Journal of NeuroVirology, 7: 250–264, 2001
 ISSN 1355–0284/01 $12.00+.00

JC virus T′ proteins encoded by alternatively spliced early mRNAs enhance T antigen-mediated viral DNA replication in human cells

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Alternative splicing of the JC Virus (JCV) precursor early mRNA yields five transcripts that encode proteins that regulate the life cycle of this human polyomavirus. Large T protein (TAg) mediates viral DNA replication and oncogenic activities, and small t protein influences these functions under certain conditions. Recently, three new early proteins, T'_{135}, T'_{136}, and T'_{165}, were discovered that contain sequences overlapping amino-terminal TAg functional domains. Initial studies with the T′ proteins suggested they contribute to viral DNA replication and transformation. Mutation of a donor splice site utilized by all three T′ mRNAs creates a mutant that exhibits a 10-fold decrease in viral DNA replication compared to wild type JCV. To assess the influence that individual T′ proteins have on the replication process, a set of T′ acceptor site mutants was created in which the unique second acceptor splice site of each T′ mRNA was altered to eliminate production of one, two or all three T′ mRNAs. The patterns of early mRNA and protein expression in these seven mutants were examined, and it was found that mutation of the T'_{135} acceptor site resulted in the utilization of cryptic splice sites and the generation of new T′ species. Additional mutations were made to prevent these aberrant splicing reactions prior to measuring DNA replication potential of the mutants. DpnI assays revealed that each T′ protein contributes to TAg-mediated DNA replication activity. The three single mutants that express two T′ proteins and the double mutant that only produces T'_{136}, exhibited levels of replication equivalent to that of wild type virus, whereas the two double mutants that fail to express T'_{136} replicated about twofold less efficiently than wild-type JCV. Replication activity of the triple acceptor site mutant, like that of the T′ donor site mutant from an earlier study, was impaired significantly. *Journal of NeuroVirology* (2001) **7,** 250–264.

Keywords: tumor proteins; cell cycle regulation; LXCXE and J domains; alternative splicing

Introduction

The multifunctional JCV large T protein (TAg) regulates viral DNA replication in a permissive cell environment; in nonpermissive cells TAg may demonstrate oncogenic potential (reviewed in Frisque and White III, 1992). These activities are mediated through interactions with viral and cellular DNA and a number of cellular proteins. TAg functional domains have been identified by genetic and biochemical analyses of wild type (WT), mutant and chimeric proteins and by comparisons with the closely related and more thoroughly studied SV40 TAg (Pipas, 1992; Frisque, 1999; Kim, Henson and Frisque, submitted). While their high degree of sequence homology dictates that JCV and SV40 TAgs will interact with many of the same cellular proteins and recognize the same DNA sequences, the two viruses do exhibit significant differences in host range, tissue tropic, DNA replication, and oncogenic properties (Frisque and White III, 1992). These differences have been attributed, in part, to the viral early regions that encode the JCV and SV40 regulatory proteins, including

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Received 15 November 2000; revised 31 January 2001; accepted 7 February 2001.

TAg (Chuke *et al*, 1986; Bollag *et al*, 1989; Haggerty *et al*, 1989; Sock *et al*, 1993; Trowbridge and Frisque, 1993; Lynch *et al*, 1994; Sullivan *et al*, 2000b).

Recently, three new alternatively spliced JCV early mRNAs were identified in infected primary human fetal glial (PHFG) cells by RT-PCR, and were shown to encode TAg-related proteins that were named T'_{135}, T'_{136}, and T'_{165} (Trowbridge and Frisque, 1995). However, during the analysis of the viral proteins immunoprecipitated from lysates of the infected cells, a band representing a 23 kD protein was observed on the SDS-polyacrylamide gel that could not be accounted for by the RT-PCR data. The identity of this protein was not determined, but it was suggested that it either represented a fourth T′ protein, or a hyperphosphorylated form of the 22 kD T'_{165} protein. T'_{135}, T'_{136}, T'_{165} and the 23 kD protein were all found to be phosphorylated and to be recognized by monoclonal antibodies (MAb) directed against the amino terminus of TAg; a MAb directed against the carboxy terminus of TAg only recognized T'_{165} and the 23 kD protein (Trowbridge and Frisque, 1995). These observations were explained, in part, by sequence information that revealed the three known T′ proteins share their amino-terminal 132 amino acids with those of TAg, and T'_{165} also shares its carboxy terminal 33 amino acids with this major regulatory protein (Trowbridge and Frisque, 1995).

A number of functional domains located within the amino terminus of TAg (the pRB, p107, p130, DNA polymerase α [pol α], hsc70, and Tst-1 binding domains, a cluster of serine/threonine phosphorylation sites and a nuclear localization signal) are predicted to have direct or indirect effects on viral DNA replication and cellular transformation (Brodsky and Pipas, 1998; Bollag *et al*, 2000; Kim, Henson, and Frisque, submitted). The ability of TAg to bind to members of the pRB tumor suppressor protein family via its LXCXE motif and to interact with the molecular chaperone hsc70 via its DnaJ (J) domain results in the release of the heterodimeric transcription factor E2F-DP and the promotion of cell cycle progression from G1 to S phase (Sheng *et al*, 1997; Sullivan *et al*, 2000a). In a permissive cell this outcome is advantageous to a simple virus like JCV that depends upon the host cell DNA replication machinery to propagate its genome. On the other hand, in a nonpermissive cell this outcome might lead to unregulated proliferation and a transformed phenotype. Although it has been shown that TAg alone is capable of mediating viral DNA replication and cellular transformation, a second early viral protein, small t antigen (tAg), also influences these processes under certain conditions (Bikel *et al*, 1986; Fanning, 1992; Pipas, 1992; Brodsky and Pipas, 1998). In addition to their interactions with hsc70 and members of the pRB family, polyomavirus TAgs may interact with Tst-1, a Pou transcription factor found in myelinating cell types, and with pol α. Tst-1 acts synergistically with JCV TAg to promote early and late viral transcription, thereby indirectly affecting DNA replication (Wegner *et al*, 1993), while SV40 TAg, and presumably its JCV counterpart, interacts with pol α to directly affect the initiation and elongation steps of the replication process (Smale and Tjian, 1986; Dornreiter *et al*, 1990; Pipas, 1992; Stillman, 1994). Finally, these events are influenced in both positive and negative ways by phosphorylation of specific serine and threonine residues in the amino-terminus of TAg (Prives, 1990; Fanning, 1992; Cegielska *et al*, 1994a; Weisshart *et al*, 1999).

The contributions of the JCV T′ proteins, and the related SV40 17kT and polyoma virus Tiny T proteins, to viral DNA replication and cellular transformation have not been well characterized. It has been suggested the JCV T'_{136} may enhance JCV transformation of rodent cells (Trowbridge and Frisque, 1995). Furthermore, the three T′ proteins differentially bind pRB, p107, and p130, and the latter protein appears to be degraded in cells expressing all five JCV early proteins or T'_{135} by itself (Bollag *et al*, 2000; unpublished data). Using a genetic approach, Trowbridge and Frisque (1995) determined that at least one of the T′ proteins enhances TAg-mediated replication. In that study, a T′ donor site mutant, JCVΔT′, was created to eliminate expression of the three T′ mRNAs by altering the common T′ donor splice site. Transfection of PHFG cells with JCVΔT′ and WT JCV DNA revealed that the loss of the three T′ proteins resulted in a $\geq$10-fold decrease in viral DNA replication activity.

The present study examines the contributions of individual T′ proteins to JCV DNA replication by creating seven T′ acceptor splice site mutants and measuring their replication potential in permissive PHFG cells. Mutation of the T'_{135} splice site led to the utilization of cryptic splice acceptor sites that necessitated the introduction of several additional mutations to prevent the production of new T′ species. All mutants were designed so that the WT TAg coding sequence was left intact. Finally, the identity of the 23 kD protein band observed in JCV-infected cells in the initial T′ study was determined.

Results

Construction of the A series of T′ acceptor site mutants

The individual contributions made by JCV T′ proteins to DNA replication were investigated by creating a set of seven acceptor site mutants (A series) to eliminate production of the T′ proteins singly, in pairs, or all together. Mutants were constructed using a PCR-based mutagenesis scheme to alter the T′ acceptor splice sites without changing the TAg amino acid sequence. Almost invariably, mutations to the conserved 3′ AG dinucleotide in the consensus acceptor sequence $U(C)_nNC(U)AG|G$ eliminate splicing (Mount, 1982; Aebi *et al*, 1986). Based on this information, T′ acceptor site mutants were created by altering these AG residues and, where

