Journal of NeuroVirology (1996) 2, 78–86

Replication activity of JC virus large T antigen phosphorylation and zinc finger domain mutants

Jennifer J Swenson, Pamela W Trowbridge* and Richard J Frisque

Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA

The replication potential of the human polyomavirus JC virus (JCV) relative to that of the related monkey virus SV40 is limited, in part, by differences in the multifunctional T antigen (T Ag). Earlier genetic analyses of the SV40 T protein indicated that specific phosphorylation sites and a zinc finger motif are involved in the regulation of viral replication. The JCV and SV40 T Ags differ with respect to sequences encoding these functional domains, and in the present study mutational analysis of the JCV protein was conducted to assess the role that unconserved residues might play in the restricted lytic behavior of JCV. Amino acids Asn316 and His317 in the zinc finger domain and Thr664 and Glu666 in the carboxy-terminal phosphorylation domain were mutated to either an SV40-like residue or an alanine. Each of the mutant JCV genomes replicated with wild type efficiency suggesting that, unlike the case for SV40 T Ag, these amino acids are not critical to the regulation of viral replication. On the other hand, mutation of amino acid Thr125 within the amino-terminal phosphorylation domain abolished JCV DNA replication and viability. This site is conserved in the SV40 T Ag, and previous results have revealed that phosphorylation of this residue (Thr124) is required for T Ag replication function.

Keywords: human polyomavirus; viral DNA replication; T antigen mutants

Introduction

Infection by the primate polyomavirus JC virus (JCV) is common in the human population and the virus persists in the kidneys, brain and lymphocytes of many individuals. Upon immunosuppression, JCV may be reactivated and cause the fatal brain disease progressive multifocal leukoencephalopathy (Arthur and Shah, 1989; Frisque and White, 1992; Berger and Concha, 1995 and references therein). Extensive homology exists between members of the primate polyomavirus group with JCV exhibiting approximately 70% sequence identity with SV40 and BK virus (BKV) (Frisque *et al*, 1984). Despite this homology, the viruses are biologically distinct. Compared to the other two viruses, JCV is highly restricted in its host range with efficient virus production limited to human kidney and brain tissues *in vivo* and human fetal glial cells *in vitro* (Padgett *et al*, 1977a; Feigenbaum *et al*, 1987; Frisque and White, 1992). The DNA replication activity of JCV, as well as its ability to transform cells in culture, are also diminished as compared to those of SV40 and BKV (Walker and Frisque, 1986; Bollag *et al*, 1989; Haggerty *et al*, 1989; Lynch and Frisque, 1991; Trowbridge and Frisque, 1993; Lynch *et al*, 1994).

T antigen (T Ag), the major regulatory protein produced by the polyomaviruses, is a multifunctional phosphoprotein which is essential for viral replication. Biochemical and mutational analyses of SV40 T Ag indicate that it binds to specific sequences within the viral origin of replication (ori), and in the presence of ATP, forms a double hexamer structure capable of unwinding the ori region (Borowiec *et al*, 1990; Fanning and Knippers, 1992; Pipas, 1992). T Ag directly interacts with DNA polymerase α and initiates the elongation process via its helicase-ATPase activity (DePamphilis and Bradley, 1986; Fanning and Knippers, 1992; Stillman, 1994).

Although JCV and SV40 T Ags are similar at the genetic level, these proteins contribute to differences between the viruses at the biological level. Studies employing JCV-SV40 hybrid genomes indicate that the restricted behavior of JCV is due, in part, to the activities of its T Ag (Chuke *et al*, 1986; Bollag *et al*, 1989; Haggerty *et al*, 1989; Lynch and Frisque, 1991; Tavis and Frisque, 1991; Lynch *et al*, 1994). Sequences within the central region of JCV T Ag recognize minor differences between the

Correspondence: RJ Frisque
*Present address: Department of Biology, University of Delaware, Newark, DE 19716, USA
Received 21 November 1995

JCV and SV40 ori sequences and prevent the protein from productively interacting with ori sequences other than its own (Lynch and Frisque, 1991; Tavis and Frisque, 1991; Lynch *et al*, 1994). The basis for this non-productive interaction involves a step subsequent to specific binding of JCV T Ag to the SV40 ori sequences (Lynch and Frisque, 1991). The central region of T Ag contains the DNA binding, Zn finger and putative N-terminal phosphorylation domains (Lynch *et al*, 1994).

