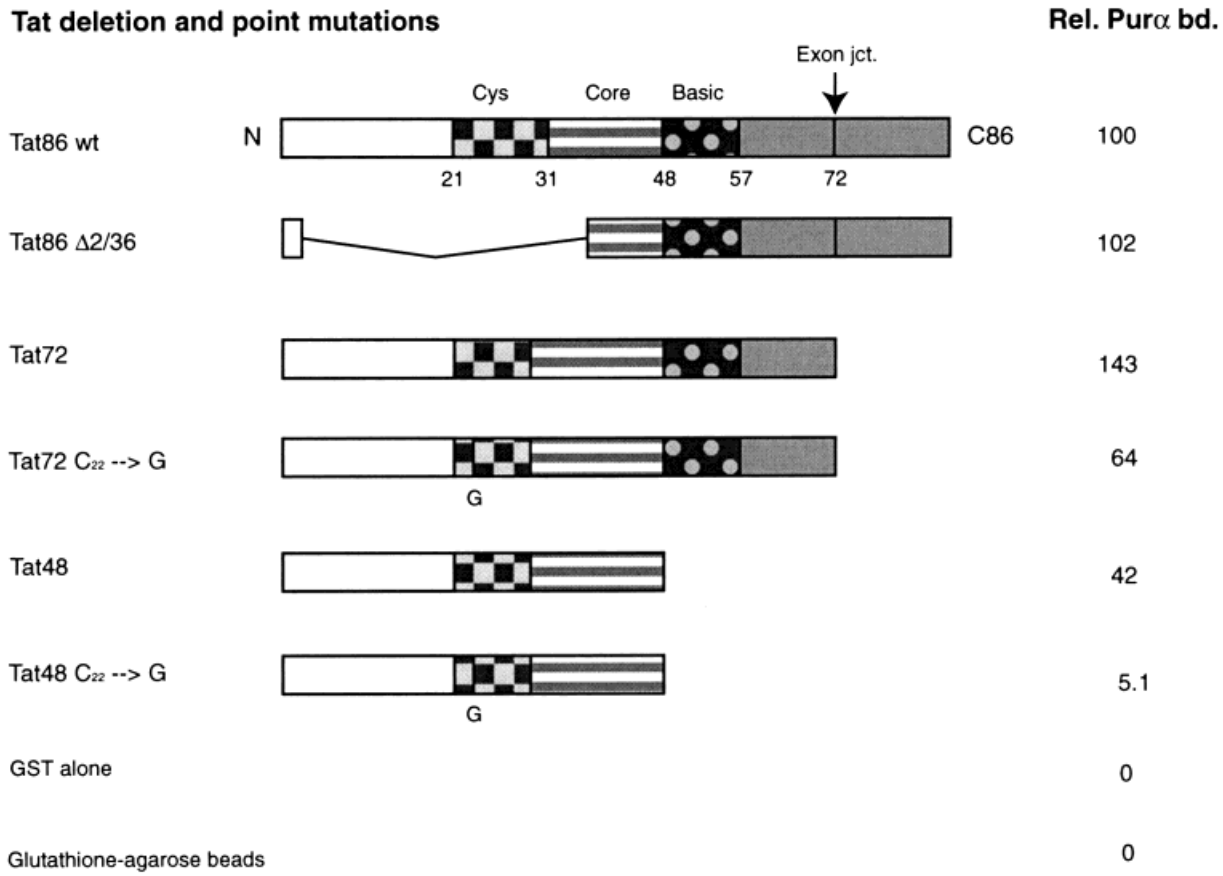


Fig. 2. Mutational analysis of Tat protein domains involved in binding to Purα. Characteristics of the Tat mutants are described in the text. GST is GST alone coupled to beads. Tat 72 and Tat 48 are C-terminal truncations of full-length Tat 86 (86 aa). The mutant Δ2/36 has had aa 2–36 removed. Mutants C_{22} and →G have had Cys at aa 22 altered to Gly.

[Rhim et al., 1994]. In addition, whereas Tat48 is capable of binding to transcription factor TFIIH in vitro, Tat48C_{22}→G is incapable of binding [Parada and Roeder, 1996].