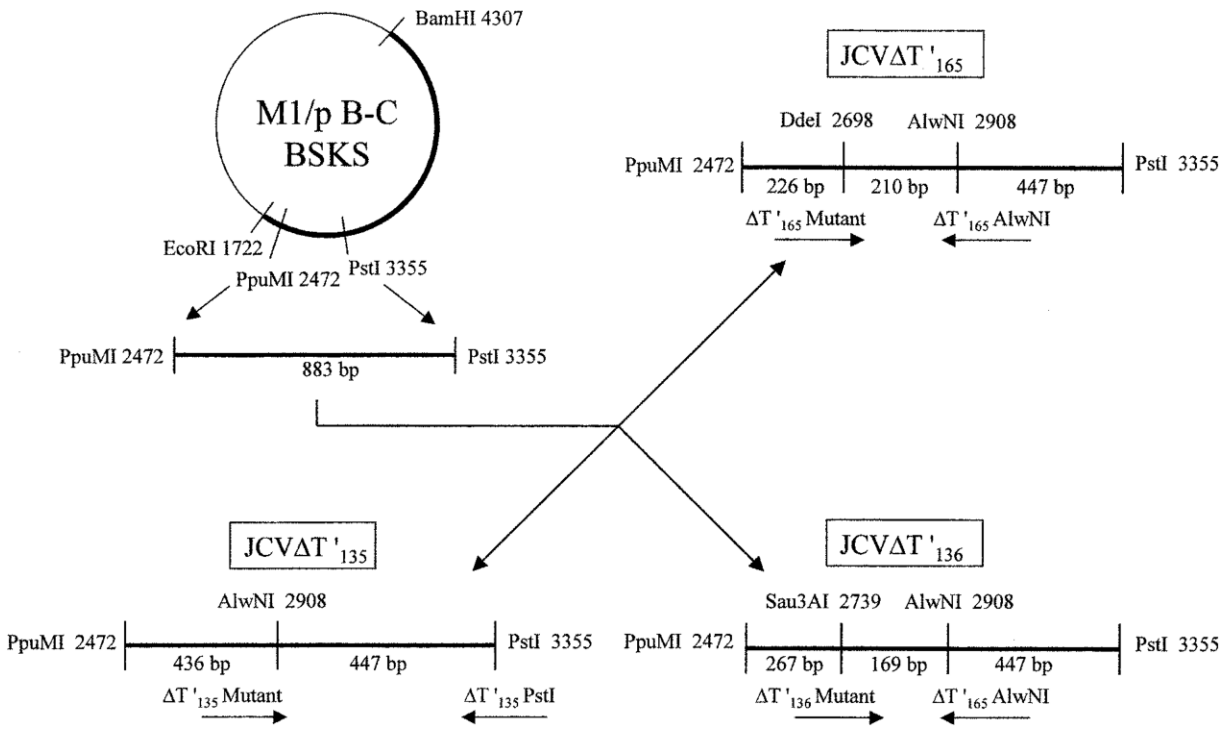

Figure 1 Mutagenesis scheme to create T′ splice acceptor site mutants. The clone M1/pB-C BSKS contains JCV sequences (in bold) cloned into pBluescript II KS (Stratagene) at *Eco*RI (nt 1722) and *Bam*HI (nt 4307) restriction sites. M1/pB-C BSKS DNA was digested with *Ppu*MI and *Pst*I to yield an 883 bp fragment for use in PCR mutagenesis. Reactions utilized to create mutants JCVΔT′$_{135}$, JCVΔT′$_{136}$, and JCVΔT′$_{165}$ are shown and are described in detail in Materials and methods. Arrows below the primer names indicate the direction of the PCR priming. Recognition sites for the restriction enzymes used to isolate PCR products are indicated and nucleotide numbering refers to that used for the JCV genome (Frisque *et al*, 1984).

possible, by also altering the acceptor nucleotide. To create JCVΔT′$_{135}$A, JCVΔT′$_{136}$A, and JCVΔT′$_{165}$A, the acceptor sites were changed from AG|G to CG|C, from AG|A to CG|C, and from AG|G to AA|G, respectively. Three double and one triple T′ acceptor site mutants were generated from these three single mutants (Figures 1, 2; Materials and methods).

Viral DNA replication of the A series of T′ mutants

PHFG cells were transfected with DNA from the seven T′ acceptor site mutants (A series), the T′ donor site mutant JCVΔT′, WT JCV(Mad1), or calf thymus, the latter serving as a negative control. Viral DNA was isolated at days 0, 7, 14, and 21 posttransfection (p.t.), and replicated DNA was visualized by Southern blotting (Figure 3). Replication of the donor site mutant, JCVΔT′, at day 21 p.t. was approximately 10-fold less than that of JCV(Mad1), confirming previous results (Trowbridge and Frisque, 1995). However, the average replication level of the triple acceptor site mutant JCVΔT′$_{135/136/165}$A at day 21 p.t. was much higher than that of the donor site mutant. This result was unexpected since both mutants were designed to eliminate production of all T′ proteins. This finding might be explained by: (i) differences in splicing patterns in the two mutants due to the alteration of the donor splice site versus the acceptor splice sites, and subsequent altered expression of TAg and tAg, or (ii) the production of a fourth, as yet unidentified, T′ protein by the triple mutant which has positive influence on viral DNA replication.

Analysis of T′ mRNA production by the A series of T′ mutants

To determine whether the predicted mRNA patterns were produced by the T′ acceptor site mutants (A series), mRNA was isolated at day 21 postinfection (p.i.) from infected cells. The mRNA was reverse transcribed and the resulting cDNA was subjected to PCR amplification using primers T′#1 and T′#3 (Table 1, Figure 4). In the lane labeled Mad1, cDNAs migrating at 434, 293, and 220 bp represented T′$_{135}$, T′$_{136}$, and T′$_{165}$ transcripts, respectively. In the lane marked ΔT′, the cDNA band seen migrating at 263 bp had previously been identified as an artifact of PCR (Trowbridge and Frisque, 1995). The expected cDNA patterns were seen in lanes ΔT′$_{136}$, ΔT′$_{165}$, and ΔT′$_{136/165}$, and no T′ cDNA bands were observed in the lane labeled ΔT′. In contrast, cDNA bands migrating at approximately 434 bp, the size of the T′$_{135}$ cDNA, were visible in lanes ΔT′$_{135}$, ΔT′$_{135/136}$, ΔT′$_{135/165}$, and ΔT′$_{135/136/165}$. These RT-PCR products were cloned, sequenced, and shown to represent a new T′ mRNA that uses the shared T′ donor site at nucleotide (nt) 4274 and a unique acceptor site at nt 2913, 5 nt downstream from the altered T′$_{135}$ acceptor

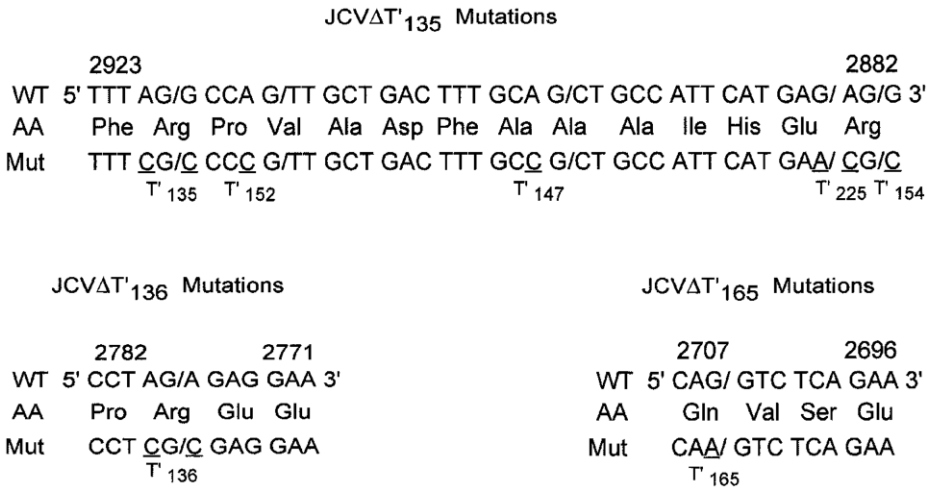

Figure 2 Mutations made to the JCV early coding region to create T′ acceptor site mutants. PCR mutagenesis was utilized to introduce mutations at (i) nt 2920 (A → C) and nt 2918 (G → C) to alter the T'_{135} splice acceptor site, (ii) nt 2915 (A → C) to alter the T'_{152} splice acceptor, (iii) nt 2900 (A → C) to alter the T'_{147} splice acceptor, and (iv) nt 2885 (G → A), nt 2884 (A → C), and nt 2882 (G → C) to prevent splicing at these potential cryptic acceptor sites. Mutations were made at nt 2779 (A → C) and 2777 (A → C) to eliminate production of T'_{136}, and at nt 2705 (G → A) to abolish expression of T'_{165}. The wild type (WT) and mutant (Mut) DNA sequences and unaltered amino acid (AA) sequences of the early coding region are shown. Mutated sequences are underlined and are associated with the name of a specific acceptor site (e.g., T'_{135}). A slash identifies the location of an exon/intron boundary, and numbers above the sequence refer to nucleotide positions within the JCV(Mad1) genome (Frisque et al, 1984).

site. This transcript encodes a 152 amino acid protein named T'_{152} (Figure 5). The carboxy-terminal amino acid sequences of T'_{152} are in a different reading frame than those of TAg and the three authentic T′ proteins.