The phosphorylation and Zn finger domains of SV40 T Ag have been shown to play essential roles in the viral DNA replication and transforming abilities of the protein (Loeber *et al*, 1989, 1991; Höss *et al*, 1990; Prives, 1990; Fanning, 1992; Fanning and Knippers, 1992). SV40 T Ag is phosphorylated at nine major sites on both serine and threonine residues (Van Roy *et al*, 1983; Scheidtmann *et al*, 1984; Prives, 1990); T Ag function is influenced by the combination of residues which are modified (Prives, 1990; Fanning, 1992; Fanning and Knippers, 1992). Phosphorylation of Thr124 is essential for stable ori binding, ori unwinding and DNA replication (Scheidtmann *et al*, 1984, 1991; McVey *et al*, 1993; Moarefi *et al*, 1993). Phosphorylation of Ser677 influences the modification of several N-terminal regulatory phosphorylation sites, which in turn affects DNA binding and replication activities of T Ag (Schneider and Fanning, 1988; Scheidtmann *et al*, 1991). Mutation of the Ser677 residue also alters T Ag conformation as well as its transforming ability; some of these changes may be due to altered oligomerization of the mutant protein (Schneider and Fanning, 1988; Scheidtmann *et al*, 1991).

The Zn finger motif is highly conserved in the polyomavirus T Ags (Tooze, 1981; Frisque *et al*, 1984; Berg, 1986), and mutational analysis of this domain in SV40 T Ag demonstrates its importance in the formation of stable hexamers and modulation of T Ag DNA binding specificity at ori sequences (Arthur *et al*, 1988; Höss *et al*, 1990; Loeber *et al*, 1991). Alterations to any of the presumed zinc coordinating residues, or the nearby histidine residue in the major loop, diminish the transforming ability of T Ag and abolish viral infectivity (Loeber *et al*, 1989).

Mutant SV40 T Ags altered at key phosphorylation sites or Zn finger residues exhibit activities similar to those displayed by wild type JCV T Ag. The present study sought to determine whether differences within these regions play a role in the diminished ability of JCV to efficiently replicate its genome and produce infectious virions in cell culture. Our approach was to genetically alter JCV T Ag coding sequences within these domains to be more SV40-like, and to then determine whether these alterations enhanced JCV's replication potential. Furthermore, since the central region of JCV T Ag is involved in discriminating between the JCV ori and other polyomavirus ori sequences, we tested the ability of JCV T Ag Zn finger mutants to productively interact with the SV40 ori.

Results

Viral DNA replication and virion production of JCV T Ag phosphorylation mutants

Post-translational modifications influence the activity of the multifunctional polyomavirus T Ags (Figure 1); phosphorylation regulates many functions of SV40 T Ag and its effects depend upon the specific amino acid (a.a.) residues which are modified (Prives, 1990; Scheidtmann *et al*, 1991; Cegielska *et al*, 1994 and references therein). DNA replication activities of SV40 T Ag affected by phosphorylation include ori binding, stable double hexamer formation at ori sequences and DNA unwinding (Scheidtmann *et al*, 1984, 1991; Schneider and Fanning, 1988; Cegielska and Virshup, 1993; McVey *et al*, 1993; Moarefi *et al*, 1993; Virshup *et al*, 1993). Thr124 is a critical site of phosphorylation (McVey *et al*, 1989; Scheidtmann *et al*, 1991); modification of this residue stabilizes T Ag binding to the ori sequences and promotes DNA unwinding (Scheidtmann *et al*, 1984; McVey *et al*, 1993; Moarefi *et al*, 1993). A comparison of the SV40 and JCV T Ags indicates that six of the nine major phosphorylation site residues identified in the SV40 protein are conserved in JCV T Ag, while a seventh residue is represented by a different,