Binding of Purα to Tat Requires Purα Complexed With Single-Stranded DNA or RNA

Purα, isolated from either eukaryotic cells or bacterial sources, is complexed with RNA. It has been reported that this RNA presence is important for binding to DNA [Herault et al., 1995] and to the HIV-1 Tat protein [Gallia et al., 1999]. With regard to Tat, this raises the question of whether association with Purα involves binding of both proteins to a given RNA species or whether RNA optimizes a direct interaction of Purα with Tat. To address this question, we have removed the RNA initially complexed with GST-Purα and replaced it with a

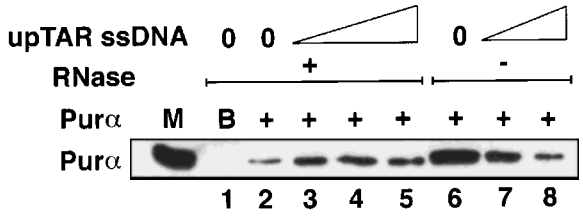


Fig. 3. Elimination of Purα Tat-binding activity by RNase and restoration with a single-stranded DNA Purα recognition element. Binding of Purα to full-length Tat protein coupled to GST-glutathione-agarose was as described under Materials and Methods. **Left lane**, **M**, is a Purα marker. **Lane 1**, **B**, Purα was present, but the binding reaction contained GST beads alone. **Lanes 2–5**, GST-Purα on glutathione-agarose beads had been treated with RNase A + T_1 before purification; **lanes 6–8**, were not treated with RNase. **Lanes 2, 6**, no ssDNA was added to the binding reaction; **lanes 3–5**, the single-stranded 24-mer *PUR* recognition element, upTAR, representing the JCV late-promoter Tat-responsive element ([ggaggc]$_4$)[Chowdhury et al., 1993] was added at 1×, 10×, and 100× molar equivalents to Purα, respectively. **Lanes 7, 8**, upTAR was added at 1× and 10× molar equivalents, respectively.

single-stranded oligonucleotide to which Tat does not bind. Figure 3 shows that treatment of GST-Purα with RNases A and T1 significantly restricts the ability of Purα to bind Tat (cf. lanes 2 and 6). The addition of the single-stranded DNA, purine-rich upTAR element, an excellent Purα recognition element, partially restores Purα Tat-binding activity (lanes 3–5), although only slightly less than 50% of original activity is restored (cf. lanes 4, 5 with lane 6). It has previously been reported that Tat does not itself bind upTAR [Krachmarov et al., 1996]. This result demonstrates that oligonucleotide binding optimizes a direct interaction between Purα and Tat. The addition of the DNA oligonucleotide to non-RNase-treated Purα inhibits binding to Tat (lanes 7, 8). Since this DNA does not interfere with the Purα-Tat interaction (lanes 3–5), it is likely that it is interfering with RNA binding to Purα. This result would suggest that RNA is more effective than DNA at optimizing the Purα-Tat interaction. It is clear that Tat and Purα interact directly rather than through an RNA or DNA intermediate, since upTAR does not bind Tat, and that either polynucleotide has the capacity to configure Purα to bind Tat. The functional importance of structural stabilization by binding to RNA has recently been noted for several proteins [Frankel and Smith, 1998].

A Purα Repeat II Peptide Inhibits Binding of Purα to Tat

Previous studies in which GST-Tat immobilized on glutathione-agarose beads was used to bind to Purα deletion mutants showed that Tat interacts with two acidic, leucine-rich repeats of Purα. The centers of these motifs comprise similar amino acid sequences: EFRDYL, aa 108–114, and EFRDAL, aa 197–202. That these central regions could be contact points for Tat was suggested by structural studies of leucine-rich repeats in other proteins in which the central regions were postulated to be exposed for protein-protein interaction [Kobe and Deisenhofer, 1994]. This was tested by introducing peptides from these and other regions of Purα into Tat-Purα binding assays. The sequences and positions of the Purα peptides, ranging from 8 to 22 amino acids are presented in Figure 5. Figure 4A presents results of addition of 7 synthetic peptides to the Purα-Tat binding assay. Only peptide 2 exhibited significant inhibitory activity. This peptide represents an