Construction of the B and C series of T′ mutants

A second set of acceptor site mutants (B series) was created to eliminate production of T'_{152}. However, alteration of the T'_{152} splice acceptor led to production of another new T′ mRNA that utilizes the T′ donor site

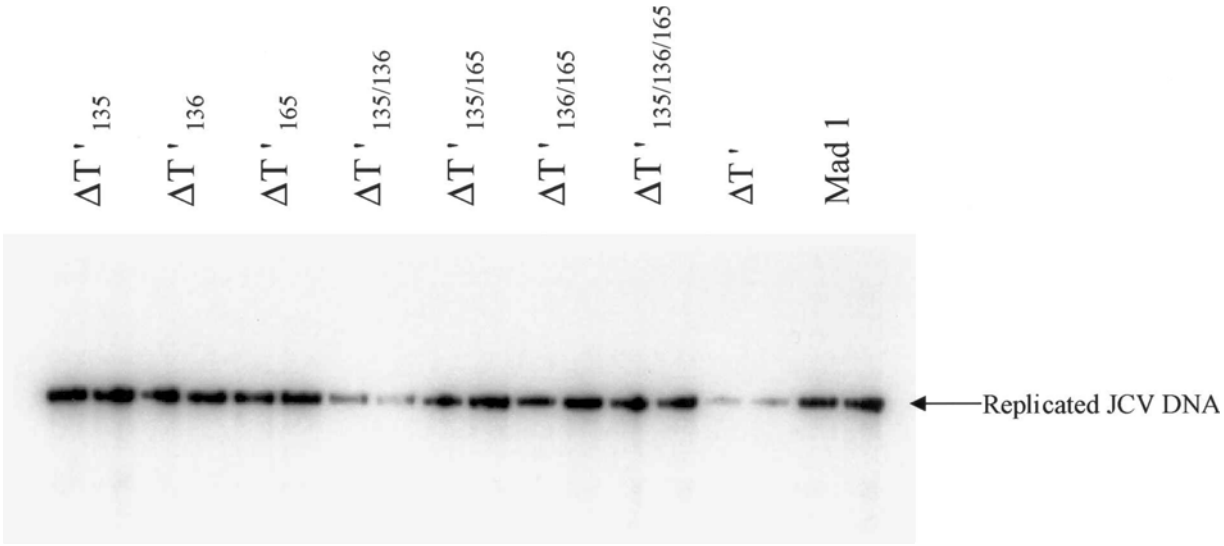

Figure 3 DNA replication of the A series of JCV T′mutants in PHFG cells. Viral DNA was isolated at day 21 p.t. from PHFG cells transfected with three single acceptor sites, (JCVΔT'_{135}A, JCVΔT'_{136}A, JCVΔT'_{165}A), three double acceptor sites (JCVΔ$T'_{135/136}$A, JCVΔ$T'_{135/165}$A, JCVΔ$T'_{136/165}$A), one triple acceptor site (JCVΔ$T'_{135/136/165}$A), and one donor site (JCVΔT′) mutant DNAs and WT JCV(Mad1) DNA. A double digest with *Eco*RI and *Dpn*I was used to linearize viral DNA (*Eco*RI) and to separate replicated DNA from input DNA (*Dpn*I). Duplicate samples were electrophoresed on a 0.8% agarose gel at 20 V overnight. DNA was transferred to a nylon membrane, probed with ^{32}P-labeled JCV(Mad1) DNA, and visualized by autoradiography. The position of the replicated viral DNA is indicated.

Table 1 Oligonucleotides used to created the T′ splice acceptor site mutants

Name	*Nucleotides*[a]	*Mutation*[b]	*Sequence*[c]
ΔT′135 mutant	2902–2925	2918 C/G 2920 T/G	5′-CAAAGTCAGCAACTGG**G**C**G**AAACC-3′
ΔT′135 PstI	3392–3375		5′-GGGGCCAATAGACAGTGG-3′
ΔT′136 mutant	2733–2782	2777 T/G 2779 T/G	5′-GTGCTTGATCCATGTCCAGAGTCTTCTG CTTCAGAATCTTCCTC**G**C**G**AGG-3′
ΔT′165 AlwNI	2927–2910		5′-CTGGTTTAGGCCAGTTGC-3′
ΔT′165 mutant	2691–2709	2705 C/T	5′-GAGGCTTCTGAGAC**T**TGGG-3′
RT-PCR #6	3407–3382		5′-GTACTGGCTATTCAAGGGGCCAATAG-3′
RT-PCR #7	2893–2917	2914 C/G	5′-TGGCAGCTGCAAAGTCAGCAA**G**TGG-3′
dT′147	2895–2917	2900 T/G 2915 T/G	5′-GCAGC**G**GCAAAGTCAGCAAC**G**GG-3′
Set-11L	2391–2410		5′-GAGTTGATGGGCAGCCTATG-3′
dT′154	2901–2878	2882 G/C 2884 A/C 2885 G/A	5′-CAGCTGCCATTCATGA**AC**G**C**ATTG-3′

[a]Nucleotide position of the oligonucleotide according to the JCV (Mad1) numbering scheme (Frisque *et al*, 1984).
[b]Position of the mutated nucleotide.
[c]Mutations within the oligonucleotide sequences are underlined and in bold.

(nt 4274) and another cryptic acceptor site (nt 2898) (Figure 2). The protein translated from this mRNA (T'_{147}) contains 147 amino acids with a carboxy-terminal region in the same reading frame as that of T'_{152} (Figure 5).

Inspection of JCV early coding sequences revealed two additional potential cryptic splice acceptor sites downstream of the T'_{135}, T'_{152}, and T'_{147} acceptor sites that, if utilized, would give rise to a T'_{154} and a T'_{225} protein. Therefore, mutations were introduced at these sites as well as the T'_{147} acceptor site (Figure 2). This third set of T′ acceptor site mutants were differentiated from the previous two sets by the addition of the latter "C" to their names. All further studies were conducted using the C series of mutants.

Viral DNA replication of the C series of T′ mutants

To investigate the replication activity of the T′ proteins, PHFG cells were transfected with DNA from seven T′ acceptor site mutants (C series), JCVΔT′, JCV(Mad1), or calf thymus. Viral DNA was isolated at days 0, 7, 10, 14, and 21 p.t. Replicated viral DNA was visualized by Southern blotting (Figure 6) and relative replication levels (Table 2) were determined by analyzing the blots with a phosphorimager. JCV(Mad1) DNA replication was arbitrarily assigned a value of 100 for each time point, and replication activities of the mutant viral DNAs were calculated relative to that value. Replication of the donor site mutant JCVΔT′ at day 21 p.t. was approximately 10-fold less than that of JCV(Mad1), confirming results obtained in the original study of this mutant (Trowbridge and Frisque, 1995). The average replication level of the triple acceptor site mutant, JCV$\Delta T'_{135/136/165}$C, was about 10-fold less than JCV(Mad1) at each time point in the experiment, suggesting that like JCVΔT′, the triple T′ mutant did not produce new T′ proteins. Replication of the

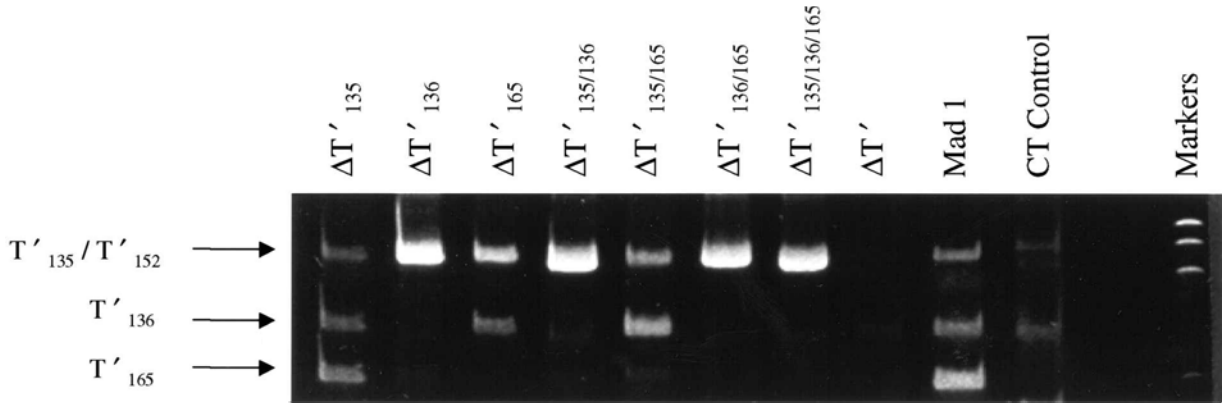

Figure 4 RT-PCR analysis of RNA isolated from PHFG cells infected with the A series of T′ mutant viruses. Total RNA was isolated at day 21 p.i. from PHFG cells infected with the mutants identified in Figure 3. RNA samples were treated with DNaseI, reverse transcribed, and PCR-amplified using JCV primers T′#1 and T′#3. RT-PCR products were separated on a 7% polyacrylamide gel. The positions of T'_{135}, T'_{136}, T'_{165}, and T'_{152} cDNAs are indicated. A *Rsa*I digest of pM1TCR1A (recombinant JCV(Mad1) DNA) was included as a marker to estimate the sizes of the cDNA bands. Positive and negative control samples were electrophoresed in the lanes labeled Mad1 (JCV(Mad1)) and CT (calf thymus) Control, respectively.