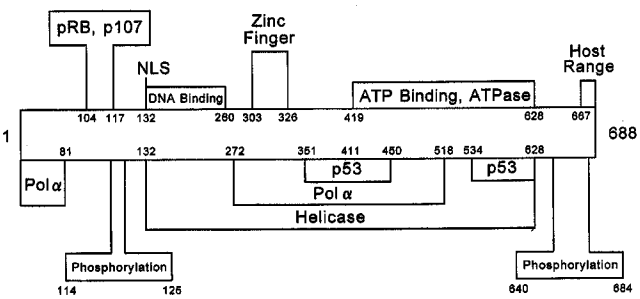

Figure 1 Functional domains of JCV T Ag. Identification of JCV T Ag functional domains as predicted by mutational analysis (Tavis and Frisque, 1991; Tavis *et al*, 1994; Swenson and Frisque, 1995) and sequence homology with SV40 T Ag (Frisque *et al*, 1984; Fanning and Knippers, 1992; Pipas, 1992). The numbers within the central box represent a.a. residues which lie at the functional domain boundaries of the 688-a.a. protein. The JCV T Ag has been divided into amino, central and carboxy regions at a.a. 81 and 411 based on studies employing JCV-SV40 hybrid T Ag genomes (Haggerty *et al*, 1989; Lynch *et al*, 1994; Tavis *et al*, 1994). The phosphorylation and Zn finger domains lie within the central-carboxy and central regions of T Ag, respectively. pRB, p107, binding domains for the cellular proteins pRB and p107; NLS, nuclear localization signal; p53, binding domain for the cellular protein p53; Pol α, DNA polymerase α binding domain. The figure was adapted from Lynch *et al* (1994).

potentially phosphorylatable residue (Ser in SV40 *versus* Thr in JCV; Figure 2). To determine whether differences in the phosphorylation state of JCV T Ag contribute to its diminished activity relative to that of SV40 T Ag, JCV T Ag mutants altered at potential phosphorylation sites (Table 1) were tested for their ability to support viral DNA replication and to produce infectious virions in primary human fetal glial (PHFG) cells. JCV-T664A/S and JCV-E666A/S replicated their viral DNA at levels similar to that of wild type JCV (Figure 3A, Table 2). In contrast, viral DNA replication was not detected for JCV-T125A (Figure 3B, Table 2). Accumulation of increasing amounts of replicated viral DNA in the transfected cells suggested that infectious virions were produced by viral genomes containing T Ag mutations at a.a. 664 and 666, but not at a.a. 125. To confirm the presence of infectious virions, extracts of transfected cells were added to fresh cultures of PHFG cells. The presence of replicated DNA in the infected cells indicated that wild type JCV, JCV-T664A/S and JCV-E666A/S were able to establish a productive infection (Table 2, data not shown).

Table 1 Viral genomes containing JCV T antigen mutations

Viral DNA[a]	*RR*[b]	*T Ag Mutation*[c]	*T Ag Domain*[d]
JCV-T125A	JCV	125 Thr→Ala	Phosphorylation
JCV-T664A	JCV	664 Thr→Ala	Phosphorylation
JCV-T664S	JCV	664 Thr→Ser	Phosphorylation
JCV-E666A	JCV	666 Glu→Ala	Phosphorylation
JCV-E666S	JCV	666 Glu→Ser	Phosphorylation
JCV-N316K	JCV	316 Asn→Lys	Zinc finger
JCV-H317Y	JCV	317 His→Tyr	Zinc finger
JCV-NHKY	JCV	316 Asn→Lys 317 His→Tyr	Zinc finger
SRJCV-N316K	SV40	316 Asn→Lys	Zinc finger
SRJCV-H317Y	SV40	317 His→Tyr	Zinc finger
SRJCV-NHKY	SV40	316 Asn→Lys 317 His→Tyr	Zinc finger

[a]Reference to both JCV-T664A and JCV-T664S viral genomes is denoted as JCV-T664A/S within the text; reference to both JCV-E666A and JCV-E666S viral genomes is denoted as JCV-E666A/S within the text
[b]Viral source of regulatory region (RR) sequences
[c]Amino acid position and residue mutated in JCV T antigen
[d]JCV T antigen domain in which mutation occurs

Viral DNA replication and virion production of JCV T Ag Zn finger mutants

Zn finger motifs are found within many regulatory DNA-binding proteins and these motifs have been shown to contribute to both protein-DNA and protein-protein interactions (Miller *et al*, 1985; Kadonaga *et al*, 1987; Frankel and Pabo, 1988; Rose and Schaffhausen, 1995). SV40 T Ag contains a single Zn finger motif within its central region (a.a. 302–320), and this sequence has been shown to influence several T Ag interactions with the core ori: binding site selection, hexamer formation and unwinding functions (Loeber *et al*, 1989, 1991; Höss *et al*, 1990). Mutational analysis of the SV40 T Ag

