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# The POU domain protein Tst-1 and papovaviral large tumor antigen function synergistically to stimulate glia-specific gene expression of JC virus

(coactivator/Oct-6/progressive multifocal leukoencephalopathy/SCIP/synergism)

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ABSTRACT **Synergism between transcriptional activators is a powerful way of potentiating their function. Here we show that the glial POU domain protein Tst-1 (also known as Oct-6 and SCIP) and large tumor antigen (T antigen) synergistically increased transcription from both the early and the late promoters of papovavirus JC in glial cells. Synergism between both proteins did not require T-antigen-mediated DNA replication or direct binding of T antigen to the promoter. The ability of T antigen to functionally cooperate with Tst-1 was contained within its N-terminal region, shown by the fact that small tumor antigen (t antigen) could substitute for T antigen in transfection experiments. In addition to this functional synergism, a direct interaction between Tst-1 and T antigen was observed *in vitro*. Using deletion mutants of Tst-1 and T antigen, the POU domain of Tst-1 and the N-terminal region of T antigen were found to participate in this interaction. Because of the low levels of Tst-1 present in oligodendrocytes, synergism between Tst-1 and T antigen could be an important factor in establishing the lytic infection of oligodendrocytes by JC virus during the course of the fatal demyelinating disease progressive multifocal leukoencephalopathy.**

Papovaviruses have long been used as model systems for cellular transcription and replication events (1). A short region of the viral genome contains all cis-acting elements necessary to control viral DNA replication, transcription, host range, and tissue tropism. Several strains of the human papovavirus JC (2) exhibit a marked tropism for myelinating glia that correlates well with the glia specificity of their gene expression (3, 4). It is this glia specificity that might be a key factor in the selective destruction of central nervous system oligodendrocytes caused by JC virus in the course of progressive multifocal leukoencephalopathy (PML) (5). A drastic increase in PML cases has been recently observed as a result of the AIDS epidemic, lending increased clinical importance to this once rare disease and its etiologic agent, JC virus (5).

We have previously shown that both early and late transcription of JC virus are stimulated by the POU domain protein Tst-1 (6), also known as Oct-6 or SCIP (7–12). This stimulation was conferred by a binding site for Tst-1 present twice within the viral regulatory region, which had only little resemblance to the octamer motif commonly recognized by most POU domain proteins (13–15). Importantly, myelinating glia, which are the targets of JC viral infection, are one of the few cell types in which Tst-1 is expressed after birth (10, 16–18). This very restricted postnatal expression pattern contrasts strongly with its widespread prenatal expression throughout the developing nervous system (8, 11). Here we show that synergism with viral large tumor antigen (T antigen) is one potential mechanism for the function of Tst-1 during PML.

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## MATERIALS AND METHODS

**Plasmid Constructs.** Reporter plasmids $pJCluc_{early}$ and $pJCluc_{late}$ contain the transcriptional control sequences of JC virus strain Mad-1 [map position (mp) 5029–279] in both possible orientations (6). Plasmids p36-luc, siteA-luc, and HSVoct-luc as well as the expression plasmids pCMV/Tst-1, pRSV-SV40T, and pRSV-JCT have been described (6, 19). Derived from pRSV-JCT, which expressed both T antigen and small tumor antigen (t antigen) of JC virus, were two other vectors: pRSVt-ag (mp 5081–4243 of Mad-1) selectively expressed t antigen, whereas pRSVT-Ag, created by removing all intron sequences, selectively expressed T antigen. An N-terminal deletion mutant of T antigen (TΔN; mp 4426–2442 of Mad-1) was constructed by introducing a Kozak consensus sequence at the beginning of exon 2 of the viral early gene and was cloned into pCMV1, yielding pCMV/TΔN. For *in vitro* transcription/translation in reticulocyte lysates (Promega), the fragments coding for t antigen, T antigen, TΔN, and various Tst-1 mutants were also inserted into pBKS II+ (Stratagene). Tst-1 mutants ΔN (missing amino acids 4–240), ΔC (missing amino acids 396–448), and ΔNC (missing amino acids 4–240 and 396–448) have been described (6). Additional Tst-1 mutants were created by deleting the whole POU domain (amino acids 241–395; ΔPOU), the POU-specific domain (amino acids 241–319; $\Delta POU_S$), or the POU homeodomain (amino acids 336–395; $\Delta POU_{HD}$). The bacterial expression vector pGEX-Tst-1 expressed amino acids 96–448 of Tst-1 as described (6). From this plasmid, pGEX-Tst-1Δ353 was derived by deleting all Tst-1 sequences downstream of an internal *Xho* I site.