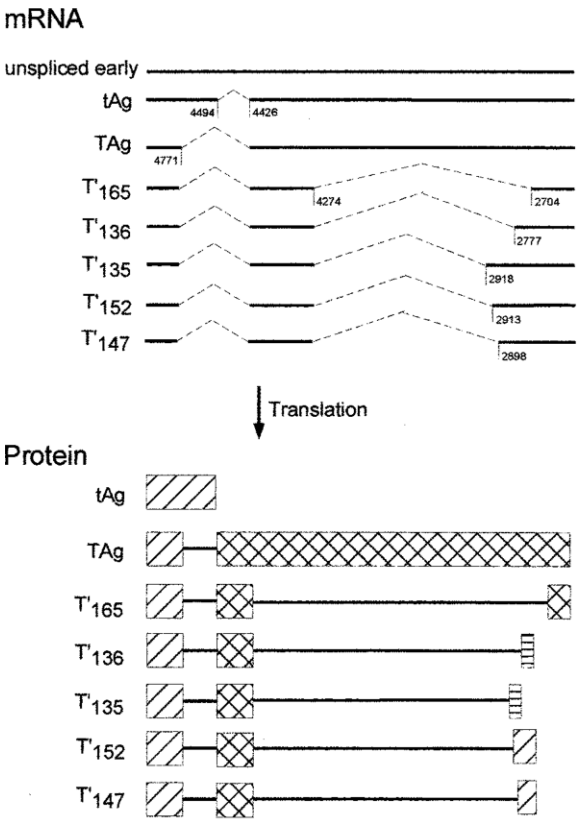

Figure 5 Transcription and translation products detected in PHFG cells infected with the T′ acceptor site mutants. Five early mRNAs are produced by alternative splicing of a single JCV precursor mRNA (**A**). All three T′ mRNAs use the same donor and acceptor splice sites as the TAg transcript. The T′ mRNAs are then spliced again at a shared donor site at nt 4274, and a unique second acceptor site. Translation of these transcripts yields three distinct T′ proteins (**B**). T'_{135} contains 135 amino acids, and has a carboxy terminus in a different reading frame (indicated by horizontal hatching) than TAg (cross hatching). T'_{136} contains 136 amino acids and has a carboxy terminus in the same reading frame as T'_{135}. T'_{165} contains 165 amino acids and shares its carboxy terminus with TAg. The T'_{152} mRNA is produced when a cryptic splice acceptor site at nt 2913 is used in the absence of T'_{135} splicing. The T'_{147} mRNA is produced when a cryptic splice acceptor site at nt 2898 is used in the absence of T'_{135} and T'_{152} splicing. Sequences representing the first two exons of T'_{152} and T'_{147} (amino-terminal 132 amino acids) are shared with TAg and the other T′ proteins. The carboxy-terminal 20 amino acids of T'_{152} and 15 amino acids of T'_{147} (diagonal hatching) are in a different reading frame than those of TAg, T'_{165}, T'_{136}, and T'_{135}.

single acceptor site mutants at the early time points was about twofold lower than that of JCV(Mad1). However, at day 21 p.t., average replication levels of the single acceptor site mutants were slightly higher than JCV(Mad1) levels. Replication of the double acceptor site mutant JCV$\Delta T'_{135/165}$C was higher than that of JCV$\Delta T'_{135/136}$C and JCV$\Delta T'_{136/165}$C at all time points examined.

Analysis of T′mRNA production by the C series of T′ mutants

To determine whether the predicted mRNA patterns were produced by the C series of T′ acceptor site mutants, mRNA was isolated at day 28 p.i. from infected cells. The mRNA was reverse transcribed and the resulting cDNA was subjected to PCR amplification using primers T′#1 and T′#3 (Figure 7). In the lane labeled Mad1, cDNAs migrating at 434, 293, and 220 bp represented T'_{135}, T'_{136}, and T'_{165} transcripts, respectively. In the lanes marked $\Delta T'$ and $\Delta T'_{136/165}$, the artifact band migrating at 263 bp was again observed. The expected cDNA patterns were seen in the lanes representing each of the T′ acceptor site mutants. In this experiment an unidentified band was observed in the $T'_{135/136}$ lane migrating slightly slower than the T'_{165} cDNA band. In addition, faint bands migrating slightly faster or slower than the T'_{135} cDNA were observed in some lanes upon longer exposure of the gels. Based on other RT-PCR experiments and the results from Western blot analyses (Figure 8), we predict that these bands represent artifacts of the RT-PCR procedure, but confirmation of this prediction will require cloning and sequencing of these rare cDNAs.

Table 2 DNA replication activity of the C series of JCV T′ acceptor site mutants[a]

DNA	*Day 7*	*Day 10*	*Day 14*	*Day 21*
JCV$\Delta T'_{135}$C	29 ± 9[b]	64 ± 20	74 ± 23	106 ± 29
JCV$\Delta T'_{136}$C	42 ± 13	106 ± 27	94 ± 31	139 ± 34
JCV$\Delta T'_{165}$C	47 ± 10	93 ± 34	69 ± 24	155 ± 25
JCV$\Delta T'_{135/136}$C	31 ± 6	59 ± 14	64 ± 40	54 ± 10
JCV$\Delta T'_{135/165}$C	51 ± 6	71 ± 14	66 ± 23	96 ± 17
JCV$\Delta T'_{136/165}$C	42 ± 3	48 ± 15	31 ± 7	65 ± 13
JCV$\Delta T'_{135/136/165}$C	9 ± 14	9 ± 14	10 ± 3	11 ± 3
JCV$\Delta T'$	36 ± 10	29 ± 8	15 ± 0	11 ± 3
JCV(Mad 1)	100[c]	100	100	100

[a] Average DNA replication values were obtained from either three (days 7, 10, 21 p.t.) or two (day 14 p.t.) DpnI replication assays.
[b] Replication value ± standard error of the mean (σ_M); $\sigma_M = \sigma/\sqrt{N}$, where σ = standard deviation of the distribution and N = sample size.
[c] JCV(Mad1) DNA replication activity was arbitrarily assigned a value of 100. Replication values of mutant DNAs were based on comparisons with JCV(Mad1) activity.

Production of early proteins by the C series of T′ mutants

Lysates of cells transfected with the C series of T′ acceptor site mutant DNAs were prepared at day 21 p.t., immunoprecipitated with PAb 416, Western blotted, and probed with PAb 2003 to verify the production of the expected T′ proteins (Figure 8). No T′ proteins were detected in cells transfected with JCV$\Delta T'_{135/136/165}$C or JCV$\Delta T'$ DNA, supporting the RT-PCR results (Figure 7) that new alternatively spliced early transcripts were not generated by these mutants. In addition, cells transfected with the other DNAs containing the T'_{135} mutation (i.e., JCV$\Delta T'_{135}$C, JCV$\Delta T'_{135/136}$C, and JCV$\Delta T'_{135/165}$C), only produced the expected viral proteins. Differences were noted in the expression levels of individual

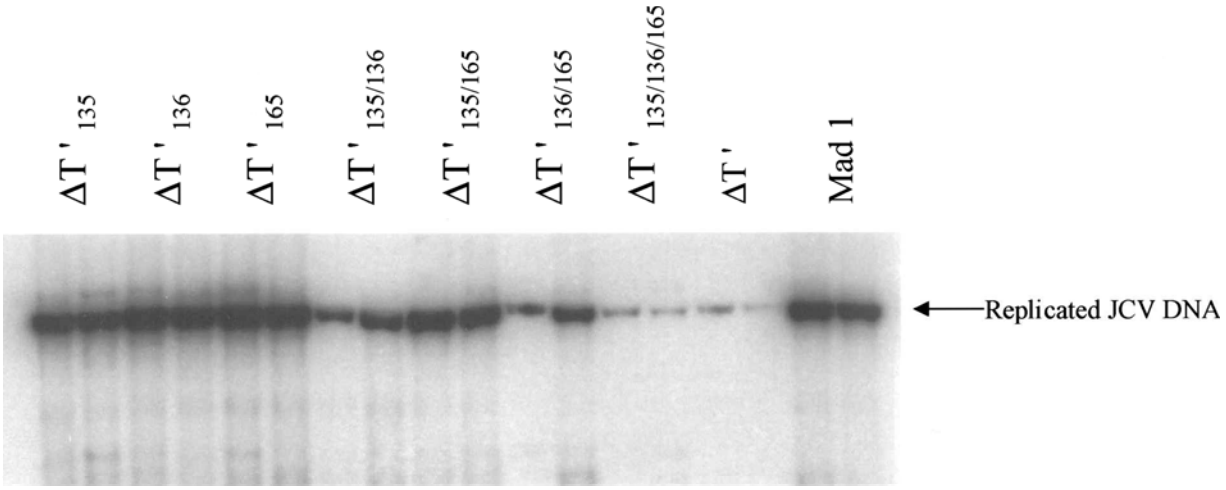

Figure 6 DNA Replication of the C series of JCV T′ mutants in PHFG cells. Viral DNA was isolated at day 21 p.t. from PHFG cells transfected with three single acceptor sites (JCVΔT′$_{135}$C, JCVΔT′$_{136}$C, JCVΔT′$_{165}$C), three double acceptor sites (JCVΔT′$_{135/136}$C, JCVΔT′$_{135/165}$C, JCVΔT′$_{136/165}$C), one triple acceptor site (JCVΔT′$_{135/136/165}$C), and one donor site (JCVΔT′) mutant DNAs and WT JCV(Mad1) DNA. A double digest with *Eco*RI and *Dpn*I was used to linearize viral DNA (*Eco*RI) and to separate replicated DNA from input DNA (*Dpn*I). Duplicate samples were electrophoresed on a 0.8% agarose gel at 20 V overnight. DNA was transferred to a nylon membrane, probed with ^{32}P-labeled JCV(Mad1), and visualized by autoradiography. The position of replicated viral DNA is indicated.

T′ proteins in cells transfected with some of the C series mutant DNAs. For example, T′$_{135}$ and T′$_{136}$ levels were highest in cells transfected with the double mutants JCVΔT′$_{136/165}$C and JCVΔT′$_{135/165}$C, respectively. It was difficult to compare levels of T′$_{135}$ and T′$_{136}$ produced in JCV(Mad1)- and JCVΔT′$_{165}$C-transfected cells because these proteins migrate as a doublet on the gels. T′$_{165}$ was readily detected in JCV(Mad1)-transfected cells, but only low levels were observed in cells transfected with acceptor site mutant genomes designed to retain the ability to express this protein (i.e. JCVΔT′$_{135}$C, JCVΔT′$_{136}$C, and JCVΔT′$_{135/136}$C).

