Interaction of the Single-stranded DNA-binding Protein Purα with the Human Polyomavirus JC Virus Early Protein T-antigen*

(Received for publication, July 17, 1998, and in revised form, September 23, 1998)

Gary L. Gallia‡, Mahmut Safak, and Kamel Khalili§

From the Center for NeuroVirology and NeuroOncology, Allegheny University of the Health Sciences, Philadelphia, Pennsylvania 19102

Large T-antigen, the major regulatory protein encoded by polyomaviruses, including Simian Virus 40 (SV40) and JC virus (JCV), is a multifunctional phosphoprotein that is involved in many viral and cellular events. In addition to its integral role in viral replication and cellular transformation, T-antigen also regulates transcription of both viral and cellular genes. In particular, the viral late promoter has been used as a model for the analysis of T-antigen-mediated transcriptional activation. Earlier studies have demonstrated that the cellular protein Purα is able to attenuate the transcriptional activity of JCV T-antigen. We investigated the mechanism whereby Purα affects T-antigen function. Co-immunoprecipitation studies demonstrated that Purα and JCV T-antigen interact in vitro, and glutathione S-transferase affinity binding assays revealed that these two proteins interact in vitro. Moreover, we localized the sequences of Purα that are important for the interaction between Purα and JCV T-antigen. In addition, we demonstrated that Purα interacts with the SV40 T-antigen. Transient transfection studies demonstrated that Purα and JCV T-antigen interact functionally as well. More specifically, Purα and a deletion mutant that interacts with T-antigen attenuated T-antigen-mediated transcriptional activation. A Purα deletion mutant that is unable to interact with JCV T-antigen, however, was found to be incapable of abrogating JCV T-antigen transactivation. Taken together, these data demonstrate that Purα and T-antigen interact both physically and functionally and that this interaction modulates T-antigen-mediated transcriptional activation. The implication of these findings with respect to the cellular role of Purα is discussed.

Polyomaviruses have proven to be powerful tools in the identification of key cellular regulatory proteins and the mechanisms that underlie their biological activities. The dependence on cellular machinery for a successful infection and the alterations in cellular processes promoting optimal conditions for viral multiplication have contributed to the utility of these viruses in identifying cellular regulatory proteins and pathways (1). In particular, studies of the major regulatory protein encoded by polyomaviruses, large T-antigen (T-antigen), have provided major insights into cellular biochemical activities including regulation of viral DNA replication, transcriptional activation of both viral and cellular promoters, and cellular transformation (2).

The human polyomavirus, JC virus (JCV), 1 is the etiologic agent of the fatal subacute human neurodegenerative disease progressive multifocal leukoencephalopathy (reviewed in Ref. 3). Unlike SV40 and other polyomaviruses, JCV has a narrow tissue tropism. In immunocompromised patients, JCV lytically infects oligodendrocytes, the myelin-producing cells in the central nervous system, and in tissue culture, JCV propagates efficiently only in primary human fetal glial cells. Studies from our and other laboratories have indicated that the cell-specific activation of the JCV promoter is determined at the level of viral gene transcription and requires multiple cellular proteins present in glial cells (reviewed in Ref. 4). One such cellular factor that influences JCV is Purα.

Puro is a 322-amino acid sequence-specific single-stranded DNA-binding protein that has been implicated in the control of both DNA replication and transcription. There are several lines of evidence supporting a role for Puro in DNA replication. Purα was initially characterized as a HeLa cell nuclear protein that binds a sequence element, called the PUR element, adjacent to a region of stably bent DNA 1.6 kilobases upstream of the human c-myc gene (5, 6). This element is near the center of a region implicated as an initiation zone for chromosomal DNA replication. Moreover, PUR elements are present at several eukaryotic origins of DNA replication (5). Purα has also been shown to interact with viral origins of DNA replication. Purα has been shown to bind the JCV and bovine papillomavirus origins of replication (7, 8). Although the biological activity of Purα on eukaryotic and bovine papillomavirus replication is yet undescribed, Purα has been shown to inhibit JCV DNA replication in glial cells (7).

Puro has also been implicated in control of gene transcription involving both viral and cellular promoters. Purα has been shown to activate several promoters, including the JCV early gene promoter (9), the human immunodeficiency virus type I (10), the myelin basic protein promoter (11), and the neuron-specific FE65 gene promoter (12). In addition, Purα has also been implicated in the expression of the neuronal nicotinic acetylcholine receptor gene promoter (13), the single-stranded cAMP response element (14), and the vascular smooth muscle α-actin gene promoter (15).

In addition to its role in transcription and replication, there is evidence suggesting that Purα is involved in the control of cell growth and proliferation. Johnson et al. (16) have demonstrated that Puro binds the hypophosphorylated form of the human retinoblastoma tumor suppressor gene product pRB.

1 The abbreviations used are: JCV, JC virus; JCV, JCV late; SV40, Simian Virus 40; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase.
Although the functional consequence of the interaction between these two proteins is yet undescribed, the selective interaction between Pur and the hypophosphorylated form of a protein intimately involved in cell cycle progression suggests a potentially crucial role in the cell cycle.