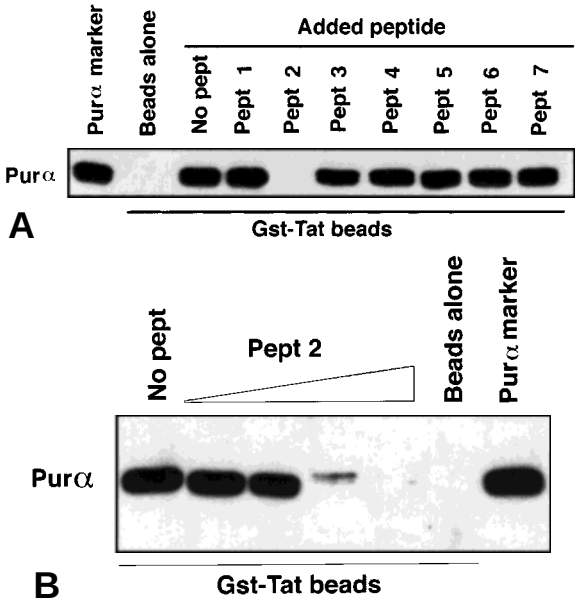
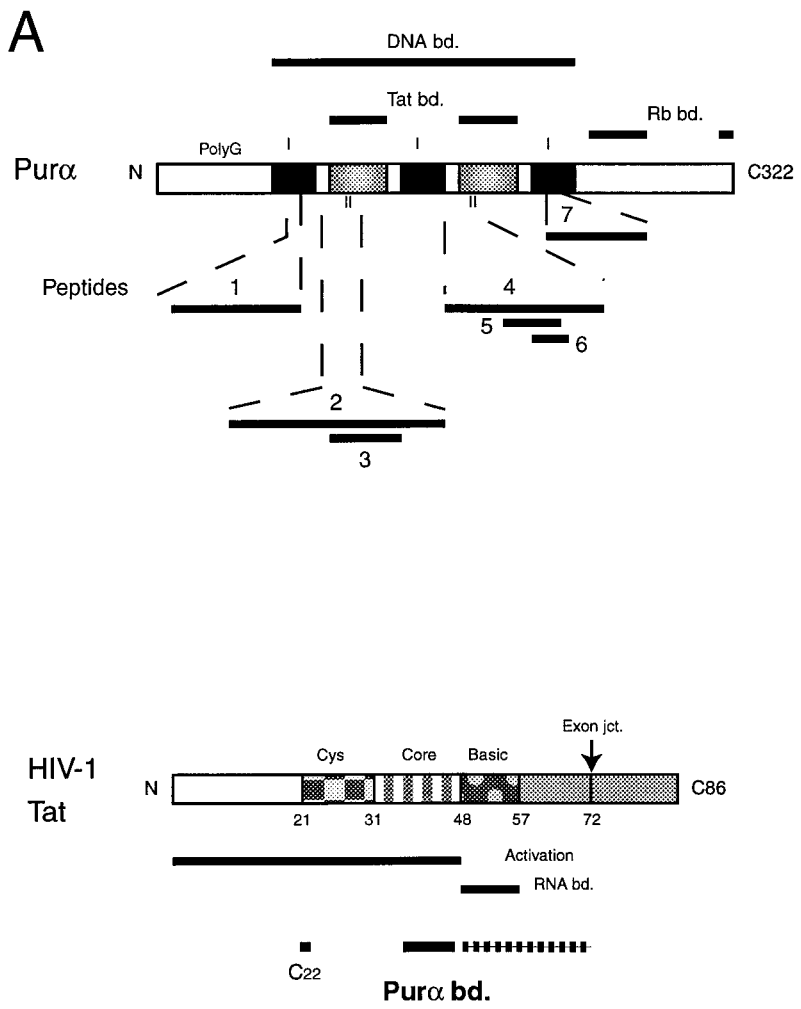


Fig. 4. Effects of synthetic peptides on binding of Purα to Tat and dose-response inhibition of binding by a Purα repeat II peptide. Binding of Purα to GST-Tat coupled to glutathione-agarose beads was performed as described under Materials and Methods. **A:** Effects of added peptides upon Purα-Tat binding. Positions and sequences of peptides 1–7 are presented in Fig. 5. **B:** Dose-response effect of added peptide 2 upon Purα-Tat binding. Purα and GST-Tat were reacted at 10^{-6} M each. Peptide 2 was added to the binding reaction at 0 (**lane 5**), 7.5×10^{-7}M, 3×10^{-6}M, 1.2×10^{-5} M, 5×10^{-5} M (**lanes 1–4**).