**Transfections, Luciferase, and DNA Replication Assays.** U138 human glioblastoma cells were propagated in RPMI medium supplemented with 10% fetal calf serum (FCS). One day before transfection, cells were plated at a density of $5 \times 10^5$ per 60-mm plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Cells were transfected by the calcium phosphate technique (6) with 2 μg of luciferase reporter plasmid and various amounts of Tst-1 and T-antigen expression plasmids. The total amount of plasmid was kept constant. At 24 h posttransfection, cells were treated for 1 min with 30% (vol/vol) glycerol in PBS and placed in fresh DMEM/10% FCS. Cells were harvested after an additional 24 h. Extracts were assayed for luciferase activity as described (6) or analyzed for the presence of newly replicated, *Dpn* I-resistant plasmid DNA. The isolation of

Abbreviations: PML, progressive multifocal leukoencephalopathy; T antigen, large tumor antigen; t antigen, small tumor antigen; mp, map position; GST, glutathion *S*-transferase; SV40, simian virus 40.
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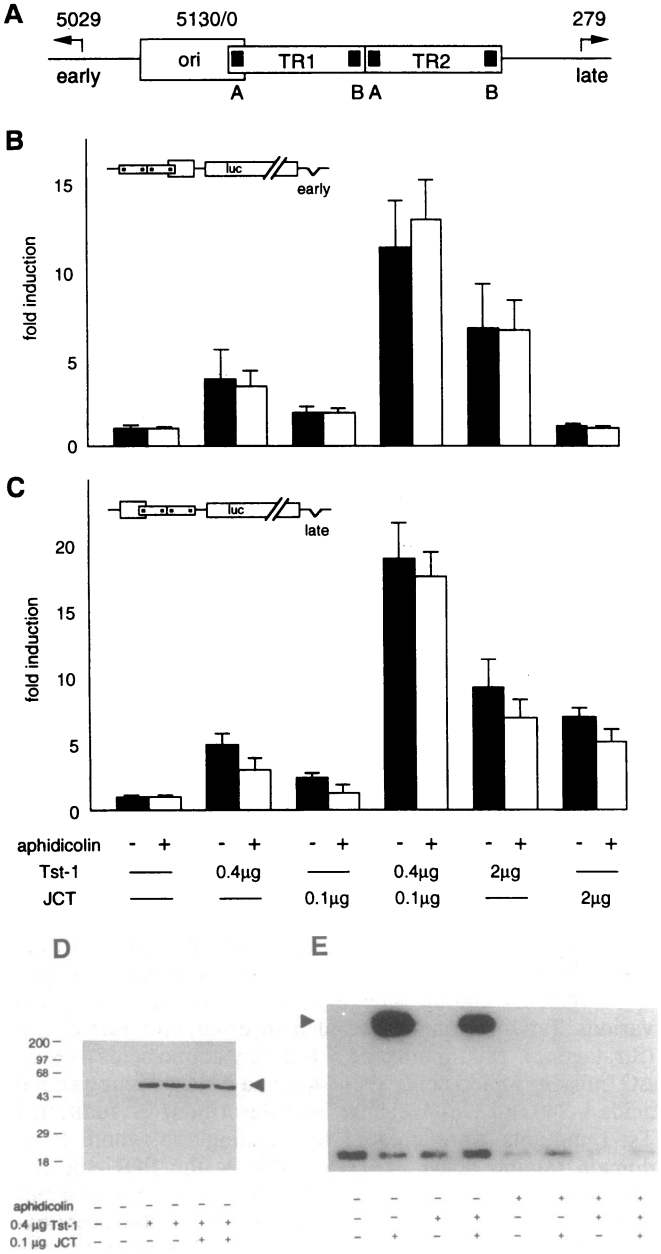