With respect to JCV, we have previously demonstrated that Pur and T-antigen exert antagonistic effects on each other's transcriptional activity on the JCV promoter (9). Like other polymaviruses, the genome of JCV can be divided into an early region, which is expressed prior to the onset of viral DNA replication and encodes the viral regulatory proteins (the T-antigens), and the late region, which is expressed after viral DNA replication and encodes the viral capsid proteins (VP1, VP2, and VP3) (reviewed in Ref. 17). The noncoding regulatory region, which contains the origin of viral DNA replication and the transcriptional control region, lies between the two coding regions. This transcriptional control region is composed of a bidirectional promoter/enhancer, with the JCV early promoter controlling expression of the early genes and the JCV late (JCV₂) promoter directing transcription of the late genes. Pur has been shown to activate the JCV early promoter, and co-expression of JCV T-antigen decreases this Pur-induced increase in the level of JCV early gene transcription (9). Likewise, Pur has been shown to abrogate JCV T-antigen-mediated transcriptional activation of the JCV₂ promoter (9).

In this report, we examine the mechanism underlying the ability of Pur to abrogate JCV T-antigen-mediated transcriptional activation of JCV₂. Using co-immunoprecipitation and in vitro affinity chromatography assays, we demonstrate that Pur associates with JCV T-antigen. In addition, we localize the region of Pur that is involved in this interaction. Transient transfection studies demonstrate that Pur and a Pur deletion mutant that retains the ability to interact with T-antigen abrogate T-antigen-mediated transcriptional activation of the JCV₂ promoter. A mutant Pur that cannot interact with JCV T-antigen, however, is unable to attenuate JCV T-antigen-mediated transcriptional activation of the JCV₂ promoter. Taken together, these data suggest that Pur and JCV T-antigen interact with one another and that this interaction modulates T-antigen-mediated transcriptional activation of the late promoter of JCV virus.

**MATERIALS AND METHODS**

**Cell Culture**—HJC-15b cells (4) were derived from a JCV virus-induced hamster brain tumor (18) and were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. U-87 MG cells (ATCC HTB-14), a human glioblastoma cell line, and SVG cells, human fetal astroglial cells immortalized with SV40 T-antigen (19), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and the same antibiotics. All cells were maintained at 37 °C in a humidified atmosphere containing 7% CO₂.

**Western Blot Analysis**—Ten μg of whole cell lysate was loaded on the gels as migration standards. After electrophoresis, the membranes were incubated for 1 h with a 1:1000 dilution of peroxidase-linked goat anti-mouse antibody at room temperature. Antibody detection was achieved by ECL according to the manufacturer’s recommendations (Amersham Pharmacia Biotech). For immunoprecipitation, 100 μg of lysate was incubated with normal mouse serum (preimmune), a monoclonal antibody against SV40 T-antigen (pAb416), Oncogene Sciences), or a monoclonal antibody against the histidine epitope tag (anti-T7) (Novagen) for 1 h at 4 °C. Immune complexes were precipitated with 20 μl of protein A-Sepharose and washed 5 times with 1 ml of 1× PBS and 1 ml of 1% Triton-X in Laemmli buffer, and separated on SDS-polyacrylamide gels. Proteins were transferred to supported nitrocellulose membranes (Schleicher and Schuell) in Western transfer buffer (192 mM glycine, 25 mM Tris base, and 20% methanol). For Western blot analysis, the membranes were blocked for 30 min in 10% nonfat dry milk in PBS-T (1× PBS, 0.1% Tween-20) and then incubated for 1 h with a 1:1000 dilution of the primary antibody at room temperature. After washing, the membranes were incubated for 1 h with a horseradish peroxidase-linked goat anti-mouse antibody at room temperature. Antibody detection was achieved by ECL according to the manufacturer's recommendations (Amersham Pharmacia Biotech).

**EMMUNITY**—The JCV T-antigen-expressing cell line HJC-15b was transfected with 30 μg of pEBV, pEBV-Pur, or pEBV-LacZ. Forty-eight h after transfection, cells were washed three times in 1× PBS and lysed in lysis 150 buffer (LB 150) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 20 min on ice. Lysates were scraped and collected into microcentrifuge tubes, vortexed briefly, and microcentrifuged for 20 min at 4 °C. For immunoprecipitations, 100 μg of lysate was incubated with normal mouse serum (preimmune), a monoclonal antibody against SV40 T-antigen (pAb416) (Oncogene Sciences), or a monoclonal antibody against the histidine epitope tag (anti-T7) (Novagen) for 1 h at 4 °C. Immune complexes were precipitated with 20 μl of protein A-Sepharose and washed 5 times with 1 ml of 1% Triton-X in Laemmli buffer, and separated on SDS-polyacrylamide gels.

**Gel Electrophoresis**—Proteins were transferred to supported nitrocellulose membranes (Schleicher and Schuell) in Western transfer buffer (192 mM glycine, 25 mM Tris base, and 20% methanol). For Western blot analysis, the membranes were blocked for 30 min in 10% nonfat dry milk in PBS-T (1× PBS, 0.1% Tween-20) and then incubated for 1 h with a 1:1000 dilution of the primary antibody at room temperature. After washing, the membranes were incubated for 1 h with a horseradish peroxidase-linked goat anti-mouse antibody at room temperature. Antibody detection was achieved by ECL according to the manufacturer’s recommendations (Amersham Pharmacia Biotech).