TAg levels produced by the C series of T′ mutants

Cells transfected with the T′ acceptor site mutants (C series) were examined to compare levels of TAg produced by the mutants. JCV early proteins present in cell lysates isolated at day 21 p.t. were immunoprecipitated with PAb 962 and detected by Western blotting using PAb 962 as the primary antibody and a goat α-mouse alkaline phosphatase (AP) conjugate as

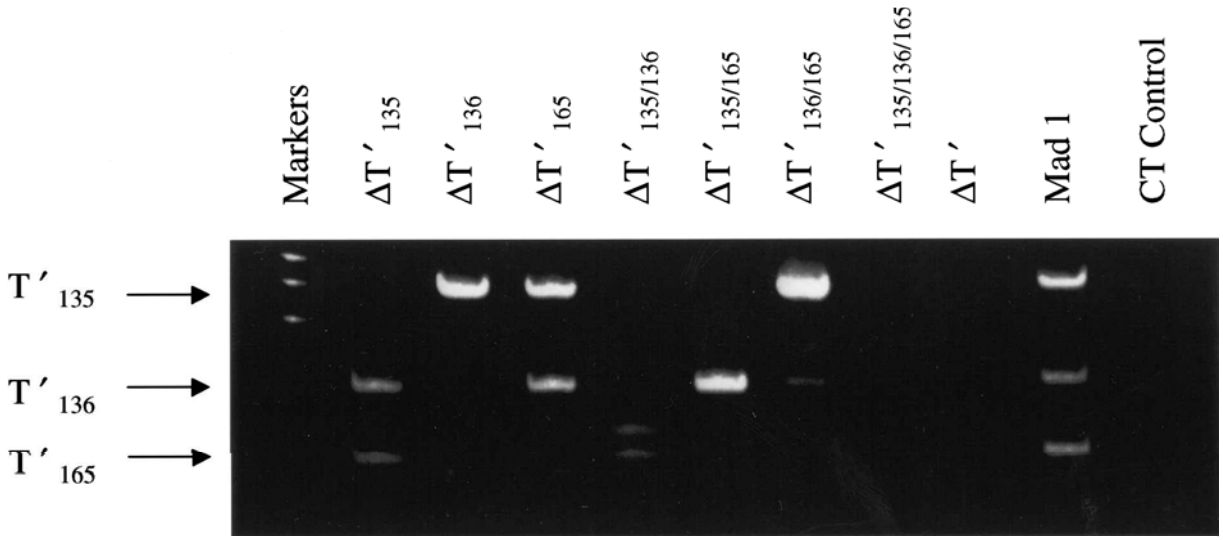

Figure 7 RT-PCR analysis of RNA isolated from PHFG cells infected with the C series of T′ mutant viruses. Total RNA was isolated from infected PHFG cells at day 28 p.i. from PHFG cells infected with the mutants identified in Figure 6. RNA samples were treated with DNaseI, reverse transcribed, and PCR-amplified using JCV primers T′#1 and T′#3. RT-PCR products were separated on a 7% polyacrylamide gel. The positions of T′$_{135}$, T′$_{136}$, and T′$_{165}$ cDNAs are indicated. A *Rsa*I digest of pM1TCR1A was included as a marker to estimate the sizes of the cDNA bands. Positive and negative control samples were electrophoresed in the lanes labeled Mad1 and CT Control, respectively.

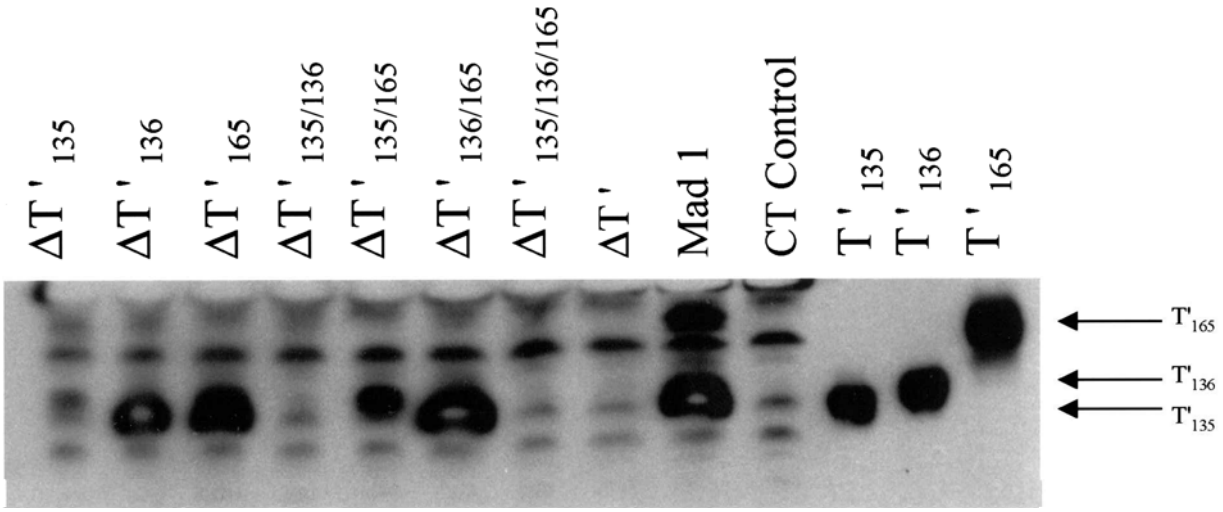

Figure 8 Detection of early viral proteins in PHFG cells transfected with the JCV T′ acceptor site mutants. Cell lysates were isolated at day 21 p.t. from PHFG cells transfected with the mutant and WT JCV DNAs identified in Figure 6. JCV early proteins were immunoprecipitated with the monoclonal antibody PAb 416, separated on an 18% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Western blot analysis was performed using PAb 2003 as the primary antibody and a goat α-mouse HRP conjugate as the secondary antibody. Note that the T'_{135} and T'_{136} bands migrate as a doublet in sample lanes containing both proteins, and that the T'_{165} band is easily detected only in the Mad1 lane; it is uniformly faint in the lanes labeled $\Delta T'_{135}$, $\Delta T'_{136}$, and $\Delta T'_{135/136}$. Purified T'_{135}, T'_{136}, and T'_{165} were included as size markers, and positive and negative control samples were electrophoresed in the lanes labeled Mad1 and CT Control, respectively.

the secondary antibody (Figure 9). Levels of TAg in cells transfected with the donor site mutant JCVΔT′ or the triple acceptor site mutant JCV$\Delta T'_{135/136/165}$C were low compared to the levels of TAg in JCV(Mad1)-transfected cells. The single acceptor site mutants had the highest levels of TAg, slightly higher than those of JCV(Mad1). Levels of TAg in cells transfected with JCV$\Delta T'_{135/165}$C cDNA were higher than those observed in JCV$\Delta T'_{135/136}$C- or $\Delta T'_{136/165}$C-transfected cells. As expected, TAg levels correlated

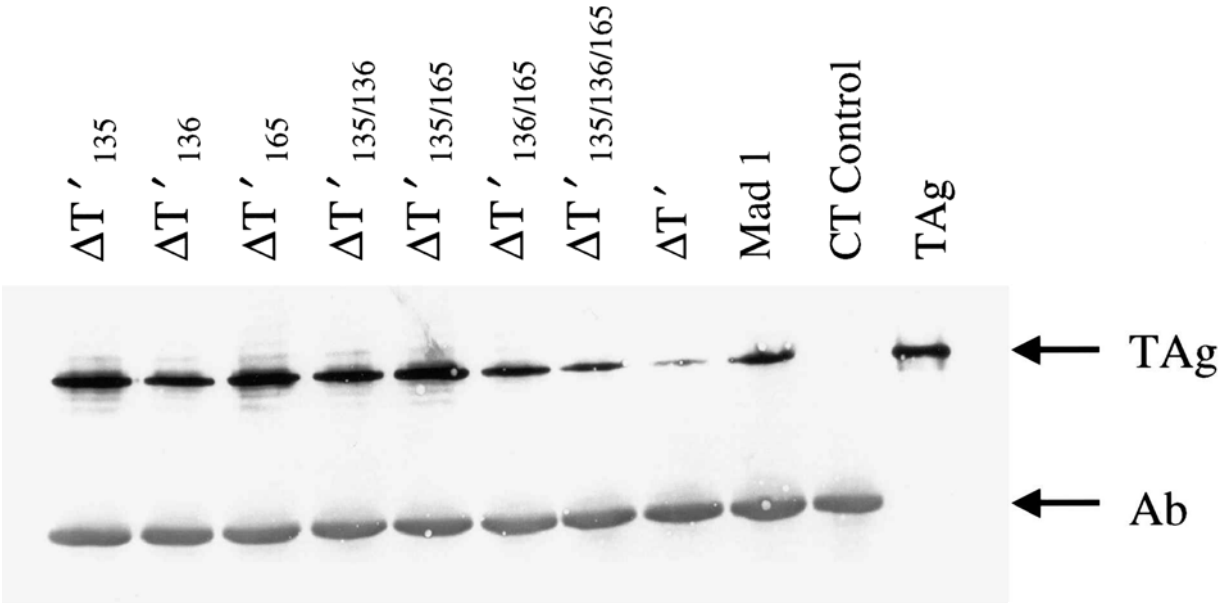

Figure 9 Detection of TAg in PHFG cells transfected with the C series of JCV mutants. Cell lysates were isolated at day 21 p.t. from PHFG cells transfected with mutant and WT JCV DNAs identified in Figure 6. JCV early proteins were immunoprecipitated with the monoclonal antibody PAb 962, separated on an 8.75% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Western blot analysis was performed using PAb 962 as the primary antibody and a goat α-mouse AP conjugate as the secondary antibody. PAb 962 recognizes the amino terminus of each JCV early protein (Bollag *et al*, 2000); the absence of tAg and the T′proteins on this blot is a result of their migration off the bottom of the 8.75% SDS-polyacrylamide gel. Purified TAg was included as a size marker, and positive and negative control samples were electrophoresed in the lanes labeled Mad1 and CT Control, respectively. The faster migrating band represents the heavy chain of the PAb 962 antibody (Ab).