FIG. 1. (*A*) Regulatory region of JC virus (mp 5029–279) comprising origin of DNA replication (ori) and 98-bp tandem repeats (TR1 and TR2). Solid boxes represent Tst-1 binding sites A and B. Early and late genes are transcribed in opposite directions as indicated by arrows. (*B* and *C*) Synergistic activation of viral transcription by Tst-1 and JC T antigen. Transient transfection experiments were performed in human U138 cells using luciferase reporter plasmids containing viral early ($pJCluc_{early}$ in *B*) or late ($pJCluc_{late}$ in *C*) promoters. pCMV1/Tst-1 and pRSV-JCT were cotransfected in the indicated amounts. After transfection, cells were kept in the absence (solid bars) or presence (open bars) of aphidicolin (10 μg/ml) as indicated. Transcription from the reporter was determined by the luciferase activity of cellular extracts in three independent experiments, each performed in duplicate. Results are presented as -fold inductions ± SEM above the level of luciferase activity obtained in transfections with the reporter plasmids alone, which was given an arbitrary value of 1. The basal activity of the JC early promoter ranged between 300 and 400, and the basal activity of the JC late promoter was between 30 and 40 light units per 10 sec per μg of protein. (*D*) Tst-1 levels in transfected cells. Western blots on extracts from cells transfected with pCMV1/Tst-1, pRSV/JCT, or a combination thereof using polyclonal anti-Tst-1 antiserum. Numbers on left are kDa. (*E*) Replication of reporter plasmids. Low molecular weight DNA was isolated from cells transfected with plasmid $pJCluc_{early}$ in combination with pCMV1/Tst-1 or pRSV/JCT and kept in the presence or absence of aphidicolin (10 μg/ml) as indicated. Newly replicated DNA (arrowhead) was separated from unreplicated bacterial input DNA because of its resistance to digestion with *Dpn* I.

low molecular weight DNA, digestion by *Dpn* I and *Hin*dIII, and Southern blot analysis was performed as described using $^{32}P$-labeled $pJCluc_{early}$ as a probe (19).

**Glutathion *S*-Transferase (GST) Chromatography.** GST as well as GST/Tst-1 fusion proteins were generated in *Escherichia coli* DH5α using pGEX, pGEX-Tst-1, and pGEX-Tst-1Δ353. GST proteins were purified by adsorption to glutathione-agarose resins (Sigma). Resins carrying equal amounts of GST, GST/Tst-1, or GST/Tst-1Δ353 were incubated for 2 h at 4°C with 50 ng of simian virus 40 (SV40) T antigen in B buffer (100 mM NaCl/20 mM Hepes, pH 7.8/2 mM EDTA/0.2% Nonidet P-40/5 mM dithiothreitol/10% glycerol/0.5% nonfat dry milk/1 μg of leupeptin per ml/1 μg of aprotinin per ml). After extensive washing resin-bound proteins were separated on SDS/10% polyacrylamide gels, which were either Coomassie stained or blotted onto nitrocellulose. Resin-bound SV40 T antigen was detected by Western blot analysis using the ECL detection kit (Amersham) in combination with pAb416 as the primary antibody and goat anti-mouse IgG antibody coupled to horseradish peroxidase as the secondary antibody.

**Coimmunoprecipitation.** $^{35}S$-labeled Tst-1 or T-antigen proteins were generated in reticulocyte lysates programmed with *in vitro* transcribed RNA. $^{35}S$-labeled Tst-1 was incubated with baculovirus-produced SV40 T antigen at 4°C in B buffer, whereas $^{35}S$-labeled JC T antigen was incubated with bacterially expressed GST/Tst-1 fusion protein. After 30 min, monoclonal pAb416 preadsorbed to protein A-Sepharose (Sigma) was added to reaction mixtures containing $^{35}S$-labeled Tst-1 proteins, while polyclonal anti-Tst-1 antiserum preadsorbed to protein A-Sepharose was added to those reaction mixtures that contained $^{35}S$-labeled JC T antigen. Incubation continued for 2 h at 4°C. Coimmunoprecipitated $^{35}S$-labeled proteins were detected after extensive washing by electrophoresis on SDS/10% polyacrylamide gels, fluorography, and autoradiography.