**Immunoprecipitations and Immunoblot Analysis**—The JCV T-antigen-expressing cell line HJC-15b was transfected with 30 μg of pEBV, pEBV-Pur, or pEBV-LacZ. Forty-eight h after transfection, cells were washed three times in 1× PBS and lysed in lysis 150 buffer (LB 150) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 20 min on ice. Lysates were scraped and collected into microcentrifuge tubes, vortexed briefly, and microcentrifuged for 20 min at 4 °C. For immunoprecipitations, 100 μg of lysate was incubated with normal mouse serum (preimmune), a monoclonal antibody against SV40 T-antigen (pAb416) (Oncogene Sciences), or a monoclonal antibody against the histidine epitope tag (anti-T7) (Novagen) for 1 h at 4 °C. Immune complexes were precipitated with 20 μl of protein A-Sepharose and washed 5 times with 1 ml of 1% Triton-X in Laemmli buffer, and separated on SDS-polyacrylamide gels. Proteins were transferred to supported nitrocellulose membranes (Schleicher and Schuell) in Western transfer buffer (192 mM glycine, 25 mM Tris base, and 20% methanol). For Western blot analysis, the membranes were blocked for 30 min in 10% nonfat dry milk in PBS-T (1× PBS, 0.1% Tween-20) and then incubated for 1 h with a 1:1000 dilution of the primary antibody at room temperature. After washing, the membranes were incubated for 1 h with a horseradish peroxidase-linked goat anti-mouse antibody at room temperature. Antibody detection was achieved by ECL according to the manufacturer’s recommendations (Amersham Pharmacia Biotech).

**Gel Electrophoresis**—Proteins were transferred to supported nitrocellulose membranes (Schleicher and Schuell) in Western transfer buffer (192 mM glycine, 25 mM Tris base, and 20% methanol). For Western blot analysis, the membranes were blocked for 30 min in 10% nonfat dry milk in PBS-T (1× PBS, 0.1% Tween-20) and then incubated for 1 h with a 1:1000 dilution of the primary antibody at room temperature. After washing, the membranes were incubated for 1 h with a horseradish peroxidase-linked goat anti-mouse antibody at room temperature. Antibody detection was achieved by ECL according to the manufacturer’s recommendations (Amersham Pharmacia Biotech).
themselves in vitro from XhoI linearized pCDNA3-Pura using the TNT-coupled transcription-translation wheat germ extract (Promega) following the manufacturer’s instructions.

**Protein Purification—** GST fusion proteins were expressed and purified as described previously (21). Briefly, bacteria were grown overnight at 37 °C in Luria Bertani (LB) medium supplemented with 100 mg/liter ampicillin. The following morning, the cells were diluted 1:10 in fresh LB medium, grown to an absorbance of 0.6–0.7, and induced for 2 h at 37 °C with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were pelleted at 6,500 × g at 4 °C; resuspended in NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) containing 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride; and sonicated on ice. The bacterial lysate was centrifuged at 40,000 × g at 4 °C to remove insoluble material. Glutathione-Sepharose beads (Amersham Pharmacia Biotech) were added to the supernatant, and binding of the GST fusion proteins was allowed to occur for 3 h at 4 °C. Beads were pelleted and washed three times with 50 volumes of NETN buffer each time. The integrity and purity of the GST fusion proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining.

**Transfections and CAT Assays—** Transient transfection assays were carried out by the calcium phosphate method as described previously (22). Briefly, 4 × 10⁵ cells were plated on a 60-mm plate and grown overnight. Three h prior to transfection, the cells were fed with new growth medium. Transfections were carried out with 3 μg of the reporter plasmid pJCVA5xCAT, alone or in combination with 2.5 μg of CMV-JCV T-antigen, and 10 μg of pEBV-Pura, pEBV-Pura (1–154), or pEBV-Pura (216–322). Experiments were designed to be promoter controlled with either pCMV-X or pEBV-His B plasmids added to equalize the total amounts of promoter in each reaction mixture. The precipitate was removed after 3 h, and a glycerol shock was applied. Forty-eight h posttransfection, the cells were harvested, and a crude protein extract was prepared by repeated cycles of freezing and thawing. Extracts were quantitated by the Bio-Rad Bradford assay, and 75 μg of protein was assayed for CAT activity (23). The fold transactivation was measured by scintillation counting of the spots cut from the thin-layer chromatography plates. Each experiment was repeated three or more times with different plasmid preparations.

**RESULTS**

**Pura Associates with JCV T-antigen in Vivo and in Vitro**—In the first series of experiments, co-immunoprecipitation experiments were performed to determine whether Pura and JCV T-antigen associate with each other in cells. To this end, the JCV T-antigen-expressing cell line HJC-15b was transfected with a control plasmid (pEBV) or a plasmid encoding a histidine epitope-tagged Pura (pEBV-Pura). Cellular extracts obtained from these transfected HJC-15b cells were immunoprecipitated with preimmune serum (normal mouse serum) or an antibody that recognizes the histidine epitope tag (anti-T7), and immune complexes were analyzed by Western blot analysis for the presence of JCV T-antigen. In extracts from HJC-15b cells transfected with the epitope-tagged Pura, anti-T7 antibody co-immunoprecipitated JCV T-antigen (Fig. 1A, lane 5). This co-immunoprecipitation was specific because normal mouse serum co-immunoprecipitated JCV T-antigen (Fig. 1A, lane 5).

