Regulation of JC virus by the POU-domain transcription factor Tst-1: Implications for progressive multifocal leukoencephalopathy

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ABSTRACT Progressive multifocal leukoencephalopathy results from an opportunistic infection of myelin-producing oligodendrocytes by the glia-specific human papovavirus JC. In this report, evidence is presented that the viral transcription factor Tst-1, a member of the POU-domain family, stimulates transcription of both early and late viral genes. Stimulation depended on site-specific binding of Tst-1 to the late viral regulatory region and on the presence of an intact amino-terminal transactivation domain within Tst-1. Because of its ability to increase the expression of viral large tumor antigen, Tst-1 stimulated viral DNA replication, without participating directly in the replication event. Our results suggest that Tst-1 is one of the determining factors in the glia specificity of JC virus.

The occurrence of progressive multifocal leukoencephalopathy (PML) has recently increased dramatically as a result of the AIDS epidemic (refs. 1 and 2; ref. 3 and references therein). This demyelinating disease of the human brain is caused by the human papovavirus JC (4). Exposure to JC virus usually occurs early in life and results in the persistence of an archetypic virus in a large percentage of the human population (5, 6). Reactivation of the latent virus in individuals suffering from immunosuppression can lead to selective infection and cytolytic destruction of the myelin-producing oligodendrocyte of the central nervous system and results in severe demyelination (ref. 3 and refs. therein). Viral isolates from infected brain tissue, such as the prototypic JC viral strain MAD-1 (7), exhibit a marked specificity for glial cells (8, 9). The tissue tropism of JC virus is defined on the level of transcription and is conferred by the hypervariable non-coding region, which not only serves as origin of DNA replication (10, 11) but also as promoter for both the early regulatory and the late capsid genes (8) (see Fig. 1A). Understanding the pathophysiology of PML might be greatly enhanced by identifying the transcription factors that are involved in determining the glia specificity of JC virus.

One of the few cell-type-selective transcriptional regulators that has been identified in myelinating glial cells is Tst-1, a member of the POU-domain family, also referred to as SCIP or Oct-6, which was originally identified by PCR in PEC-RCS RNA (12-15). In the adult, this potential regulator of early embryogenesis and neural development (12, 13) is mainly expressed in the myelin-producing Schwann cells of the peripheral nervous system and the oligodendrocytes of the central nervous system (15, 16). Expression of Tst-1 is highest before differentiation and stays at a reduced basal level thereafter (17, 18). Since oligodendrocytes are the physiological target of viral infection during PML and since both Schwann cells and oligodendrocytes are permissive for infection by JC virus in culture (9), there is a striking correlation between expression of Tst-1 and susceptibility for JC virus. This correlation prompted us to analyze the influence of Tst-1 on various processes of the life cycle of JC virus.

MATERIALS AND METHODS

Plasmid Constructs. Plasmid pBKS-MAD1 contained the complete JC viral genome inserted into the EcoRI site of pBKCS (Stratagene). pJC433 has the viral regulatory region inserted into the polylinker of pUC18 and has been described (11).

Luciferase reporter plasmids pJClucEearly and pJCluclate were created by inserting the regulatory region of JC viral strain MAD-1 (spanning map position 5029–279 according to ref. 7) into the Bgl II site of the promoterless luciferase plasmid pGL2 basic (Promega) in both possible directions. For the functional analysis of isolated Tst-1 binding sites identified within the JC viral regulatory region, oligonucleotides containing these sites were inserted into a luciferase reporter in front of the rat prolactin minimal promoter (from position −36 to 33), which drove the transcription of the luciferase gene (19). Site A from the regulatory region of JC viral strain MAD-1 was cloned in various orientations and copy numbers into the Xho I site of the reporter plasmid; site B and the herpes simplex virus octamer motif (5′-GCAATTCGATATCTCTT-3′) were cloned into the BamHI site.