## RESULTS

The possible interplay between Tst-1 and other transcription factors in the regulation of JC viral gene expression was investigated in transient transfection experiments (Fig. 1). Reporter plasmids with the firefly luciferase gene under control of the early or late promoter of JC virus were cotransfected with suboptimal amounts of Tst-1 in U138 glioblastoma cells, which did not express endogenous Tst-1 (6). Under these conditions, both early and late promoter were activated <5-fold. When, in addition, low levels of JC T antigen were also present, expression from both viral promoters increased dramatically, on average 11-fold for the early and 18-fold for the late promoter (Fig. 1 *B* and *C*). The only significant effect observed for JC T antigen alone was an ≈6-fold induction of late gene expression at high levels (Fig. 1*C*), in agreement with previous findings on SV40 (20, 21). Importantly, cotransfection of Tst-1 with JC viral T antigen did not increase the amount of Tst-1 detectable in the transfected cells (Fig. 1*D*), nor did it change the predominantly nuclear localization of Tst-1 (data not shown).

Because JC T antigen is essential for viral DNA replication and because our reporter plasmids contained a functional origin (19, 22, 23), we analyzed the replication status of reporter plasmids. We could indeed detect replication as judged by the presence of *Dpn* I-resistant reporter plasmid (Fig. 1*E*). Part of the observed change in luciferase activity

could therefore be due to increased amounts of reporter plasmid or to replication-dependent, facilitated enhancer function (24). To address this issue, we repeated transfections in the presence of aphidicolin, which suppressed DNA replication (Fig. 1*E*). Still we observed a strong stimulation of viral gene expression by limiting amounts of Tst-1 and JC viral T antigen, showing that this effect can be regarded as a true transcriptional synergism.

Next we tested whether the T antigens of the related papovaviruses SV40 and BK virus could substitute for JC T antigen. SV40 and BK T antigens are much more potent inducers of DNA replication than JC T antigen (19), so that experiments had to be carried out in the presence of aphidicolin (Fig. 2*A*). Low levels of SV40 or BK T antigen exhibited no significant effect on the expression of either early or late viral genes. In the presence of Tst-1, however, SV40 and BK T antigen induced a 9- to 10-fold activation of both viral promoters compared to a 10- to 13-fold stimulation observed for JC T antigen (Fig. 2*A*; data not shown).