**Fig. 1. JCV T-antigen co-immunoprecipitates with Pura.** A and C, whole cell extracts from hamster glial cells expressing JCV T-antigen (HJC-15b) transfected (Tfxn) with pEBV-B (control) (A and C), pEBV-Pura (A), or pEBV-LacZ (C) were immunoprecipitated with normal mouse serum (NMS) (lanes 2 and 4) or anti-T7 antibody (αT7) (lanes 3 and 5). The immunocomplexes were resolved under reducing conditions by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed for JCV T-antigen by Western blot analysis using an anti-T-antigen antibody. HJC-15b whole cell extract was loaded as a migration control (lane 1). Bracket indicates JCV T-antigen. Asterisks indicate immunoglobulin G heavy and light chains. B and D, whole cell extracts from HJC-15b cells transfected with pEBV-Pura (B) or pEBV-LacZ (D) were immunoprecipitated with normal mouse serum (NMS) (lane 3) or anti-T7 antibody (αT7) (lane 4). The immunocomplexes were resolved under nonreducing conditions by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed for either Pura (B) or β-galactosidase (D) by Western blot analysis using an anti-T7 antibody. Lanes 1 and 2 represent Western blot analysis of cells mock-transfected (lane 1) or transfected with pEBV-Pura (B, lane 2) or pEBV-LacZ (D, lane 2). Protein A-Sepharose (Psa) (lane 5), normal mouse serum (NMS) (lane 6), and anti-T7 antibody (αT7) (lane 7) were loaded as additional controls. Arrows indicate Pura (B) and β-galactosidase (β-gal) (D). In B, the band directly above the Pura band (lane 4) is due to a protein cross-reacting with the anti-T7 antibody (double asterisks). The positions of molecular mass markers (in kilodaltons) are shown on the left of each panel.
mouse serum did not immunoprecipitate JCV T-antigen (lane 4), and the anti-T7 antibody that recognizes the histidine tagged Purα did not immunoprecipitate JCV T-antigen from cells that were not transfected with Purα (lane 3). The expression and immunoprecipitation of Purα in HJC-15b cells transfected with pEBV-Purα was confirmed by immunoprecipitation followed by Western blot analysis with the anti-T7 antibody (Fig. 1B). To rule out any possible contribution of the histidine epitope tag to the interaction between Purα and JCV T-antigen, HJC-15b cells were transfected with the control plasmid pEBV and a plasmid encoding a histidine epitope-tagged β-galactosidase protein (pEBV-LacZ). Cellular extracts obtained from these transfected HJC-15b cells were immunoprecipitated with preimmune serum or anti-T7 antibody, and immune complexes were analyzed by Western blot analysis for the presence of JCV T-antigen. In extracts from HJC-15b cells transfected with pEBV-LacZ, the anti-T7 antibody did not co-immunoprecipitate JCV T-antigen (Fig. 1C, lane 5) indicating that the interaction between Purα and JCV T-antigen is not mediated via the histidine epitope tag. The expression and immunoprecipitation of β-galactosidase in HJC-15b cells transfected with pEBV-LacZ was confirmed by immunoprecipitation followed by Western blot analysis with the anti-T7 antibody (Fig. 1D). Taken together, these results demonstrate that Purα and JCV T-antigen are able to form a complex in cells in which both proteins are present.

To provide additional evidence for the interaction between Purα and JCV T-antigen, in vitro GST pulldown experiments were performed. JCV T-antigen containing HJC-15b cellular extract was incubated with Escherichia coli-purified GST or GST-Purα proteins immobilized on glutathione-Sepharose beads. After washing, proteins retained on the beads were analyzed by immunoblot analysis with an antibody that recognizes JCV T-antigen. As shown in Fig. 2A, JCV T-antigen was specifically retained on the Sepharose column containing GST-Purα (lane 3) but not on the column containing GST alone (lane 2). In another series of experiments, in vitro translated 35S-labeled Purα was incubated with E. coli-purified GST or GST-JCV T-antigen immobilized on glutathione-Sepharose beads. As shown in Fig. 2B, 35S-labeled Purα was specifically retained on the Sepharose column containing GST-JCV T-antigen (lane 3) but not on the column containing GST alone (lane 2). Taken together, these results demonstrate that Purα and JCV T-antigen interact both in vivo and in vitro.

Because JCV T-antigen possesses greater than 70% amino acid homology with the SV40 large T-antigen (24), the ability of Purα to interact with SV40 T-antigen was assessed. To this end, GST pulldown experiments were performed with cellular extract from cells constitutively expressing SV40 T-antigen. As shown in Fig. 2C, SV40 T-antigen was specifically retained on the Sepharose column containing GST-Purα (lane 2) but not on the column containing GST alone (lane 2). Thus, Purα is able to interact with T-antigen produced not only by JCV but also by SV40.