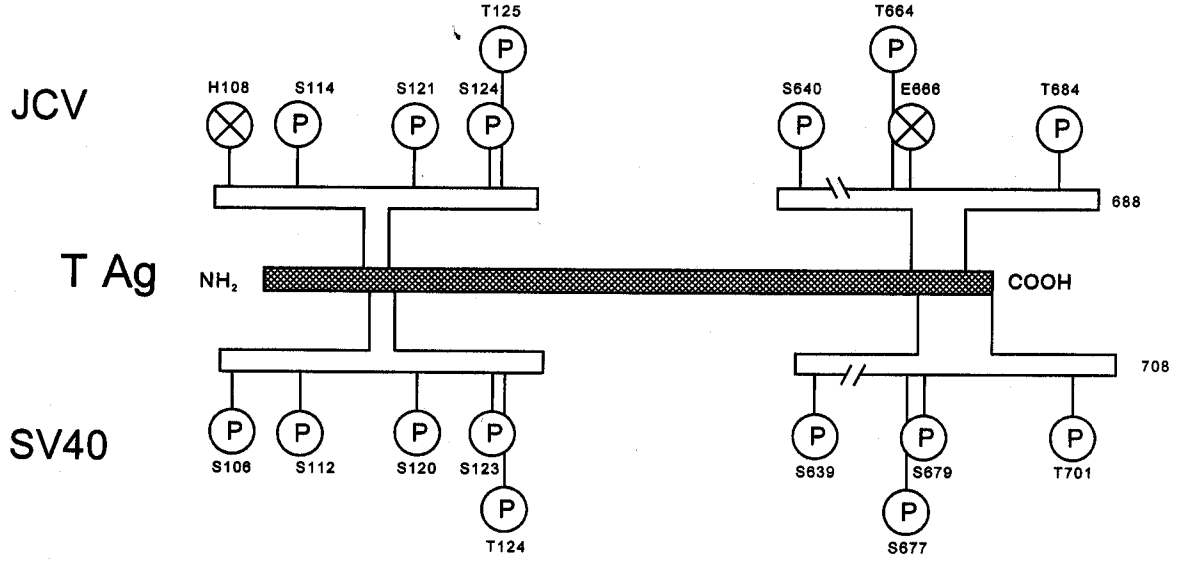

Figure 2 Comparison of the SV40 and the putative JCV T Ag phosphorylation sites. The central box represents an alignment of the 688-a.a. JCV T Ag and the 708-a.a. SV40 T Ag sequences. Regions of T Ag containing known or putative phosphorylation sites have been enlarged; JCV and SV40 sequences are indicated above and below the central box, respectively. The nine major phosphorylation sites identified in SV40 T Ag expressed in infected monkey and transformed rat cells are indicated by a 'P' within a circle (Scheidtmann *et al*, 1984, 1991). JCV T Ag a.a. residues corresponding in position to the SV40 T Ag phosphorylation sites are denoted by a 'P', non-phosphorylatable residues are indicated by an 'X'. Recent work indicates that Thr664 in JCV T Ag is not phosphorylated *in vivo* (Swenson and Frisque, 1995). All sites are identified by the single letter a.a. code and its numbered a.a. position. Figure was adapted from Swenson and Frisque (1995).

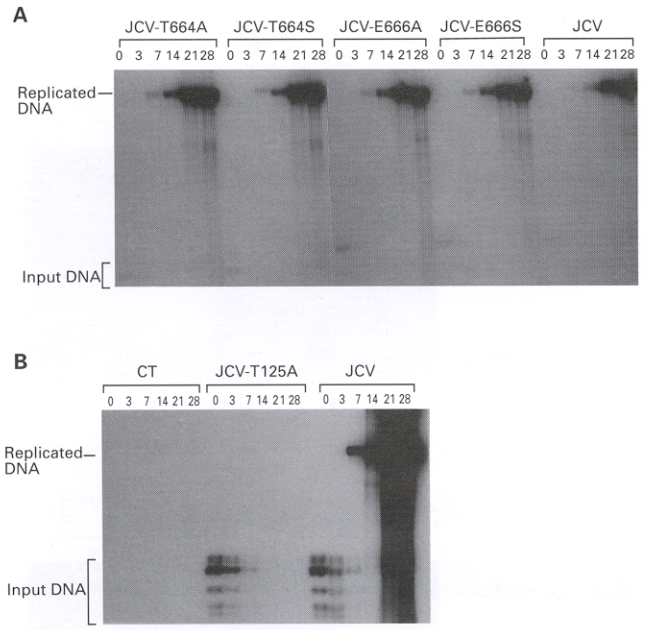

Figure 3 Viral DNA replication of JCV T Ag phosphorylation site mutants in PHFG cells. Low molecular weight DNA was isolated from transfected PHFG cells at various times post-transfection by the method of Hirt (1967). Purified DNA was digested with *EcoRI* and *DpnI*, separated on a 0.8% agarose gel, transferred to a nitrocellulose membrane and probed with linearized ^{32}P-labeled pM1TCR1A; numbers above the lanes denote days post-transfection. Samples were visualized by autoradiography after a 20 min (A) or 16-h (B) exposure; 'CT' indicates calf thymus DNA. The positions of linear replicated DNA and input DNA are indicated.