The expression plasmid for Tst-1 was constructed by inserting an Apa I/Xba I fragment from the mouse genomic Tst-1 gene, which contained the whole coding region, into pCMV, yielding pCMV/Tst-1. The control plasmid pCMV/asTst-1 contained the same fragment inserted in the opposite direction. Expression plasmids for mutant Tst-1 proteins were created by deleting the region between amino acids 3 and 241 to obtain pCMV/Tst-1 ΔN, by introducing a stop codon after amino acid 395 to obtain pCMV/Tst-1 ΔC, or by changing amino acids 383 and 384 from tryptophan and phenylalanine to cysteine and serine to obtain pCMV/Tst-1 mt. pCMV/Tst-1 ΔNC was constructed by combining the deletion of amino acids 4–240 with the introduction of a stop codon after amino acid 395. The expression plasmids pCMV/Pit-1 and pCMV/Oct-1 and the Oct-2-expressing plasmid pOEV1 have been described (20–22). pRSV-JCT, an expression vector for JC viral large tumor antigen (T antigen), was generated by placing a 2.6-kb fragment containing the gene for JC viral T antigen under the control of the Rous sarcoma virus long terminal repeat. For that purpose, an Xba I and an EcoRI site were introduced at the start and the end of the gene, respectively.

Luciferase Assays. In general, 2 μg of reporter plasmid was cotransfected with an equal amount of effector plasmid into

Abbreviations: PML, progressive multifocal leukoencephalopathy; T antigen, large tumor antigen.

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the human glioblastoma cell line U138. 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% (vol/vol) fetal calf serum and transfected by using the calcium phosphate technique (23). To control for transfection efficiency, the total amount of plasmid was kept constant at 4 \(\mu\)g, adding pCMV1 where necessary. After 24 h, cells were treated for 1 min with 30% (vol/vol) glycerol in DMEM, harvested after an additional 24 h, and assayed for luciferase activity as described (24).

Replication Assays. Reporter plasmids pJC433 (2 \(\mu\)g) and pBKS-MAD1 (3 \(\mu\)g) were transfected in various combinations with pR5V-ICT (1 \(\mu\)g) and expression plasmids for Tst-1 or Oct-1 (2 \(\mu\)g) into U138 cells as described above. Cells were harvested 3 (for pJC433) or 5 (for pBKS-MAD1) days posttransfection. The isolation of low molecular weight DNA, digestion by Dpn I and EcoRI (or BamHI), agarose gel electrophoresis, transfer to Hybond membrane, and Southern analysis were performed as described (11). \(^{32}\)P-labeled pJC433 was used as a probe.

DNase I Footprinting. DNA fragments containing the complete regulatory region of JC viral strain MAD-1 or the isolated sites A and B were generated by a PCR strategy in which only one of the input oligonucleotide primers was radiolabeled using T4 kinase and \([\gamma-\text{P}]\ATP\). Plasmids pJC433 (11), pBKS/site A, and pBKS/site B served as templates. DNase I footprinting was performed as described (25) with 5000-10,000 cpm of the radiolabeled fragment, 2 \(\mu\)g of poly(dl-dC), and increasing amounts of a purified Tst-1 fusion protein. The fusion protein was produced in the bacterial strain DH5\(\alpha\) with the expression vector pGEX-KG (26) and contained amino acids 96–448 of Tst-1 fused to the carboxyl terminus of glutathione S-transferase. Purified protein was obtained using glutathione-agarose affinity chromatography.

Electrophoretic Mobility Shift Assay. Double-stranded oligonucleotides containing the sequence of either site A or site B (see Fig. 2) were generated and radiolabeled by filling in the Xho I/Sal I (site A) or BamHI/Bgl II (site B) overhangs using Klenow enzyme and [\(\alpha-\text{P}\)]CTP. For electrophoretic mobility shift assays, 0.5 ng of labeled probe was incubated with Tst-1 protein for 20 min at room temperature in a 20-\(\mu\)l reaction mixture containing 10 mM Hepes (pH 8.0), 5% glycerol, 100 mM NaCl, 5 mM MgCl\(_2\), 2 mM dithiothreitol, 0.1 mM EDTA, 2 \(\mu\)g of poly(dl-dC), and 0.5 \(\mu\)g of bovine serum albumin. The bacterially expressed glutathione S-transferase fusion protein as well as nuclear extracts of COS cells transfected with pCMV/Tst-1 were used as a source of Tst-1. One-third of each reaction mixture was loaded onto a 5% nondenaturing acrylamide gel. Electrophoresis was in 0.5× TBE (1 \(\times\) TBE = 89 mM Tris/89 mM boric acid/2 mM EDTA, pH 8.3) at 200 V for 3 h. For competition analyses, radiolabeled probe and competitor DNA were added simultaneously to the protein. Competition curves were generated after Phosphorlmager (Molecular Dynamics)-assisted quantification using the GraphPad INPLOT program (GraphPad Software, San Diego). Data were fitted to a four-parameter logistic equation with the slope factor set to -1.