Localization of Sequences in Purα Important for Interaction with JCV T-antigen—Structurally, Purα is composed of a several modular domains (see Fig. 4A). In particular, the central region of Purα is composed of three aromatic and basic repeats (class I) interspersed with two acidic leucine-rich repeats (class II). Other notable structural features of Purα include an aminoterminal glycine-rich region and an amphipathic α-helix and a glutamate-glutamine-rich domain near the carboxyl terminus. To identify the region(s) of Purα necessary for the interaction with JCV T-antigen, a series of amino-terminal, carboxyl-terminal, and internal deletion mutants fused to GST was constructed (see Fig. 4A), and GST pulldown experiments utilizing HJC-15b cellular extract and these various deletion mutants were performed. Consistent with the data presented above, full-length Purα fused to GST bound to JCV T-antigen (Fig. 3A, lane 3), whereas GST alone did not (Fig. 3A, lane 2). An amino-terminal GST-Purα deletion construct removing the amino-terminal 54 amino acids encompassing the glycine-rich region of Purα, GST-Pura (54–322), showed a slightly reduced ability to interact with JCV T-antigen compared with the full-length GST-Purα (Fig. 3A, compare lanes 3 and 4). Removal of the amino-terminal 85 amino acids, including the glycine-rich region and the first class I repeat, GST-Purα (1–322) (Fig. 3A, lane 5). Further amino-terminal deletion mutants, GST-Pura (167–322), GST-Purα (216–322), and GST Purα (274–322) were unable to interact with JCV T-antigen (Fig. 3A, compare lanes 3 and 5). These results indicate that sequences amino-terminal to amino acid 167 are involved in the interaction between Purα and JCV T-antigen.

To further define the sequences within Purα that are important for its interaction with JCV T-antigen, similar experiments were performed with a series of carboxyl-terminal GST-
Puro deletion mutants. Carboxyl-terminal deletion mutants GST-Puro (1–215), GST-Puro (1–174), GST-Puro (1–154), and GST-Puro (1–123) all interacted with JCV T-antigen to the same extent as full-length GST-Puro (1–322) (Fig. 3B, lanes 3–7). The smallest carboxyl-terminal deletion mutant containing only the amino-terminal 71 amino acids, GST-Puro (1–71), was unable to interact with JCV T-antigen (Fig. 3B, lane 8). These results are consistent with those obtained with the amino-terminal GST-Puro deletion mutants and further refine the region of Puro that is involved in the interaction with JCV T-antigen to amino acids 72–123.

Two additional Puro deletion mutants were tested for the ability to interact with JCV T-antigen. One of these mutants, GST-Puro (Δ72–229), removes the central region of Puro; the other, GST-Puro (85–229), contains only the central region of Puro. As shown in Fig. 3C, both of these GST-Puro deletion constructs were able to interact with JCV T-antigen (lanes 4 and 5), albeit to a lesser extent when compared with full-length GST-Puro (1–322) (lane 3). The interaction between the central region of Puro, GST-Puro (85–229), is not surprising considering the previous demonstration that GST-Puro (85–229) was able to interact with JCV T-antigen (Fig. 3A, lane 5) and that the carboxyl-terminal deletion construct GST-Puro (1–123) was able to interact with JCV T-antigen (Fig. 3B, lane 7). The observation that GST-Puro (Δ72–229) interacts with JCV T-antigen is somewhat surprising because neither the deletion construct containing only the first 71 amino acids, GST-Puro (1–71), nor the amino-terminal deletion constructs GST-Puro (167–322), GST-Puro (216–322), and GST-Puro (274–322) interact with JCV T-antigen. Upon closer evaluation of GST-Puro (Δ72–229), an interesting observation can be made. This deletion construct fuses the first one-third of the first class I repeat (Ia) with the last two-thirds of the last class I repeat (Ic), essentially reconstituting a class I repeat (hybrid) (Fig. 4B). Taken together, these observations indicate that a class I repeat is involved in the interaction with JCV T-antigen. Other sequences, however, are also important for the interaction of Puro with JCV T-antigen. This is supported by the observations that GST-Puro (167–322), which contains an intact class I repeat, is unable to interact with JCV T-antigen and that GST-Puro (85–322), which contains two intact class I repeats, interacts weakly with JCV T-antigen. Nonetheless, the above studies demonstrate that the minimal region of Puro, which is important in the interaction with JCV T-antigen, resides between amino acids 72 and 123. Additional studies involving smaller deletions as well as point mutations will be necessary to help define the amino acid contacts between Puro and JCV T-antigen.

**Puro Inhibits JCV T-antigen-mediated Transcriptional Activation**—Earlier studies have demonstrated that Puro has the ability to interfere with the stimulatory action of JCV T-antigen on JCV late gene transcription (9). To investigate the functional significance of the physical interaction between Puro and JCV T-antigen, transient transfection assays were performed. In these studies, the plasmid pJCVLΔB, which contains 286 base pairs of DNA sequence of the JCV control region upstream of the reporter CAT gene in the late orientation, was introduced into U-87 MG human glial cells singly or in combination with plasmids expressing JCV T-antigen, Puro, or mutant Puro proteins.