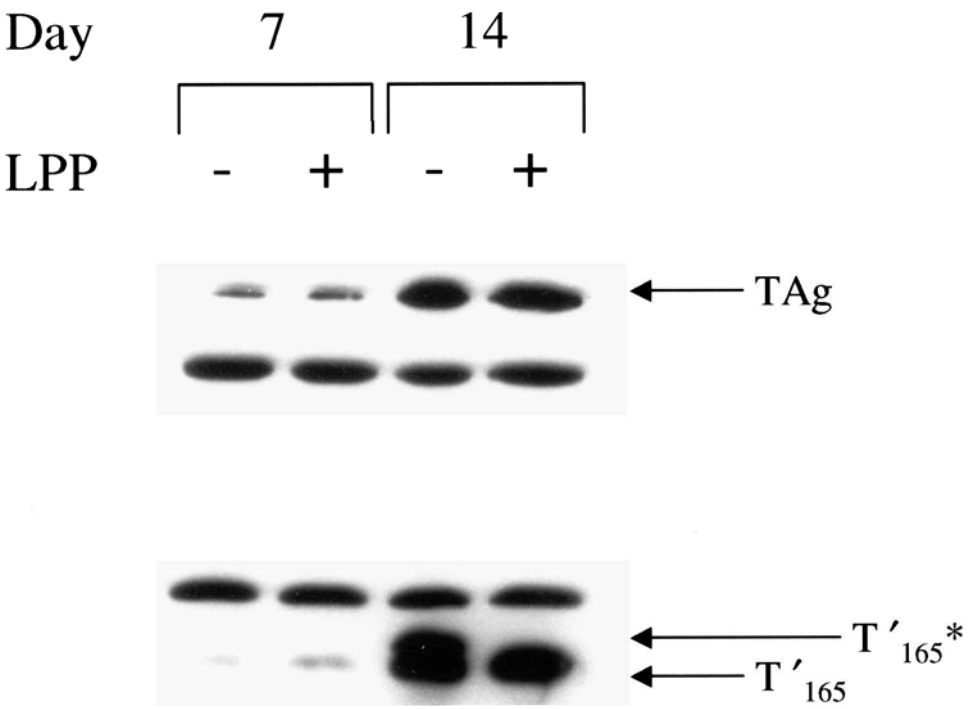

Figure 10 Phosphatase treatment of T'_{165} protein. PHFG cells were infected with 100 hemagglutination units of JCV(Mad1), and lysates were prepared at days 7 and 14 p.i. Lysates were treated with 800 units of lambda protein phosphatase ("with LPP") or left untreated as a control ("No LPP"). JCV early proteins were immunoprecipitated with the monoclonal antibody PAb 962, separated on an 18% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Western blot analysis was performed using PAb 2003 as the primary antibody and a goat α-mouse HRP conjugate as the secondary antibody. Arrows indicate the positions of TAg, phosphorylated T'_{165} (T'^{*}_{165}), and un(der)phosphorylated T'_{165}.

with viral replication efficiencies that had been measured on day 21 p.t. (Table 2).

JCV T'_{165} protein is phosphorylated

A doublet band of 22–23 kD is detected in JCV-infected PHFG cells following immunoprecipitation with anti-TAg antibodies. This doublet had been noted previously (Trowbridge and Frisque, 1995) and was proposed to represent either a differentially phosphorylated form(s) of T'_{165} or a new T′ species. To examine the first possibility, lysates were prepared from JCV-infected PHFG cells and treated with lambda protein phosphatase (LPP), which cleaves phosphates from serine, threonine, and tyrosine residues (Figure 10). Untreated lysates served as controls. At day 7 p.i., only one species of T'_{165} was present, and it was not affected by LPP treatment. At day 14 p.i., a distinct doublet band appeared in the untreated day 14 p.i. sample. The upper band in this doublet disappeared, and the lower band, which co-migrates with purified T'_{165}, became more intense when the sample was treated with LPP. These results indicate that differentially phosphorylated forms of T'_{165} are produced in infected cells at day 14 p.i.

Discussion

TAg functions are necessary but not sufficient for establishing WT levels of JCV DNA replication; the T′ proteins augment this process *in vivo* (Trowbridge and Frisque, 1995; this study). The SV40 17kT protein, a counterpart to the JCV T′ proteins, has not been demonstrated to enhance TAg-mediated DNA replication activity, however, under certain conditions SV40 tAg does influence this activity (Scheidtmann *et al*, 1991a,b; Cegielska *et al*, 1994b). The initial investigation of JCV T′ proteins found that at least one of these proteins complements TAg-mediated viral DNA replication (Trowbridge and Frisque, 1995). The purpose of the present study was to assess the contributions of individual T′ proteins to this process by employing a genetic approach. Using two different mutants, we have confirmed our initial findings that the inability to express all three T′ proteins results in a 10-fold decrease in replication potential. Furthermore, mutants that only produce a single T′ protein (i.e., T'_{135} or T'_{165}) showed a twofold reduction in activity, while a third double mutant producing only T'_{136} replicated nearly as well as WT JCV DNA. In addition, the three mutants that were capable of expressing two out of the three T′ proteins replicated two- to threefold less efficiently than the parental genome at an early time point (day 7 p.t.), but slightly better than the WT genome at the latest time point (day 21 p.t.; Table 2). Interestingly, SV40 TAg J domain mutants show a similar time-dependent replication defect relative to WT virus (Campbell *et al*, 1997). Finally, we found that two T′ proteins,

T'_{152} and T'_{147}, not normally expressed in JCV-infected cells, are capable of influencing replication potential (Figure 3, data not shown). These results indicate that the amino-terminal 132 amino acids shared by each individual T′ protein possess DNA replication activity, and that the unique carboxy-terminal sequences of one of these proteins (T'_{136}) may make additional contributions to replication function.

Our C series of T′ mutants exhibited some differences in alternative splicing patterns in addition to altered replication potentials. When examined at late time points, we consistently observed lower levels of T'_{165} mRNA and protein production in the JCV$\Delta T'_{136}$ acceptor splice site mutant, and higher levels of T'_{135} products in the JCV$\Delta T'_{136}$ and JCV$\Delta T'_{136/165}$ acceptor splice site mutants relative to their expression in cells transfected with the JCV(Mad1) genome (Figures 4, 7). Splice site selection is governed by a number of factors (reviewed in Stojdl and Bell, 1999; Elliot, 2000). A branch point sequence, generally located 18 to 37 nucleotides upstream of the 3′ splice acceptor site, is the recognition site for the U2 small nuclear ribonucleoprotein particle (snRNP) (Reed and Maniatis, 1985; Lopez, 1998). The degree of sequence similarity between the branch point sequence of a splice acceptor and the consensus branch point sequence (YNYURAY) determines the strength of the interaction of the U2 snRNP with the branch point sequence, and thus regulates the selection of alternative splice acceptor sites (Padgett *et al*, 1985; Reed and Maniatis, 1985; Ruskin *et al*, 1985; Noble *et al*, 1987). The first AG dinucleotide positioned at least 18 nucleotides downstream from the branch point is the 3′ splice junction. A survey of mutations at 3′ splice sites of several human genes reveals that the use of cryptic splice acceptors due to mutation of a known 3′ splice acceptor is not uncommon, and that the selection of the cryptic splice acceptor may be influenced more by the branch point sequence and polypyrimidine tract than by the similarity of the acceptor site to the consensus acceptor site sequence (Krawczak *et al*, 1992). The AG dinucleotide preceding the 3′ splice acceptor site is invariant, and in noncryptic 3′ splice sites, approximately 50% of the human genes surveyed utilized a G as the acceptor nucleotide; 28%, 13%, and 10% utilized an A, C, and T, respectively (Krawczak *et al*, 1992). The T'_{135} and T'_{165} splice acceptor nucleotides are both G's, while the T'_{136}, T'_{147}, and T'_{152} splice acceptors are an A, C, and T, respectively.

Cell-specific splicing factors regulate mRNA processing through the recognition of splicing enhancer elements within intron and exon sequences. These splicing factors include SR proteins, a family of RNA-binding proteins that contain a carboxy-terminal serine-rich motif involved in protein-protein interactions, and the protein U2 snRNP auxiliary factor (U2AF) that binds to the polypyrimidine tract located in the intron downstream of the branch point sequence. This latter factor may facilitate recognition of the branch point sequence by the U2 snRNP (Lopez, 1998). The phosphorylation status and/or levels of these splicing factors may influence changes in the alternative splicing patterns of adenovirus transcripts during the course of a viral lytic cycle (Molin and Akusjärvi, 2000). Interestingly, over the course of a JCV infection of PHFG cells, we have observed the time-dependent appearance of the five early transcripts, with the T'_{135} and T'_{136} mRNAs appearing first (Prins, Jones, and Frisque, unpublished data). Furthermore, significant differences in splicing patterns are observed in JCV-infected versus JCV-transformed cells (Jones and Frisque, unpublished data). It will be important to examine the status of the cellular splicing factors under these various conditions.