Table 2 Viral DNA replication and viability of JCV T antigen phosphorylation mutants

Viral DNA	*Day p.t.*[a]	*Experiment*[b] *1*	*2*	*Viability*[c]
JCV-T125A	7	–	–	
	21	–	–	–
JCV	7	1.0	1.0	
	21	15.0 (15.0)	27.6 (27.6)	+[d]
JCV-T664A	7	2.4	0.6	
	21	40.0 (16.7)	4.6 (7.7)	+
JCV-T664S	7	1.4	0.5	
	21	31.8 (22.7)	3.4 (6.8)	+
JCV-E666A	7	1.1	0.5	
	21	17.7 (16.1)	9.2 (18.4)	+
JCV-E666S	7	1.4	0.4	
	21	21.1 (15.1)	8.7 (21.8)	+
JCV	7	1.0	1.0	
	21	25.3 (25.3)	15.0 (15.0)	+

[a]Day p.t. indicates day post-transfection; viral spread initiates at day 10 to 11 post-transfection
[b]Numerical values for DNA replication were obtained by βetascope analysis of Southern blots containing *DpnI–EcoRI* digested DNAs; after background subtraction, values were normalized for input and then to JCV day 7 post-transfection (arbitrarily set to 1.0). Values within the parentheses represent the fold amplification from day 7 to 21 post-transfection. '–', no DNA replication. Experiments 1 and 2 for JCV-T125A (above the dotted line) are different from Experiments 1 and 2 for JCV-T664A/S and JCV-E666A/S (below the dotted line)
[c]Viability was assigned upon the observation of increased levels of replicated DNA in cultures exposed to extracts (day 28) from transfected cells. '–', no infectious virions produced as determined by the absence of DNA replication in transfected cells
[d]Assigned on the basis of accumulation of replicated DNA in transfected cells

Zn finger motif demonstrates the importance of individual residues in DNA replication and transformation (Figure 4; Loeber *et al*, 1989). Residues in the minor loops and the N-terminal region of the major loop have no detectable effect on replication or transformation, whereas the four conserved cysteine and histidine residues (SV40 a.a. 302, 305, 317 and 320), and a neighboring histidine residue (SV40 a.a. 313), are essential for viability (Loeber *et al*, 1989). Mutations within the C-terminal region of the major loop (a.a. 312 to 316) reduce replication and transforming activities of T Ag (Loeber *et al*, 1989); these residues have been shown to influence stable hexamer formation and structural alterations at ori sequences (Loeber *et al*, 1991). The JCV T Ag sequence differs from that of SV40 T Ag at four of the five positions within the C-terminal region of the major loop (Figure 4). Analysis of an SV40 T Ag mutant at Lys315, in which the positively charged lysine was replaced with a negatively charged glutamic acid, displayed greatly diminished DNA replication activity (Loeber *et al*, 1989). The absence of a positively charged residue at the comparable site in JCV T Ag (Asn316) may contribute to the protein's diminished DNA replication activity. In addition, a histidine residue at a.a. position 317 in JCV T Ag, which lies adjacent to one of the zinc-coordinating residues, may affect the ability of the finger to correctly coordinate the zinc atom and induce the protein conformation required for DNA replication. To test these possibilities, mutations were introduced into the JCV T Ag Zn finger at a.a. 316 and 317 (Table 1). JCV T Ags containing single or double mutations at these two positions replicated the viral genomes to approximately the same level as that of the wild type protein (Figure 5A, Table 3). To analyze the ability of the Zn finger mutants to generate infectious virions, extracts from the transfected cells were added to fresh cultures of PHFG cells. The accumulation of increasing amounts of replicated DNA in transfected cells, and the presence of replicated DNA in infected cells, indicate the mutants were viable (Table 3, data not shown).