As shown in Fig. 5, the activity of the JCVL promoter is increased when cells are co-transfected with the pJCVLΔB reporter plasmid and the JCV T-antigen expressing plasmid (compare lanes 1 and 2). The stimulatory effect of JCV T-antigen is independent of an increase in DNA replication as the auxiliary sequences required for JCV DNA replication located on the early side of the origin are deleted in the reporter construct used (25). In agreement with earlier studies, the T-antigen-induced transcriptional activity of the JCVL promoter is decreased when cells are co-transfected with a plasmid expressing Puro (Fig. 5, compare lanes 2 and 3) (9). To determine whether the physical interaction between JCV T-antigen and Puro underlies their functional interaction, the ability of Puro mutants to modulate the transcriptional activity of T-antigen on the JCVL promoter was examined. One Puro mutant, Puro (1–154), retains the ability to interact with JCV T-antigen, whereas the second Puro mutant, Puro (216–322), is unable to interact with JCV T-antigen. Expression of these mutants was verified by Western blot analysis using the anti-T7 antibody, which recognizes the histidine tag on both pro-
teins (data not shown). As shown in Fig. 5, the T-antigen-mediated transcriptional activity of the JCV L late promoter is decreased when cells are co-transfected with Purα (1–154) (compare lanes 2 and 4). Alternatively, Purα (216–322) has little effect on T-antigen-mediated transcriptional activation of the JCV late promoter reporter construct (compare lanes 2 and 5). Neither full-length Purα nor either deletion mutant affected the basal level of transcription in the absence of JCV T-antigen (Fig. 5, lanes 6–8) (9). Of note, expression of JCV T-antigen remains unaltered upon expression of wild-type Purα or either mutant Purα (data not shown) (9). Thus, the ability of Purα to inhibit T-antigen-mediated transcriptional activation is correlated with its ability to physically interact with T-antigen. Taken together, these results suggest that Purα specifically inhibits T-antigen-induced transcriptional activation through its physical association with JCV T-antigen.

DISCUSSION

Previous studies have demonstrated that the cellular protein Purα plays a role in the transcriptional regulation of the JCV promoters (9). More specifically, Purα stimulates transcription from the JCV early promoter. Moreover, T-antigen attenuates the Purα-induced level of early gene transcription. Although Purα has little effect on the late promoter itself, Purα is able to decrease T-antigen-mediated transcriptional transactivation of the JCV_L promoter. In this study, we have investigated the mechanism responsible for the ability of Purα to abrogate the transcriptional activation of the JCV_L promoter by T-antigen. Results from the current studies demonstrate that Purα interacts with JCV T-antigen in vivo and in vitro. Additional studies, including co-immunoprecipitation and co-localization assays, utilizing mice transgenic for JCV T-antigen (JC-91) have also demonstrated an interaction between these two proteins.² Of note, the human and mouse Purα proteins exhibit a very high degree of conservation, differing by only two amino acids (26). Moreover, we also demonstrate the Purα is able to interact with the T-antigen produced by another polyomavirus, SV40. Transient transfection assays demonstrate that a mutant Purα protein that retains the ability to interact with JCV T-antigen is, like the wild-type Purα protein, able to abrogate the JCV T-antigen-mediated transactivation of the JCV_L promoter. A mutant Purα protein that is unable to interact with JCV T-antigen, however, is not capable of attenuating the JCV T-antigen-induced transactivation of the JCV_L promoter. Taken together, these data demonstrate that Purα and T-antigen interact both physically and functionally and that the interaction between these two protein modulates T-antigen-mediated transactivation.

² A. Tretiakova et al., unpublished observations.
transcriptional activation of the late promoter of JCV.

Several interesting observations can be made regarding the physical interaction between Purα and JCV T-antigen. JCV large T-antigen present in HJC-15b cells exists as several isoforms with different electrophoretic mobilities (Figs. 1–3). Because JCV T-antigen is a phosphoprotein with several phosphorylation sites (27), these different isoforms are believed to represent different phosphorylation states of the protein. Interestingly, Purα interacts with all of the isoforms of JCV T-antigen present in HJC-15b cells (Figs. 1–3). This is in contrast to the interaction between Purα and another protein, which exists in different phosphorylated states, the product of the retinoblastoma gene, pRB. Johnson et al. (16) have demonstrated that Purα binds selectively to the hypophosphorylated form of pRB. The phosphorylation state of JCV T-antigen, however, may not dictate the association between Purα and JCV T-antigen as in vitro translated Purα interacts with GST-JCV T-antigen, which, being produced in bacteria, does not contain phosphorylated residues (Fig. 3). Nonetheless, future experiments will be required to elucidate the contribution of the phosphorylation state of JCV T-antigen to the intermolecular complex formed between Purα and JCV T-antigen.