It is possible that differences in viral DNA replication levels of the T′ acceptor site mutants relative to JCV(Mad1) are either due to (i) the direct loss of one or more T′ protein functions or (ii) the altered splice site selection in the T′ mutants that results in changes in TAg levels. We favor the former idea given the similarity of the replication phenotypes of the donor site mutant, JCVΔT′, and the triple acceptor site mutant, JCV$\Delta T'_{135/136/165}$C, which both fail to express the three T′ proteins. One could also argue that the differences we observed in the replication activities could be explained either by (i) the loss of a T′ early function(s) that influences DNA replication potential directly or (ii) the loss of a possible T′ late function that prevents efficient viral spread, thereby reducing this activity indirectly. Our data support the former suggestion since we observed adverse effects on replication function(s) prior to the secondary infection of the cultures that occurs about day 11 p.t. (Table 2).

Several of the functional domains shared by TAg and the T′ proteins are known to enhance JCV DNA replication, although this does not necessarily indicate that the T′ proteins mediate the same effect as TAg does through these domains. For example, binding of TAg oligomers to the core origin sequences and to the DNA polymerase α-primase complex leads to synthesis of replication primers within the origin bubble (reviewed by Stillman, 1994). However, the T′ proteins do not possess the TAg domains responsible for oligomerization or DNA binding. Therefore, if the T′ proteins do bind the polymerase α-primase complex, one might predict that this interaction would impart a negative effect on DNA replication by sequestering this critical cellular replication factor. On the other hand, T′ proteins could utilize the domains shared with TAg, such as those that bind Tst-1, pRB, p107, p130, and hsc70, in their own unique ways to enhance replication. As examples of their unique properties, T′ proteins are expressed at different times and in different amounts during the JCV replication cycle. In addition, T′ proteins exhibit differential binding to some cellular proteins (Bollag *et al*, 2000), possibly because of their

altered structures and/or their altered phosphorylation states (Swenson and Frisque, 1995; Trowbridge and Frisque, 1995). Evidence for this latter suggestion is provided in the present study (Figure 10); T'_{165}, unlike T'_{135} or T'_{136}, appears to exist as two differentially-modified forms, presumably because T'_{165} contains a carboxy-terminal phosphorylation site not found in the other two T′ proteins.

Tst-1 stimulates JCV transcription, and TAg interacts with Tst-1 through its J domain (Sock *et al*, 1999) to synergistically activate the viral promoters (Wegner *et al*, 1993). The T′ proteins share the J domain of TAg, and we predict they will also interact with Tst-1 to promote transcription. Such an activity might enhance virion production, promote secondary spread of the virus, and increase DNA replication indirectly during an infection of cultured cells.

The T′ proteins differentially interact with the cellular tumor suppressor protein pRB and related proteins p107 and p130 (Bollag *et al*, 2000). In addition, T'_{135} alters the phosphorylation state of p107 and p130 and appears to cause degradation of a hyperphosphorylated form(s) of the latter protein (Bollag, Kilpatrick, and Frisque, unpublished results). These interactions, and the expected interactions of the T′ proteins with the molecular chaperone hsc70, might be necessary to prepare the cell for S phase. The most likely outcome of a T′/hsc70 interaction with pRB, p107, and p130 would be the release of the E2F family of transcription factors to promote progression of the cell cycle from G1 to S phase.

The ability of the T′ proteins to influence cell cycle progression might be a necessary event for a successful infection of PHFG cells by JCV. These cells grow slowly, and it may be important for the virus to encode multiple regulatory proteins that modulate cell cycle progression. Because the individual T′ proteins exhibit differential binding to the pRB family of proteins, and because they are differentially expressed in cultured cells, the T′ proteins may influence JCV DNA replication by enhancing and fine-tuning a cell's progression onto S phase. It is in this stage of the cell cycle that the "simple" JC virus commandeers the cellular machinery to replicate its genome.

Materials and methods

Construction of the JCV T′ acceptor site mutants

The clone M1/pB-C BSKS (Swenson *et al*, 1996) was digested with *Pst*I and *Ppu*MI to yield an 883 bp fragment that was used as a template for PCR mutagenesis (Figure 1), and a 4650 bp vector fragment that was saved to recreate the clones after the mutagenesis. Primers containing the appropriate nucleotide alterations were used to incorporate mutations into the amplified DNA (Figure 2). To alter the T'_{135} splice acceptor, the C at nt 2918 was changed to a G and the T at nt 2920 was changed to a G using the ΔT′135 Mutant and ΔT′135 PstI primers. To alter the T'_{136} splice acceptor, the T at nt 2777 was changed to a G and the T at nt 2779 was changed to a G using the ΔT′136 Mutant and ΔT′165 AlwNI primers. To alter the T'_{165} splice acceptor, the C at nt 2705 was changed to a T using the ΔT′165 Mutant and ΔT′165 AlwNI primers. The PCR product containing the T'_{135} mutations was digested with *Alw*NI and *Pst*I to generate a 447 bp fragment, which was then ligated with a WT *Alw*NI-*Ppu*MI 436 bp fragment from JCV(Mad1) DNA and the 4650 bp vector fragment to create the clone JCVΔT'_{135}A/KS ("KS" refers to the parent clone M1/pB-C BSKS).

The PCR product containing the T'_{136} mutations was cleaved with *Sau3*AI and *Alw*NI to generate a 169 bp fragment, which was then ligated with a 267 bp WT *Ppu*MI-*Sau3*AI fragment from JCV(Mad1) DNA, the 447 bp WT *Pst*I-*Alw*NI fragment, and the 4650 bp vector fragment to create the clone, JCVΔT'_{136}A/KS. The PCR product containing the T'_{165} mutation was cleaved with *Dde*I and *Alw*NI to generate a 210 bp fragment, which was then ligated with a 226 bp WT *Ppu*MI-*Dde*I fragment from JCV(Mad1) DNA, the 447 bp WT *Pst*I-*Alw*NI fragment, and the 4650 bp vector fragment to create the clone JCVΔT'_{165}A/KS. To construct the double acceptor site mutant JCVΔ$T'_{135/136}$A/KS, the 447 bp *Alw*NI-*Pst*I PCR product of the JCVΔT'_{135}A/KS mutant was ligated with the 169 bp *Sau3*AI-*Alw*NI PCR product of the JCVΔT'_{136}A/KS mutant, the 267 bp WT *Ppu*MI-*Sau3*AI fragment, and the 4650 bp vector fragment. The double acceptor site mutant JCVΔ$T'_{135/165}$A/KS was constructed by ligating the 447 bp *Pst*I-*Alw*NI PCR product of the JCVΔT'_{135}A/KS mutant with the 210 bp *Alw*NI-*Dde*I PCR product of the JCVΔT'_{165}A/KS mutant, the 226 bp WT *Ppu*MI-*Dde*I fragment, and the 4650 bp vector fragment. The clone JCVΔ$T'_{136/165}$A/KS was created by using JCVΔT'_{136}A/KS as a template, and using primers ΔT′165 Mutant and ΔT′165AlwNI to insert mutations at the T'_{165} site. This PCR product was digested with *Dde*I and *Alw*NI to generate a 210 bp fragment, which was then ligated with the 447 bp WT *Pst*I-*Alw*NI fragment, the 226 bp WT *Ppu*MI-*Dde*I fragment, and the 4650 bp vector fragment.

The triple acceptor site mutant JCVΔ$T'_{135/136/165}$ A/KS was created by ligating the 210 bp *Dde*I-*Alw*NI fragment containing the T'_{136} and T'_{165} mutations with the 447 bp *Alw*NI-*Pst*I fragment containing the T'_{135} mutation, the 226 bp wild-type *Ppu*MI-*Dde*I fragment, and the 4650 bp vector fragment. The entire *Pst*I to *Ppu*MI region for each "KS" clone was confirmed by sequencing. The JCV sequences, including the mutated sequences, located in the 1658 bp *Pst*I to *Cla*I fragments of these clones were isolated and ligated with the 4249 bp *Pst*I to *Pst*I and the 3586 bp *Pst*I to *Cla*I fragments from pM1TCR1B (the prototype JCV(Mad1) genome linked to pBR322; Frisque, 1983) to create seven intact mutant JCV genomes cloned into pBR322. Dideoxy sequencing

was performed to confirm the presence of the correct mutations in each acceptor site mutant clone. The notation "Δ" in the name of each acceptor site mutant indicates which T′ species has been eliminated in that clone. The names of the final T′ acceptor site mutants cloned in pBR322 retain the names of the T′ acceptor site mutants cloned in M1/pB-C BSKS, minus the "KS" designation.