extended version of repeat II containing the motif EFRDYL at the center. Interestingly, peptide 3, a smaller version of this repeat including EFRDYL, did not inhibit binding. This could mean either that EFRDYL is not involved in binding to Tat or that the sequences on either side of this motif are required to maintain the proper binding configuration. No repeat I analogues inhibited Purα-Tat binding. This is consistent with the observation that repeat I is involved in DNA binding, and suggests it may not be involved in protein-protein interaction. Figure 4B shows a dose-response study of inhibition of Purα-Tat binding by peptide 2. Calculations from this and similar studies indicate that half-maximal inhibition is obtained with peptide 2 at approximately 5×10^{-6} M.

DISCUSSION

The present work establishes two important points regarding the interaction of Tat and Purα, the functional relevance of which has now been extensively demonstrated. First, Tat and Purα are colocalized to nuclear, extranucleolar structures in human glial cells. Despite the fact that



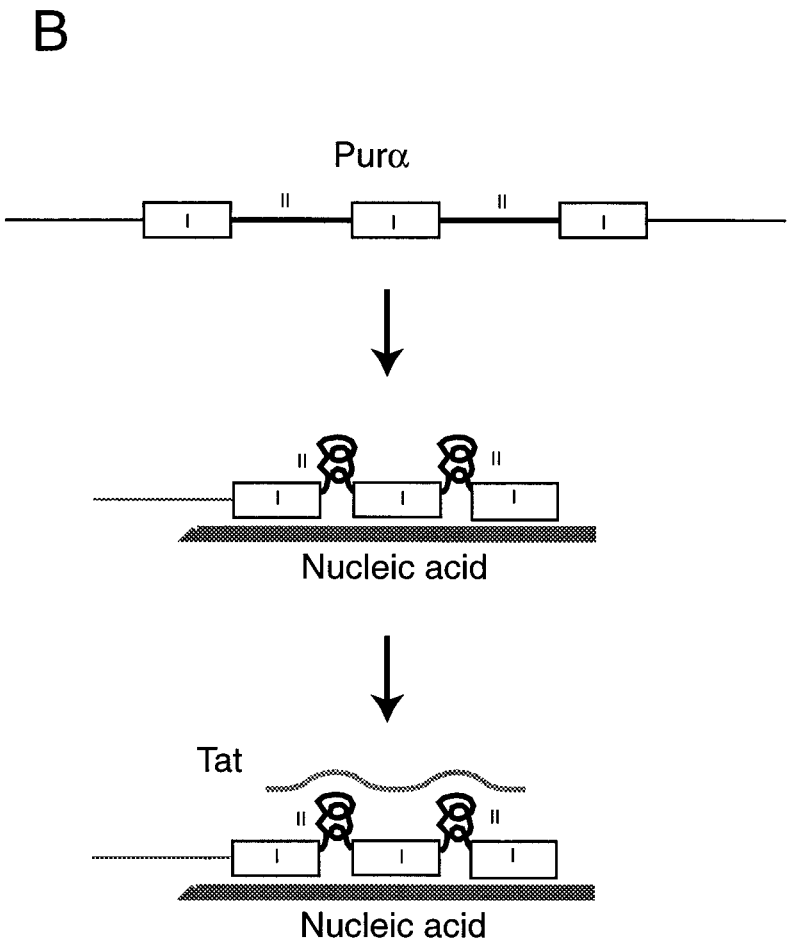


Fig. 5. Purα and Tat domains involved in Purα-Tat interaction. **A:** Purα synthetic peptides and Tat mutants employed in this study. Purα repeats I and II are indicated, and positions of synthetic peptides 1–7 are delineated. The sequence of these peptides is as follows: peptide 1: aa 69–80, QNKRFYLDVKQN; peptide 2: aa 100–121, LTLSMSVAVEFRDYLGDFIEHY; peptide 3: aa 109–116, EFRDYLGD; peptide 4: aa 188–209, IALPAQGLIEFRDALAKLIDDY; peptide 5: aa 194–203, GLIEFRDALA; peptide 6: aa 197–204, EFRDALAK; peptide 7: aa 226–236, QNKRFFFDVG. DNA, Rb, and Tat-binding regions, previously mapped [Johnson et al., 1995; Krachmarov et al., 1996], are shown. Functional regions of the Tat protein are indicated relative to aa 22, 48, and 72. Solid line for the Purα-binding region of Tat, a minimal binding domain; dashed line, sequences important for binding. **B:** Schematized mechanism of optimization of Purα-Tat interaction by binding to single-stranded RNA or DNA. In this mechanism, binding of the three Purα repeat I domains to nucleic acid helps induce proper folding of the two repeat II domains for optimal interaction with Tat.