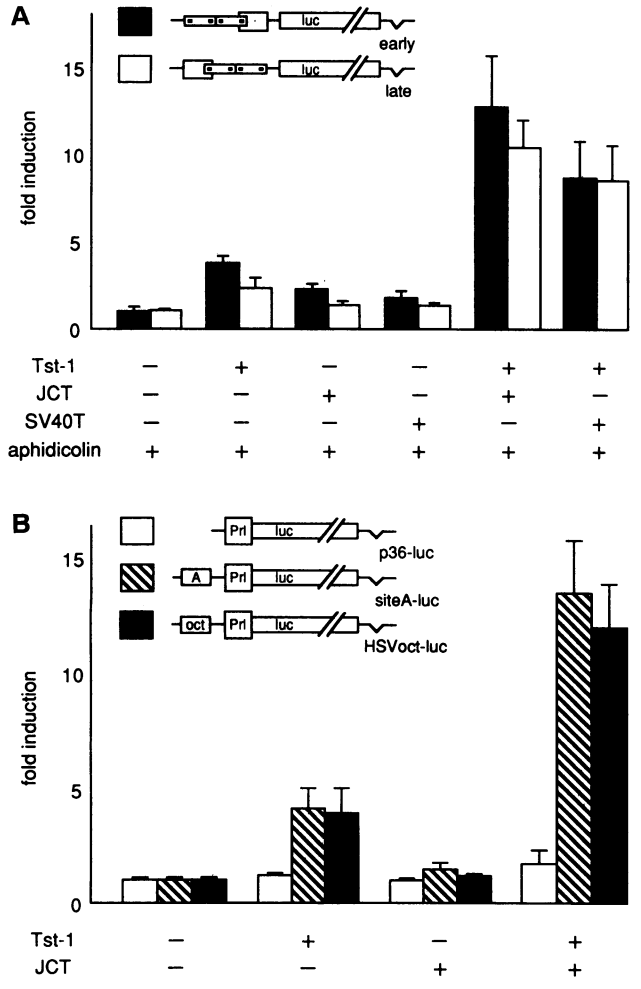


FIG. 2. (*A*) Functional comparison between JC and SV40 T antigens. Reporter plasmids pJCluc$_{early}$ (solid bars) or pJCluc$_{late}$ (open bars) were cotransfected with 0.4 μg of pCMV/Tst-1, 0.1 μg of pRSV-JCT, 0.1 μg of pRSV-SV40T, or various combinations thereof in U138 cells as indicated. (*B*) Synergistic activation of synthetic promoters by Tst-1 and JC T antigen. Plasmids carrying the luciferase gene under the control of the rat prolactin minimal promoter (from position −36 to +33) alone (p36-luc, open bars) or a combination of minimal promoter and binding site for Tst-1 (siteA-luc, hatched bars; HSVoct-luc, solid bars) were cotransfected with 0.4 μg of pCMV/Tst-1, 0.1 μg of pRSV-JCT, or various combinations thereof in U138 cells as indicated. Basal activity of all synthetic promoters ranged between 10 and 20 light units per 10 sec per μg of protein. Transcription levels in *A* and *B* were determined as luciferase activities in three independent experiments, each performed in duplicate, and are presented as -fold inductions ± SEM (see Fig. 1).

One copy of the functionally important Tst-1 binding site A within the regulatory region of JC virus (6) is situated in close proximity to T-antigen binding site II, overlapping with both TATA box and origin of DNA replication (4, 22, 23). Synergism between Tst-1 and JC T antigen could thus require both proteins to bind to adjacent sites. To address this issue, we performed transient transfection experiments with luciferase reporters containing a synthetic modular promoter consisting of one copy of site A (5′-CTCCTGTATATA-TAAAAA-3′) in front of a TATA box. As shown in Fig. 2*B*, one copy of site A was sufficient to confer synergistic activation by Tst-1 and JC T antigen as evidenced by a significant 13-fold stimulation. Site A from the JC regulatory region could be replaced by a different binding site for POU domain proteins (HSVoct, 5′-GCATGCTAATGATAT-TCTTT-3′) without loss of synergism. In contrast, no synergism was detected in the absence of a Tst-1 binding site or in the presence of the functionally inactive site B (6) from the viral regulatory region (Fig. 2*B*; data not shown). Thus, synergism does not require a binding site for JC T antigen in the presence of a functional binding site for Tst-1. This does not rule out, however, that in the context of the viral regulatory region T-antigen binding sites participate in mediating the synergism.

The expression plasmid for JC T antigen used so far expressed both T and t antigens (T/t-ag in Fig. 3). To perform cotransfections of Tst-1 with t or T antigen alone, vectors were generated that selectively expressed either T antigen (T-ag) or t antigen (t-ag). T antigen interacted synergistically with Tst-1 on both the early and the late promoters (Fig. 3; data not shown). Synergism was also observed between t antigen and Tst-1. Coexpression of t and T antigens from different expression plasmids together with Tst-1 led to no additional increase. In contrast, a T-antigen mutant, which was created by deleting the N-terminal region coded for by the first exon was unable to cooperate synergistically with Tst-1 (TΔN in Fig. 3). Because t and T antigens share the