Alteration of the T'_{152} splice acceptor site

Four acceptor site mutants, JCVΔT'_{135}A, JCVΔ$T'_{135/136}$A, JCVΔ$T'_{135/165}$A, and JCVΔ$T'_{135/136/165}$A, were subjected to PCR mutagenesis a second time to alter the cryptic T'_{152} splice acceptor site. Primers RT-PCR#6 and RT-PCR#7 and the JCVΔT'_{135}A/KS template were used in PCR mutagenesis to alter the C at nt 2914 to a G. The PCR product was cleaved with *Pst*I and *Pvu*II to generate a 459 bp fragment. JCVΔT'_{135}A/KS, JCVΔT'_{136}A/KS, JCVΔT'_{165}A/KS, and JCVΔ$T'_{136/165}$A/KS were cleaved with *Ppu*MI and *Pvu*II to generate a 424 bp fragment from each clone. The 459 bp PCR fragment was ligated with the 424 bp *Ppu*MI-*Pvu*II fragment of each "KS" clone and with the 4650 bp vector fragment used in the original cloning of the mutants to create JCVΔT'_{135}B/KS, JCVΔ$T'_{135/136}$B/KS, JCVΔ$T'_{135/165}$B/KS, and JCVΔ$T'_{135/136/165}$B/KS. Each clone was then subjected to restriction enzyme digests with *Pst*I and *Cla*I to remove the 1658 bp fragment containing all T′ mutations. This fragment was ligated with the 4249 bp *Pst*I-*Pst*I and 3586 bp *Pst*I-*Cla*I fragments of pM1TCR1B to create JCVΔT'_{135}B, JCVΔ$T'_{135/136}$B, JCVΔ$T'_{135/165}$B, and JCVΔ$T'_{135/136/165}$B.

Alteration of the T'_{147} splice acceptor site

Four acceptor site mutants, JCVΔT'_{135}A, JCVΔ$T'_{135/136}$A, JCVΔ$T'_{135/165}$A, and JCVΔ$T'_{135/136/165}$A, were altered a third time using PCR mutagenesis to prevent the production of T'_{147}, T'_{152}, and two other potential T′ proteins, T'_{154} and T'_{225} (Figure 2). Using primers RT-PCR#6 and dT'_{147} (Table 1) and using JCVΔT'_{135}A/KS as a template, the T at nt 2915 was changed to a G to alter the T'_{152} splice acceptor site, and the T at nt 2900 was changed to a G to alter the T'_{147} splice acceptor site. This PCR product was cleaved with *Pst*I and *Msp*A1I to yield a 459 bp fragment.

JCVΔT'_{135}A/KS, JCVΔT'_{136}A/KS, JCVΔT'_{165}A/KS, and JCVΔ$T'_{136/165}$A/KS were used as templates in PCR reactions with primers Set 11-L and dT'_{154}. In these reactions, the C at nt 2882 was changed to a G and the T at nt 2884 was changed to a G to prevent production of T'_{154}, and the C at nt 2885 was changed to a T to prevent production of T'_{225}. These PCR products were digested with *Ppu*MI and *Msp*A1I to generate a 424 bp fragment. For each clone, this fragment was ligated with the 459 bp *Pst*I-*Msp*A1I fragment and the 4650 bp M1/pB-C BSKS vector fragment to create JCVΔT'_{135}C/KS, JCVΔ$T'_{135/136}$C/KS, JCVΔ$T'_{135/165}$C/KS, and JCVΔ$T'_{135/136/165}$C/KS. The 1658 bp *Pst*I-*Cla*I fragment containing the T′ mutations was isolated from each clone and ligated with the 4249 bp *Pst*I-*Pst*I and 3586 bp *Pst*I-*Cla*I WT fragments of pM1TCR1B to create JCVΔT'_{135}C, JCVΔ$T'_{135/136}$C, JCVΔ$T'_{135/165}$C, and JCVΔ$T'_{135/136/165}$C. All T′ acceptor site mutant genomes (A and C series) encode a WT TAg.

Cell culture

PHFG cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma, Irvine Scientific) with 3 or 10% bovine calf serum (BCS; Hyclone), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) (Irvine Scientific), and were incubated at 37°C in an atmosphere of 10% CO_2.

DpnI replication assay

PHFG cells were transfected with JCV DNA (the Mad1 variant of JCV was used in all studies) using a modified DEAE-dextran method (Sompayrac and Danna, 1981; Lynch and Frisque, 1990). Low molecular weight viral DNA was extracted from PHFG cells at various time points p.t. using the method of Hirt (1967) and a portion of the extract was digested with *Eco*RI and with *Dpn*I to distinguish replicated viral DNA from the DNA used for transfection. Samples were loaded onto a 0.8% TBE agarose gel and electrophoresed at 80V for 20 min and at 20 V overnight. DNA fragments were transferred to 0.2 μm pore size nylon membrane (Schleicher and Schuell, Amersham) using the Rapid Downward Transfer System and the alkaline transfer protocol (Schleicher and Schuell). Samples were incubated overnight with a JCV-specific DNA probe, labeled with 50 μCi of α-^{32}P-dCTP (NEN, ICN) using an oligolabelling kit (Pharmacia). Bands representing JCV-specific DNA were detected by autoradiography using Kodak film. Phosphor screens exposed to the blots were scanned on a Phosphorimager (Molecular Dynamics). Relative replication levels of viral DNA were determined using the ImageQuant program (Molecular Dynamics).

JCV infection of PHFG cells

Virus was isolated from cells on day 28 p.t. Plates were freeze–thawed twice and cells were subjected to sonication two times at an output of 0.3 for 45 s on a Model 300 Sonic Dismembrator (Fisher). A volume equal to 1/10 the total volume of cellular extract was added to DMEM to a total volume of 4 ml. PHFG cells were incubated with 1 ml of this mixture for 90 min at 37°C.

Total RNA isolation

RNA was isolated from infected cells using the GlassMAX RNA Microisolation Spin Cartridge System (Gibco BRL). RNA was subjected to RT-PCR analysis using the Access RT-PCR System

(Promega) and primers T′#1 (JCV nt 2580-2602; 5′-CCAGCTTTACTTAACAGTTGCAG-3′) and T′#3 (JCV nt 4368-4345; 5′-GGGATGAAGACCTGTTTTGCCA TG-3′) (Trowbridge and Frisque, 1995). PCR products were cloned into the PCR II or PCR 2.1 vector of the TA Cloning Kit (Invitrogen) and used to transform bacterial cells (TA Cloning Kit's One Shot cells). Clones of interest were identified by restriction enzyme analysis and sequenced by Penn State's Nucleic Acid Facility.

Immunoprecipitation and Western blotting of viral proteins

To obtain lysates, cells were washed twice with 2 ml cold STE (0.15 M NaCl; 0.02 M Tris, pH 7.2; 1 mM EDTA, pH 7.2) and then treated with RIPA buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium lauryl sulfate; 0.02 M Tris, pH 7.2; 0.15 M NaCl) containing 10 μg/ml leupeptin. Plates of cells were rocked on ice for 15 min. Lysates were collected, centrifuged at 4°C at 13 000 RPM for 40 min, aliquoted as appropriate and stored at −80°C.

To immunoprecipitate viral proteins present in lysed cells, lysates were incubated on ice with 10 μl of hybridoma supernatant for 45 min. Staph A (Pansorbin) stored in RIPA buffer was pelleted by centrifugation and resuspended in RIPA buffer containing BSA (1 mg/ml). After incubating for 15 min at room temperature, 30 μl of the Staph A were added to the cell lysates and the mixture was incubated on ice for 30 min. Immune complexes were pelleted by centrifugation for 20 s, washed one time with 1 ml high salt buffer (HSB; 1.0 M NaCl; 0.01 M Tris, pH 7.2; 0.5% Triton X-100) containing leupeptin (10 μg/ml), and washed twice with 1 ml RIPA buffer containing leupeptin (10 μg/ml). Proteins were released from the complexes by addition of 18 μl of sample buffer (62 mM Tris; 10% glycerol; 2% SDS; 0.72 M βME; 0.1 mg/ml bromphenol blue). Samples were vortexed, heated to 95°C for 2 min, loaded into SDS-polyacrylamide gels and electrophoresed at 200 V for 1 h.

The SDS-PAGE gel was soaked in Hoeffer's transfer buffer (25 mM Tris; 193 mM glycine; 20% methanol; 0.1% SDS) for 20 min, and proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) at 100 V for 1 h. The membrane was rinsed with ddH_2O and soaked in blocking solution (2% nonfat dry milk; 0.1% Tween-20; 10% PBS) for 30 min. This step was repeated one time. Anti-TAg monoclonal antibody (20 μl) was added to blots in 5 ml of blocking solution and incubated at room temperature on a shaker for 1.5 to 3 h. Blots were washed six times over a 20-min period with wash buffer (0.1% Tween-20; 10X TBS). Blots were incubated with 5 μl goat α-mouse HRP conjugate, in 5 ml blocking solution for 1.5 to 3 h. Blots were washed again with wash buffer and protein bands were detected by enhanced chemiluminescence using either Supersignal West Femto or Pico Maximum Sensitivity Substrate (Pierce).

Treatment of T'_{165} protein with protein phosphatase

To determine the phosphorylation status of T'_{165}, cell lysates were prepared from JCV-infected PHFG cells at days 7 and 14 p.i. Lysates were treated with 800 units of Lambda Protein Phosphatase (LPP; New England Biolabs) in LPP buffer (50 mM Tris-HCl; 0.1 mM Na_2EDTA; 5 mM DTT; 0.01% Brij 35) supplemented with 2 mM $MnCl_2$ and incubated at 30°C for 2 h. Early viral proteins were immunoprecipitated using PAb 962 (Tevethia *et al*, 1992) and transferred to a nitrocellulose membrane. Western blotting was performed using PAb 2003 (Bollag *et al*, 2000) as the primary antibody and a goat-α-mouse IgG HRP conjugate as the secondary antibody. Proteins were visualized using the Supersignal West Pico Maximum Sensitivity Substrate.

Acknowledgements

We thank Pamela Trowbridge for assistance in the construction of the T′ cDNA clones. This study was supported by a Sigma Xi Grant-In-Aid of Research to CP and by Public Health Service Grant CA-44970 from the National Cancer Institute.

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