**RESULTS AND DISCUSSION**

To investigate the influence of Tst-1 on the transcription of JC viral genes, transient transfection experiments were performed in the human glioblastoma cell line U138, which by RNA-blot analysis does not express a significant level of endogenous Tst-1 (unpublished results). When reporter plasmids containing the firefly luciferase gene under the control of either the early or the late promoter of JC viral strain MAD-1 were cotransfected with an expression plasmid for Tst-1, a 3- to 10-fold stimulation of both promoters was observed (Fig. 1 B and D). Tst-1 did not stimulate the expression of a promoterless luciferase construct, whereas the transcription of a luciferase gene under the control of the P\(_E\) promoter was inhibited 3- to 8-fold (data not shown), as has been described in other cell lines (16, 17). Neither the early nor the late viral promoter was reproducibly stimulated by the related POU proteins Oct-1, Oct-2, or Pit-1 (ref. 27 and references therein), indicating that the stimulation of both the early and the late viral promoter was specific for Tst-1.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** (A) Schematic representation of the regulatory region of JC virus containing the origin of DNA replication (ori) and the 98-bp tandem repeats TR1 and TR2. Black boxes within the tandem repeats represent Tst-1 binding sites A and B. Early and late genes are transcribed from the regulatory region in opposite directions as indicated by the arrows. (B–E) Influence of Tst-1 on viral transcription. Transient transfection experiments were performed in the human glioblastoma cell line U138. Reporter plasmids contained the luciferase gene under the control of the viral early (pJCLucearly; B and C) or late (pJCLuclate; D and E) promoter and were cotransfected with effector plasmids expressing Tst-1, Oct-1, Oct-2, Pit-1 as well as various mutants of Tst-1 under the control of the cytomegalovirus promoter. Tst-1 mutants lacked either the region amino-terminal (\(\Delta N\)) or carboxyl-terminal (\(\Delta C\)) of the POU domain, expressed solely the POU domain (\(\Delta NC\)), or were strongly impaired in binding because of a substitution of conserved amino acids in the third helix of the POU homeodomain (\(\Delta T\)). Transcription from the reporter was determined by the luciferase activity of cellular extracts and was measured as the amount of light units per 10 sec per microgram of protein. Results represent the mean of triplicate determinations; error bars indicate the SEM. Similar results were obtained in four additional independent experiments. In all experiments the early promoter of JC virus was ~10-fold more active than the late promoter, wt, wild type.
It has been concluded from previous studies of the P_0 promoter that Tst-1 functions as an inhibitor of myelin-specific genes (17). With the early and late promoter of JC virus, glia-specific promoters that are stimulated by Tst-1 have now been identified. This indicates that, in addition to its inhibitory function on a subset of genes, Tst-1 positively regulates the expression of another group of glia-specific genes.

The stimulation of both the early and the late promoter of JC virus was dependent on DNA binding: substitution of two conserved amino acids within the POU homeodomain (Trp-383 and Phe-384), previously shown to be essential for DNA binding of the related POU-domain protein Pit-1 (28), not only rendered DNA binding of Tst-1 undetectable in vitro (unpublished observation) but also abolished its ability to activate the viral promoters (Fig. 1C and E). Deletion of the region amino-terminal to the POU domain also led to a loss of stimulation, whereas deletion of the region carboxy-terminal to the POU domain did not have any influence on the activity of Tst-1. These studies map the major transactivation domain of Tst-1 to the region amino-terminal to the POU domain, which is consistent with results recently obtained on a synthetic octamer-containing promoter (29).