The transcriptional antagonism between JCV T-antigen and Purα is reminiscent of the interaction between the related SV40 large T-antigen and the tumor suppressor p53 protein. Several studies have demonstrated that SV40 T-antigen abrogates p53-mediated transcriptional activation (28–30). Moreover, p53 has been shown to inhibit SV40 late promoter transactivation by SV40 T-antigen (31). This is similar to the case of Purα and JCV T-antigen in that T-antigen abrogates Purα-mediated transcriptional transactivation, and Purα inhibits JCV T-antigen-mediated transactivation of the JCV late promoter (Ref. 9 and this study). The interaction between SV40 T-antigen and p53 not only has functional consequences with respect to transcription, but this interaction has also been shown to regulate replication. SV40 T-antigen, when complexed with p53, has been shown to be unable to replicate an SV40 origin-containing DNA (32, 33). Interestingly, p53 has been shown to inhibit JCV DNA replication by interacting with JCV T-antigen (34). Although the functional significance of the interaction between Purα and T-antigen with respect to DNA replication is yet undeclared, the results reported here invite investigation into the functional relevance of the interaction between these two proteins in other well characterized functions of T-antigen.

Another well characterized function of viral oncoproteins including large T-antigen is cellular transformation. One mechanism by which these proteins are able to cause cellular transformation is via their interaction with the products of cellular tumor suppressor genes, such as p53 and pRb. Several viral oncoproteins have been shown to interact with p53, including JCV T-antigen (34, 35), SV40 T-antigen (36, 37), adenovirus E1B protein (38), and the E6 protein from the human papillomavirus (39). The interaction between these proteins and p53 results in functional inactivation, as in the case of T-antigen, or increased p53 turnover, as in the case of human papillomavirus E6. Similar to p53, pRB is targeted by several different transforming viruses and is complexed with JCV T-antigen (40), SV40 T-antigen (40, 41), adenovirus E1A protein (42), and human papillomavirus E7 protein (43). These observations are interesting in light of our results demonstrating an interaction between JCV and SV40 large T-antigens with the cellular protein Purα. This raises interesting questions regarding the cellular role of Purα. Interestingly, overexpression of Purα causes growth inhibition in vitro. This is particularly noteworthy in light of several observations regarding Purα. The gene encoding Purα, PURA, has been localized to human chromosome band 5q31 (44). Loss of heterozygosity at 5q31 is frequently associated with hematologic malignancies particularly myelodysplastic syndrome and myeloid leukemias (45, 46). Moreover, the recent demonstration of PURA gene deletions in many cases of myelogenous leukemia and myelodysplastic syndrome suggest that Purα may also be involved in tumor development (47).

In this report, we demonstrate that Purα interacts with the large T-antigen of JCV and SV40. In addition, we localize the region of Purα that is important for its interaction in JCV T-antigen. Using transient transfection assays, we also demonstrate that these two proteins interact functionally as well. Full-length Purα and a deletion mutant that retains the ability to interact with JCV T-antigen, however, is incapable of alternating the transcriptional activation of JCV T-antigen. Taken together, these data demonstrate that a physical interaction underlies the functional antagonism between these two proteins.

Acknowledgments—We thank Edward Johnson for insightful discussions and helpful suggestions during the course of this project. We also thank members of the Center for NeuroVirology and NeuroOncology for their support and sharing of reagents and Cynthia Schriver for editorial assistance and preparation of the manuscript.

REFERENCES

1. Moran, E. (1993) Curr. Opin. Genet. Dev. 3, 63–70
2. Fannin, E., and Knippers, R. (1992) Annu. Rev. Biochem. 61, 55–85
3. Major, E. O., Amemiya, K., Tornatore, C. S., Houff, S. A., and Berger, J. (1992) Clin. Microbiol. Rev. 5, 49–73
4. Raj, G. V., and Khalili, K. (1995) Virology 213, 283–291
5. Bergmann, A. D., and Johnson, E. M. (1992) Mol. Cell. Biol. 12, 1257–1265
6. Bergmann, A. D., Ma, Z. W., and Johnson, E. M. (1992) Mol. Cell. Biol. 12, 5673–5682