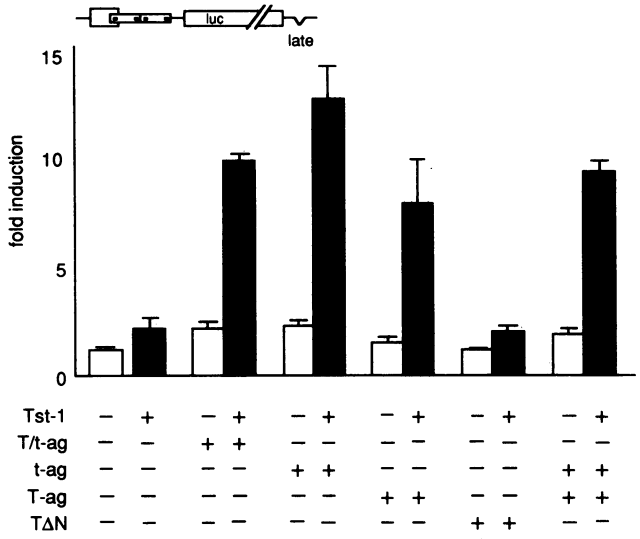


FIG. 3. Functional comparison between t and T antigens of JC virus. Reporter plasmid pJCluc$_{late}$ was transfected in U138 cells alone or in combination with 0.4 μg of pCMV/Tst-1 (solid bars) and 0.1 μg of the following plasmids: pRSV-JCT (T/t-ag), pRSVt-ag (t-ag), pRSVT-ag (T-ag), and pCMVTΔN (TΔN). Transcription levels were determined as luciferase activities in cellular extracts 48 h after transfection in three independent experiments, each performed in duplicate, and are presented as -fold inductions ± SEM as described in Fig. 1.

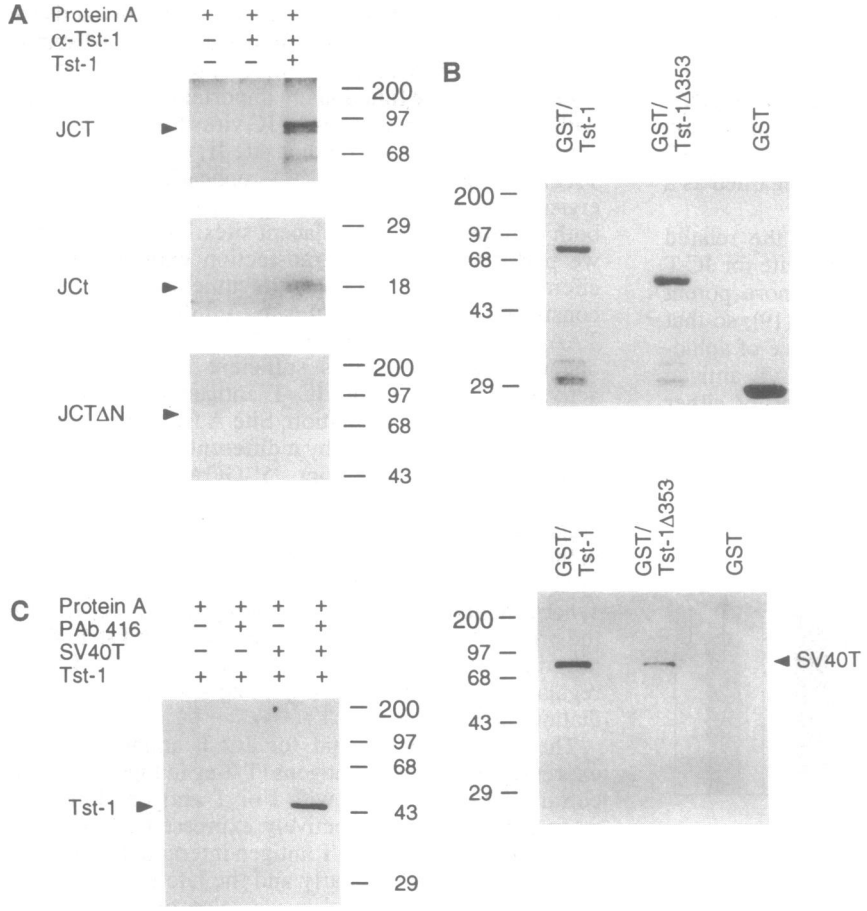


FIG. 4. (*A*) Coimmunoprecipitation of JC T antigen with polyclonal anti-Tst-1 antiserum. The following proteins were $^{35}$S-labeled by *in vitro* translation in reticulocyte lysates: JCT, JC T antigen; JCt, JC t antigen; JCTΔN, an N-terminal mutant of JC T antigen. Equal amounts of $^{35}$S-labeled proteins were used for each reaction. Both T and t antigens, but not the N-terminal mutant, were specifically immunoprecipitated in the presence of GST/Tst-1 fusion protein (Tst-1), polyclonal anti-Tst-1 antiserum (α-Tst-1), and protein A-Sepharose (Protein A). (*B*) GST chromatography of SV40 T antigen. SV40 T antigen was incubated with resins carrying comparable amounts of GST/Tst-1, GST/Tst-1Δ353, or GST alone as determined by Coomassie staining (*Upper*). T antigen retained on the respective resins after extensive washing was visualized by Western blotting. (*C*) Coimmunoprecipitation of Tst-1 with monoclonal antibodies against T antigen. $^{35}$S-labeled Tst-1 was specifically immunoprecipitated in the presence of purified baculoviral SV40 T antigen (SV40T), a monoclonal antibody directed against SV40 T antigen (pAb416), and protein A-Sepharose (Protein A). Numbers on left indicate size of molecular weight markers in kDa.