Because stimulation of the viral early and late promoters depended on the ability of Tst-1 to bind DNA, DNase I footprinting was performed to identify the binding sites for Tst-1 within the regulatory region of JC virus. Four sites were protected from DNase I digestion by purified Tst-1 fusion protein (Fig. 2). Examination of the DNA sequence within the protected regions revealed two different sequence motifs (A and B), each of which is represented twice within the regulatory region of JC virus (Fig. 1A). Both sites A and site B are A+T-rich, but otherwise they share no obvious similarity to each other, the octamer motif, or the A+T-rich regions within the P_0 promoter, which are bound by Tst-1 in vitro (13, 16, 29). Upon binding of Tst-1, DNase I hypersensitive sites were detected throughout the viral regulatory region. This might indicate the induction of major structural alterations by the binding of Tst-1 to sites A and B or the presence of additional lower affinity binding sites.

When analyzed by electrophoretic mobility shift assays, oligonucleotides containing either the sequence of site A or site B (Fig. 2), both bound Tst-1 as a purified fusion protein or as a full-length protein obtained from extracts of transfected COS cells (data not shown). To compare the affinity of Tst-1 to both sites, competition analyses were performed by challenging the binding of Tst-1 to radiolabeled site B by an excess of unlabeled site A or site B DNA as competitors (Fig. 3A). Repeated analysis showed that site A had a 2.5-fold greater affinity for Tst-1 than site B (Fig. 3B).

To identify the contribution of either site to the observed stimulation of the viral promoters, each site was tested for its ability to confer Tst-1 responsiveness to a heterologous promoter by using a transient transfection assay (Fig. 3C). A luciferase reporter plasmid containing the prolactin minimal promoter was activated less than 2-fold by the presence of ectopically expressed Tst-1 in U138 cells. After the insertion of an octamer motif, the same reporter plasmid could be stimulated 18-fold by Tst-1. Similarly, one copy of site A was sufficient to confer responsiveness to Tst-1, though a single copy of site A proved to be less efficient than the octamer motif. No difference could be found between the two possible orientations of site A (11- and 12-fold, respectively). With two or three copies of site A, the expression of the reporter plasmid could be stimulated by Tst-1 beyond the level obtained with the octamer motif. A single copy of site B, on the other hand, did not confer responsiveness to Tst-1 regardless of its orientation. In fact, site B failed to confer Tst-1 responsiveness, even when multimerized up to seven copies. No cooperativity was observed in constructs that contained a single copy of site B adjacent to a single copy of site A (data not shown). From these studies, we conclude that Tst-1 exerted its stimulatory effect on the transcription of JC virus mainly through its interaction with site A. Previous studies have identified a binding site for a glia-specific transcriptional inhibitor within the sequences directly downstream of Tst-1 binding site A (30). It is an intriguing...
Fig. 3. Functional characterization of Tst-1 binding sites within the regulatory region of JC virus. (A and B) Electrophoretic mobility shift experiments were performed to compare the binding affinities for site A and site B with each other. (A) The complex between the glutathione S-transferase–Tst-1 fusion protein and labeled site B [identified by comparing reactions with (+) and without (−) Tst-1 protein] was challenged by 0.1, 1, 10, 30, 60, 150, and 1500 ng of unlabeled competitor site A or site B, as indicated by the triangle above the lanes. (B) The relative amount of complex formed in the presence of various amounts of competitor DNA was determined in three independent experiments using the PhosphorImager and was plotted as percent maximal binding versus the logarithm of the concentration of competitor DNA. The effective concentration needed to reduce binding to 50% was determined to be $1.9 \times 10^{-8}$ M for site A and $4.9 \times 10^{-8}$ M for site B, respectively. (C) The ability of site A and site B to transfer regulation by Tst-1 to a heterologous promoter was tested by transient transfection of U138 cells. The halfarrows above sites A and B indicate their orientation. The activity of each reporter construct in the presence of an expression plasmid for Tst-1 (Tst1) was compared to its activity in the presence of an antisense control plasmid (asTst1). Transcription levels were determined as described in Fig. 2. Results represent the mean of triplicate determinations; error bars indicate the SEM. Comparable results were obtained in two additional independent experiments in U138 cells and two further experiments in HeLa cells. SV40, simian virus 40; HSV oct, herpes simplex virus octamer.