3. G. L. Gallia et al., unpublished observations.
Interaction of Purα with T-antigen

7. Chang, C. F., Gallia, G. L., Muralidharan, V., Chen, N. N., Zoltick, P., Johnson, E., and Khalili, K. (1996) *J. Virol.* 70, 4150–4156
8. Jurk, M., Weisinger, F., Lottspeich, F., Schwarz, U., and Winnacker, E. L. (1996) *Nucleic Acids Res.* 24, 2799–2806
9. Chen, N. N., and Khalili, K. (1995) *J. Virol.* 69, 5843–5848
10. Chepenik, L. G., Tretiakova, A. P., Krachmarov, C. P., Johnson, E. M., and Khalili, K. (1998) *Gene* 210, 37–44
11. Haas, S., Thatikunta, P., Steplewski, A., Johnson, E. M., Khalili, K., and Amini, S. (1995) *J. Cell Biol.* 130, 1171–1179
12. Zambrano, N., DeRenzis, S., Minopoli, G., Faraonio, R., Donini, V., Scaloni, A., Cimino, F., and Russo, T. (1997) *Biochem. J.* 328, 283–300
13. Du, Q., Tomkinson, A. E., and Gardner, P. D. (1997) *J. Biol. Chem.* 272, 14990–14995
14. Osugi, T., Natarajan, V., Strika, V., Khoury, G., and Salzman, N. P. (1984) *Science* 226, 1337–1339
15. Kelm, R. J., Jr., Elder, P. K., Strauch, A. R., and Getz, M-J. (1997) *J. Biol. Chem.* 272, 14990–14995
16. Chang, C. F., Gallia, G. L., Muralidharan, V., Chen, N. N., Zoltick, P., Johnson, E., and Khalili, K. (1996) *J. Virol.* 70, 4150–4156
17. Cheer, M., Weissinger, F., Lottspeich, F., Schwarz, U., and Winnacker, E. L. (1996) *Nucleic Acids Res.* 24, 2799–2806
18. Chen, N. N., and Khalili, K. (1995) *J. Virol.* 69, 5843–5848
19. Zambrano, N., DeRenzis, S., Minopoli, G., Faraonio, R., Donini, V., Scaloni, A., Cimino, F., and Russo, T. (1997) *Biochem. J.* 328, 283–300
20. Walker, D. L., Padgett, B. L., ZuRhein, G. M., Albert, A. E., and Marsh, R. F. (1973) *Science* 181, 674–676
21. Linzer, D. I. H., and Levine, A. J. (1979) *Cell* 17, 43–52
22. Sarnow, P., Ho, Y. S., Williams, J., and Levine, A. J. (1982) *Cell* 28, 387–394
23. Dyson, N., Bernards, R., Friend, S. H., Gooding, L. R., Hassell, J. A., Major, E. O., Pipas, J. M., Van Dyke, T., and Harlow, E. (1996) *J. Virol.* 64, 1353–1356
24. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsillo, F., Pauzauski, R., and Livingston, D. M. (1988) *Cell* 54, 275–283
25. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988) *Nature* 334, 124–129
26. Dyson, N., Bernards, R., Friend, S. H., Gooding, L. R., Hassell, J. A., Major, E. O., Pipas, J. M., Van Dyke, T., and Harlow, E. (1989) *Science* 243, 934–937
27. Swenson, J. J., and Frisque, R. J. (1995) *Virology* 212, 295–308
28. Jiang, D., Srinivasan, A., Lozano, G., and Robbins, P. D. (1993) *Oncogene* 8, 543–548
29. Meitz, J. A., Unger, T., Huibregtse, J. M., and Howley, P. M. (1992) *EMBO J.* 11, 5013–5020
30. Segawa, K., Minowa, A., Sugasawa, K., Takano, T., and Hanaoka, F. (1992) *Oncogene* 8, 543–548
31. Zambra, N., DeRenzis, S., Minopoli, G., Faraonio, R., Donini, V., Scaloni, A., Cimino, F., and Russo, T. (1997) *Biochem. J.* 328, 283–300
32. Zambra, N., DeRenzis, S., Minopoli, G., Faraonio, R., Donini, V., Scaloni, A., Cimino, F., and Russo, T. (1997) *Biochem. J.* 328, 283–300
33. Zambrano, N., DeRenzis, S., Minopoli, G., Faraonio, R., Donini, V., Scaloni, A., Cimino, F., and Russo, T. (1997) *Biochem. J.* 328, 283–300
34. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsillo, F., Pauzauski, R., and Livingston, D. M. (1988) *Cell* 54, 275–283
35. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988) *Nature* 334, 124–129
36. Dyson, N., Bernards, R., Friend, S. H., Gooding, L. R., Hassell, J. A., Major, E. O., Pipas, J. M., Van Dyke, T., and Harlow, E. (1989) *Science* 243, 934–937
37. Swenson, J. J., and Frisque, R. J. (1995) *Virology* 212, 295–308
38. Jiang, D., Srinivasan, A., Lozano, G., and Robbins, P. D. (1993) *Oncogene* 8, 2805–2812
39. Meitz, J. A., Unger, T., Huibregtse, J. M., and Howley, P. M. (1992) *EMBO J.* 11, 5013–5020
40. Segawa, K., Minowa, A., Sugasawa, K., Takano, T., and Hanaoka, F. (1992) *Oncogene* 8, 543–548
41. Zambra, N., DeRenzis, S., Minopoli, G., Faraonio, R., Donini, V., Scaloni, A., Cimino, F., and Russo, T. (1997) *Biochem. J.* 328, 283–300
42. Zambra, N., DeRenzis, S., Minopoli, G., Faraonio, R., Donini, V., Scaloni, A., Cimino, F., and Russo, T. (1997) *Biochem. J.* 328, 283–300
43. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsillo, F., Pauzauski, R., and Livingston, D. M. (1988) *Cell* 54, 275–283
44. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988) *Nature* 334, 124–129
45. Dyson, N., Bernards, R., Friend, S. H., Gooding, L. R., Hassell, J. A., Major, E. O., Pipas, J. M., Van Dyke, T., and Harlow, E. (1989) *Science* 243, 934–937
46. LeBeau, M. M., Espinosa, R., 3rd, Neuman, W. L., Stock, W., Roulston, D., Larson, R. A., Keinanen, M., and Westbrook, C. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 5484–5488
47. Lezon-Geyda, K. A., Najfeld, V., and Johnson, E. M. (1997) *FASEB J.* 11, A100
48. Lezon-Geyda, K. A., Najfeld, V., and Johnson, E. M. (1997) *FASEB J.* 11, A100