same 81 N-terminal amino acids, it is very likely that this common sequence information is responsible for the observed synergistic effect.

Its potential involvement in direct protein–protein interactions with Tst-1 was tested in coimmunoprecipitation experiments. Large JC T antigen $^{35}$S-labeled in reticulocyte lysates could be specifically precipitated under native conditions in the presence of Tst-1 and anti-Tst-1 antiserum (Fig. 4*A*). Similarly, JC t antigen was immunoprecipitated with antibodies to Tst-1, whereas a mutant T antigen without the 84 N-terminal amino acids was not. While the interaction of *in vitro* translated JC T antigen with Tst-1 proved to be specific, only weak signals could be obtained. The weak signals could be due to inefficient translation or posttranslational modification (reviewed in ref. 25) of T antigen in reticulocyte lysates. To circumvent this problem, we used baculovirus-produced SV40 T antigen in all following experiments. When baculovirus-produced SV40 T antigen was passed over a GST/Tst-1 column, it was specifically retained, while it was not detected in the eluate of a GST column (Fig. 4*B*). Interestingly, deletion of the C-terminal 96 amino acids of Tst-1, which included part of the POU homeodomain, led to a drastic reduction in the amount of T antigen retained on the column.

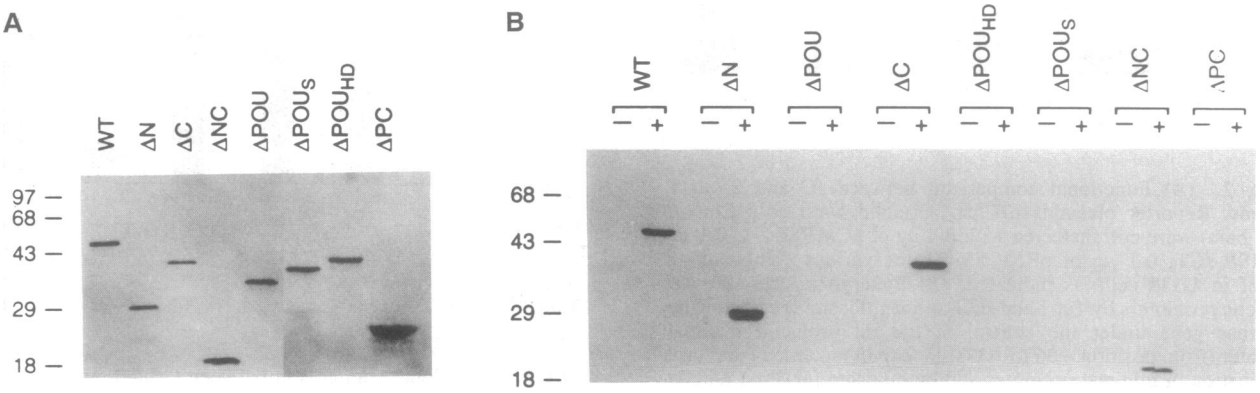


FIG. 5. Identification of the interaction domain in Tst-1. (*A*) $^{35}$S-labeled Tst-1 proteins. WT, wild-type Tst-1; ΔN, mutant Tst-1 lacking amino acids 4–240; ΔC, mutant Tst-1 lacking amino acids 396–448; ΔNC, POU domain of Tst-1; ΔPOU, mutant Tst-1 lacking amino acids 241–359; ΔPOU$_S$, mutant Tst-1 lacking amino acids 241–319; ΔPOU$_{HD}$, mutant Tst-1 lacking amino acids 319–395; ΔPC, mutant Tst-1 containing amino acids 1–240. (*B*) Coimmunoprecipitation of Tst-1 proteins. $^{35}$S-labeled Tst-1 proteins were incubated with monoclonal pAb416 and protein A-Sepharose in the absence (lanes −) or presence (lanes +) of purified baculoviral SV40 T antigen. Tst-1 proteins still bound to protein A-Sepharose beads after extensive washing were visualized by SDS/polyacrylamide gel electrophoresis, fluorography, and autoradiography. Numbers on left indicate size of molecular weight markers in kDa.