both proteins can be visualized in the cytoplasm, their colocalization is virtually exclusively nuclear, suggesting that the Tat-Purα complex has a preferential tropism for the nucleus. It is conceivable that this localization is due to binding of the Tat-Purα complex to a nuclear component, potentially RNA or DNA, as further considered in this study, but also potentially to a nuclear partner protein such as Rb or cyclin A/CDK2 [Itoh et al., 1998].

A second important point established in this investigation is that Tat and Purα bind to each other through a protein-protein interaction, rather than to a common, intermediary RNA or DNA molecule. An ability of small RNA species to copurify with Purα has previously been reported [Herault et al., 1995], and an association of Purα with cellular 7SL RNA has been noted [Tretiakova et al., 1998]. Recent studies have identified a decanucleotide motif (CCCGGCc/gc/gGG) in many cellular RNAs that bind to Purα and have noted an ability of RNA to mediate interaction of Purα and Tat [Gallia et al., 1999]. In those studies, however, the possibility was not considered that Purα and Tat could bind to a common RNA molecule rather than to each other. The data presented in Figure 3 rule out that possibility and provide a mechanism by which RNA facilitates a direct protein-protein interaction between Purα and Tat. Figure 5 presents a schematized model for how Purα-protein interactions could be optimized by interaction with nucleic acids. Purα repeats II bear certain similarities, although not exact homologies, to repeats present in two nucleic acid-binding proteins of known crystal structure, porcine ribonuclease inhibitor [Kobe and Deisenhofer, 1994] and yeast Rap1 [Konig et al., 1996]. In both cases, the sequence immediately N-terminal to a motif corresponding to EFRDYL (RFRVYL in Rap1) are postulated to be in a beta sheet configuration with the motif itself at a turn. In ribonuclease inhibitor, although not in Rap1, the sequence immediately C-terminal to the motif is in an alpha-helical configuration with hydrophobic residues interacting with similar residues in the preceding β sheet, positioning the more highly charged turn region outward [Kobe and Deisenhofer, 1994]. In both proteins of known structure, the turn region is exposed for potential interaction with other proteins. In Figure 5, interaction of Purα repeat I sequences with DNA facilitate secondary structure interactions that would keep the turn region of repeats II, including EFRDYL, positioned outward for interaction with Tat. Two regions of Tat are depicted binding to repeats II. Further work would be necessary to determine how Tat interaction incorporates important aspects of C_{22} and amino acids 37–72. Although speculative at present, such a scheme such as that in Figure 5 could help explain the finding that nucleic acid binding enhances Purα-Tat interaction, which could, in turn, be important in designing inhibitors of such interaction based on sequences in repeat II.

JC virus is the etiological agent of progressive multifocal leukoencephalopathy (PML), a neurodegenerative disease characterized by destruction of oligodendrocytes, the myelin producing cells of the central nervous system. Among HIV-infected individuals with neurological symptoms, the incidence of PML is approximately 8%. Although this disease may occur as a consequence of an impaired immune system, the interaction of Tat with Purα raises the possibility that the HIV-1 virus may play a more direct role. Several observations now indicate that Tat can be secreted from cells in which it is synthesized [Barillari et al., 1993; Ensoli et al., 1990, 1993], and that exogenous Tat can be incorporated by many different cells in a transcriptionally active form [Zauli et al., 1995]. The RGD and basic amino acid domains of Tat have been identified as important for cellular uptake of the exogenous protein [Valvatne et al., 1996], and recent work has employed an 11 aa Tat sequence to transduce a variety of fusion proteins into cells [Ezhevsky et al., 1997; Lissy et al., 1998]. These findings lend additional credence to the notion that HIV-1 infection of microglial cells or astrocytes in the brain could influence the course of JCV infection in oligodendrocytes through transmigration of the Tat protein. In the case that the Tat-Purα interaction can be considered a target for therapy, further development of the inhibitory potential of Purα-based peptides may be warranted.

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