Viral DNA replication of JCV T Ag Zn finger mutants containing SV40 ori sequences

The central region of JCV T Ag, which includes the

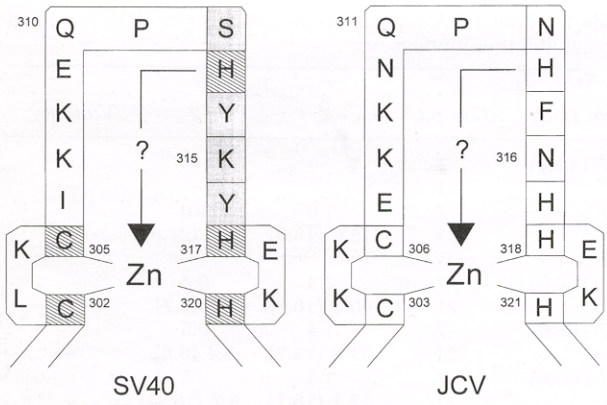

Figure 4 Comparison of the SV40 and JCV T Ag Zn finger motif. The portion of the central domain of SV40 and JCV T Ag which contains the Zn finger motif (residues 302–320 for SV40; 303–321 for JCV) has been enlarged for comparison; the a.a. are represented by the single letter a.a. code. Mutants with single substitutions at most positions in the SV40 finger have a wild type phenotype (unshaded letters). Mutations within the stippled region (residues 312–316) may affect an active site for DNA replication; mutants are replication negative but tranformation positive. Letters highlighted by diagonal lines (C at 302, 305 and H at 317, 320) represent putative zinc-binding a.a. of the SV40 finger; the histidine at position 313 (SV40) and 314 (JCV) may also be involved. SV40 mutants altered at positions 302, 305, 313, 317 and 320 transform inefficiently and fail to replicate. The figure was adapted from Loeber *et al* (1989).

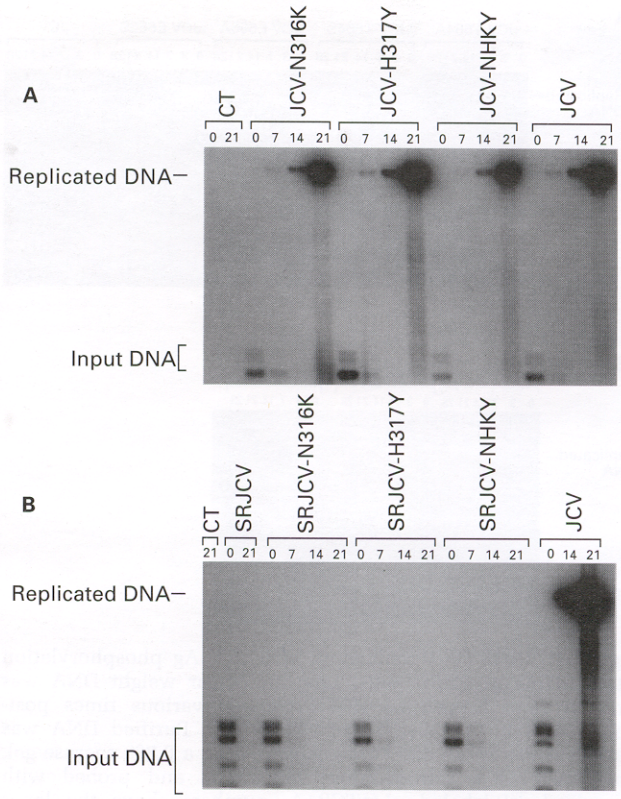

Figure 5 Viral DNA replication of JCV T Ag Zn finger mutants in PHFG cells. DNA replication of JCV T Ag Zn finger mutants containing JCV (A) or SV40 (B) ori sequences. Low molecular weight DNA was isolated from transfected PHFG cells at various times post-transfection by the method of Hirt (1967). Purified DNA was digested with *EcoRI* and *DpnI*, separated on a 0.8% agarose gel, transferred to a GeneScreen Plus membrane and probed with linearized ^{32}P-labeled pM1TCR1A; numbers above the lanes denote days post-transfection. Samples were visualized by autoradiography after a 22-h (A) or 2.5-h (B) exposure; 'CT' indicates calf thymus DNA. The positions of linear replicated DNA and input DNA are indicated.