FIG. 4. Influence of Tst-1 on the DNA replication of JC virus in vivo. Test plasmids pJC433 (A) and pBKS-MAD1 (B) were cotransfected with expression plasmids for the JC viral T antigen (JC-TAg), Oct-1, and Tst-1 [either wild type (wt) or as a mutant (mt)] in DNA binding] as indicated above the lanes. The rate of replication was determined as the amount of test plasmid that, after linearization with EcoRI (pJC433) or BamHI (pBKS-MAD1), was resistant to the restriction endonuclease Dpn I (indicated by arrows), which selectively degrades the unreplicated bacterial input DNA. Quantification of Southern blots was performed using the PhosphorImager system.

Because of the close proximity of site A to the viral origin of DNA replication (10, 11) (Fig. 1A), we examined the possibility that in addition to its effect on viral transcription, Tst-1 might also influence viral DNA replication. Such a function is conceivable, because several POU proteins, including Tst-1, play important roles in cell proliferation (17, 31, 32) and have been suggested to participate directly in DNA replication (33). Replication of plasmid DNA in mammalian cells can be determined by the amount of Dpn I-resistant plasmid DNA that can be retrieved from transfected cells, since replication alters the methylation pattern of the transfected plasmid DNA. A plasmid that contained the isolated JC viral regulatory region (pJC433 in Fig. 4A) replicated in U138 cells only in the presence of viral T antigen as previously shown (11). JC viral T antigen was supplied by ectopic expression from a cotransfected plasmid. Additional expression of Tst-1 or Oct-1 did not alter the rate of DNA replication. These data argue against a direct participation of Tst-1 in the DNA replication of JC virus.
A more indirect role of how Tst-1 might influence viral DNA replication is suggested by the results of the transcription studies. Because of its stimulatory effect on the early viral promoter, Tst-1 can upregulate the expression of the viral T antigen. Since viral T antigen is needed for both the initiation and the elongation steps of JC viral DNA replication, an increase in the amount of available T antigen should lead to a net increase in viral DNA replication, at least during the early phases of infection when T-antigen levels are still rate limiting. We tested this hypothesis by the following experiment (Fig. 4B): a plasmid containing the complete genome of JC viral strain MAD-1 (pBKS-MAD1) including an intact reading frame for the viral T antigen was transfected into U138 cells. After a 5-day period, only a very low amount of replication could be detected, which was made possible by expression of the viral T antigen from its own promoter. When Tst-1 was cotransfected with pBKS-MAD1, replication was stimulated 30-fold, indicating that Tst-1 indeed stimulated the expression of the viral T antigen from its own promoter in its natural context. The level of stimulation obtained with Tst-1 was ~5-fold lower than was observed when JC viral T antigen was supplied from a cotransfected expression plasmid. The Tst-1-dependent stimulation was no longer observed when wildtype Tst-1 was replaced by a mutant version with strongly reduced DNA-binding ability. Similarly, Oct-1 was not able to substitute for Tst-1 in the replication assay.

These studies show that Tst-1 has the ability to positively affect the crucial phases within the lytic life cycle of JC virus. First, Tst-1 stimulates the expression of the viral regulatory genes, small and large tumor antigen. These multifunctional proteins then allow the virus to subvert cellular control mechanisms by altering the cellular transcription pattern, overriding control of cellular proliferation exerted by the retinoblastoma and p53 genes and stimulating quiescent cells to synthesize DNA (34, 35). In addition, T antigen is required for DNA replication, so that a Tst-1-dependent increase in the amount of available T antigen also increases the rate of viral DNA replication. Since Tst-1 also stimulates expression of the late viral capsid proteins, the packaging of viral progeny genomes in infectious particles should also be facilitated. This positive effect of Tst-1 on various stages of the life cycle of JC virus correlates well with its expression in those cells of the central nervous system that are the preferred target of JC viral infection. Tst-1 might thus be one of the factors that determine the specificity of JC virus for oligodendrocytes. Understanding the regulation of its expression might therefore help to gain insights into the origins and pathogenesis of PML, which so far cannot successfully be treated.

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