Likewise, *in vitro* translated Tst-1 was coimmunoprecipitated in the presence of both purified SV40 T antigen and pAb416, a monoclonal antibody directed against the SV40 T antigen (Fig. 4*C*). Using $^{35}$S-labeled Tst-1 mutants (Fig. 5*A*), we mapped the interaction domain within Tst-1. Deletion of the region N-terminal to the POU domain (ΔN), which is known to contain the transactivation domain (26), or the region C-terminal to the POU domain (ΔC) did not interfere with coimmunoprecipitation (Fig. 5*B*). In agreement, a Tst-1 mutant that consisted only of the POU domain itself (ΔNC) was still capable of interacting with T antigen. When, on the other hand, the POU domain was deleted either alone (ΔPOU) or in combination with the C terminus (ΔPC), Tst-1 could no longer be coimmunoprecipitated. After selective deletion of the POU-specific ($\Delta POU_S$) or the POU homeodomain ($\Delta POU_{HD}$), Tst-1 also lost its ability to interact with T antigen (Fig. 5*B*). These results argue that a structurally intact POU domain is necessary and sufficient for the interaction between Tst-1 and JC T antigen.

## DISCUSSION

The POU domain transcription factor Tst-1, which is naturally present in oligodendrocytes (10, 16, 18), activates both promoters of JC virus, arguing for a potential role of Tst-1 in determining the glia specificity of viral gene expression (6). Synergism among POU domain proteins or between POU domain proteins and other transcriptional regulators has been described in several systems (ref. 27; for a review, see ref. 15). Here we show the existence of synergism between Tst-1 and JC T antigen. This synergism is most pronounced at low levels of both proteins and becomes less visible in the presence of higher amounts of Tst-1 (data not shown). When infected initially, mature oligodendrocytes contain only low amounts of Tst-1 (18). Given the synergism between Tst-1 and T antigen, these levels might nevertheless be important during the onset of PML.

Synergism between Tst-1 and JC T antigen did not require the presence of T-antigen binding sites, consistent with findings on SV40 T-antigen-dependent transcriptional regulation (28–31). With the exception of SV40 early gene expression (32), activation or repression of most promoters by SV40 T antigen is independent of site-specific T-antigen binding and is mediated instead by binding sites for other transcription factors (30, 31).

Although we cannot rule out that the observed synergism is caused by a T-antigen-dependent modification of Tst-1 or a T-antigen-dependent increase in the level of otherwise limiting cellular proteins, which are required for Tst-1-mediated activation, direct interaction between Tst-1 and JC T antigen seems to be the most likely mechanism. On the part of JC T antigen, interaction is mediated by its N-terminal region, which also contains the transcriptional transactivation function (33) but not the DNA-dependent helicase activity (34). Thus, it is unlikely that T antigen mediates the synergistic activation by overcoming repressive chromatin effects, as suggested for other DNA-dependent ATPases such as SWI2 (35).

Binding of Tst-1 to T antigen proved to be dependent on its POU domain, as was the transcriptional synergism (data not shown), confirming that the POU domain is a multifunctional domain. In addition to conferring site-specific high-affinity DNA binding (for a review, see refs. 13–15), the POU domain is also involved in nuclear localization (M.W. and M.G. Rosenfeld, unpublished data) and provides an interface for interactions with other proteins, including various viral or cellular coactivators (36–38), as for instance VP16 in the case of Oct-1 (38–40).

Because T antigen interacts not only with Tst-1 but also with the TATA binding factor TBP (41), T antigen could mediate contact between the transcriptional activator Tst-1 and the basal transcription machinery. The observed synergism between JC T antigen and Tst-1 might thus be yet another example of how coactivators increase the transactivation potential of POU domain proteins.

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