Zn finger motif (a.a. 303–321), permits the protein to discriminate between the JCV and SV40 ori sequences (Lynch and Frisque, 1990, 1991). JCV T Ag productively interacts only with its own ori to drive DNA replication, despite the ability of the protein to bind both JCV and SV40 ori sequences with similar efficiencies (Lynch and Frisque, 1991). It has been proposed that the non-productive interaction is a result of the JCV T Ag's inability to unwind the SV40 ori (Lynch and Frisque, 1991), and since the Zn finger affects this T Ag function, we asked whether the mutant JCV T Ags might direct replication from the SV40 ori. The regulatory sequences (ori and promoter-enhancer) of the JCV T Ag Zn finger mutants were replaced with those of SV40, and the mutant chimeras were then tested for their ability to replicate in PHFG cells. Neither the single nor the double Zn finger mutants were capable of productively interacting with the SV40 ori to replicate the viral genome (Figure 5B, Table 3).

Discussion

In this study, mutational analysis of JCV T Ag was performed to determine whether sequences within the phosphorylation and Zn finger domains contribute to the limited lytic behavior of the virus. Mutations were introduced at Thr664 and Glu666; the comparable sites in SV40 T Ag, Ser677 and Ser679, have been reported to affect DNA binding, oligomer formation and ori unwinding activities of T Ag (Schneider and Fanning, 1988; Scheidtmann *et al*, 1991), activities found to be diminished in the JCV protein (Lynch and Frisque, 1991; Tavis *et al*, 1994). An additional JCV T Ag mutant was generated at Thr125; the corresponding residue in SV40 T Ag, Thr124, is critical for DNA replication (Scheidtmann *et al*, 1991). We predicted that alterations to the unconserved phosphorylation sites at a.a. positions 664 and 666 would affect the protein's DNA binding and unwinding activities, and that these effects would be evident by altered DNA replication levels. However, viral DNA replication assays indicated that replacement of residues Thr664 and Glu666 with either the corresponding SV40 a.a. or with a non-phosphor-

Table 3 Viral DNA replication and viability of JCV T antigen Zn finger mutants

Viral DNA	*Day p.t.*[a]	*Experiment*[b] *1*	*2*	*Viability*[c]
JCV-N316K	7	0.5	0.6	
	21	76.0 (152.0)	16.3 (27.2)	+
JCV-H317Y	7	0.6	0.8	
	21	58.9 (98.2)	27.1 (33.9)	+
JCV-NHKY	7	0.5	0.9	
	21	80.7 (161.4)	17.2 (19.1)	+
JCV	7	1.0	1.0	
	21	82.5 (82.5)	27.3 (27.3)	+
SRJCV-N316K	7	–	–	
	21	–	–	–
SRJCV-H317Y	7	–	–	
	21	–	–	–
SRJCV-NHKY	7	–	–	
	21	–	–	–
SRJCV	7	–	–	
	21	–	–	–
JCV	7	ND	1.0	
	21	106.9[d]	114.5 (114.5)	+[e]

[a]Day p.t. indicates day post-transfection; viral spread initiates at day 10 to 11 post-transfection
[b]Numerical values for DNA replication were obtained by βetascope analysis of Southern blots containing *DpnI–EcoRI* digested DNAs; after background subtraction, values were normalized for input and then to JCV day 7 post-transfection (arbitrarily set to 1.0). Values within the parentheses represent the fold amplication from day 7 to 21 post-transfection. '–', no DNA replication. 'ND', not determined. Experiments 1 and 2 for the constructs containing the JCV ori (above the dotted line) are different from Experiments 1 and 2 for the constructs containing the SV40 ori (below the dotted line)
[c]Viability was assigned upon the observation of increased levels of replicated DNA in cultures exposed to extracts (day 21) from transfected cells. '–', no infectious virions produced as determined by the absence of DNA replication in transfected cells
[d]Numerical value for DNA replication was normalized to JCV day 7 post-transfection from Experiment 2
[e]Assigned on the basis of accumulation of replicated DNA in transfected cells

ylatable residue did not alter replication activity (Figure 3, Table 2). The findings that mutant and wild type JCV exhibited similar lytic behavior indicate that Thr664 and Glu666 do not play an essential role in the regulation of JCV T Ag DNA replication activities. Recently we reported that, despite its presence in a DNA protein kinase consensus site, the Thr664 residue is not phosphorylated in JCV T Ag (Swenson and Frisque, 1995). Since the phosphorylation of the comparable Ser677 residue in SV40 T Ag regulates the phosphorylation state of critical N-terminal phosphorylation sites (Scheidtmann *et al*, 1991), our results suggest an important difference in the regulatory role played by phosphorylation between JCV and SV40 T Ags.

In contrast to the C-terminal phosphorylation mutants, a mutation at Thr125 in JCV T Ag completely abolished DNA replication activity of the virus (Figure 3). The corresponding conserved Thr124 residue in SV40 T Ag is phosphorylated and mutation of this residue to an alanine completely abolishes DNA replication activity (McVey *et al*, 1989, 1993; Scheidtmann *et al*, 1991; Moarefi *et al*, 1993). $p34^{cdc2}$-cyclin B has been shown to phosphorylate Thr124 *in vitro* (McVey *et al*, 1989); an identical $p34^{cdc2}$ kinase recognition sequence is present at Thr125 in JCV T Ag. The presence of the same consensus sequence in both T Ags, and the similar functional consequences of Thr124 and Thr125 mutation, suggest that Thr125 is modified in JCV T Ag and plays a critical role in T Ag replication functions.

Relative to SV40 T Ag, JCV T Ag exhibits reduced oligomerization, DNA binding and DNA replication activities; the Zn finger domain of JCV T Ag differs from that of SV40 T Ag at the very positions known to affect these functions (Figure 4; Arthur *et al*, 1988; Lynch and Frisque, 1991; Tavis *et al*, 1994). We predicted that SV40-like alterations to JCV T Ag within the Zn finger domain might enhance its oligomerization and DNA binding activities, and this would be reflected in enhanced DNA replication activity. However, mutations to residues Asn316 and/or His317 did not enhance the replication activity of mutant genomes relative to that of wild type JCV (Figure 5). We conclude that neither the presence of an uncharged residue at a.a. 316, nor a histidine residue adjacent to a zinc-coordinating residue, is responsible for the less efficient replication potential of the wild type JCV T Ag relative to the SV40 protein. This in turn would suggest that protein-protein interactions involving the Zn finger region of JCV T Ag are altered relative to the interactions exhibited by SV40 T Ag.

Previous work indicates that JCV T Ag interacts productively with only its own ori. Genetic studies have revealed that sequences within the central region of JCV T Ag are capable of recognizing a three nucleotide difference in the late half of the JCV and SV40 core ori sequences (Lynch and Frisque, 1990). The inability of JCV T Ag to promote replication of the SV40 ori occurs at a step subsequent to DNA binding, since the protein binds both JCV and SV40 ori sequences with similar efficiency (Lynch and Frisque, 1991). Because the central region of JCV T Ag contains the Zn finger motif, and this region is involved in stable hexamer formation and T Ag-induced structural changes at the ori sequences in SV40, we tested the ability of the Zn finger mutants to productively interact with the SV40 ori. The T Ags produced by JCV-N316K, JCV-H317Y and JCV-NHKY, as well as wild type JCV, were unable to productively interact with the SV40 ori sequences indicating that SV40-like mutations at Asn316 and His317 residues are not sufficient to alter ori specificity of JCV T Ag.

The present study addressed the role that differences between the JCV and SV40 T Ag phosphorylation and Zn finger regions might play in regulating JCV DNA replication and virion production. Alterations of residues within the C-terminal phosphorylation domain and proposed Zn finger active site did not cause a detectable effect on these activities, whereas a mutation at Thr125 completely abolished DNA replication activity. These results suggest that, while global regulatory processes may be similar between JCV and SV40 T Ags, subtle differences at key regulatory sites may have evolved to dictate the specificity and unique biology of JCV.

Materials and methods

Cell culture

PHFG cells were propagated according to Padgett *et al* (1977a,b) and maintained in DMEM with penicillin (99 U/ml) and streptomycin (73 U/ml), supplemented with 3 or 10% bovine calf serum (BCS). Cells were maintained at 37°C in a humidified atmosphere containing 10% CO_2.

Plasmid DNAs

All viral constructs were cloned into the unique *EcoRI* site of pBR322. pM1TCR1A contains the JCV (Mad1 strain) genome that was derived from virus passaged in cell culture (Frisque *et al*, 1984); pSV40R1B contains the SV40 (776 strain) genome (Chuke *et al*, 1986). The chimera pM1(SV40) is composed of JCV coding and SV40 regulatory (ori and promoter-enhancer) sequences (Chuke *et al*, 1986). pJCV-T125A contains JCV T Ag coding sequences in which the Thr residue at position 125 has been mutated to an Ala. pJCV-T664A and pJCV-T664S encode JCV T Ags in which the Thr residue at position 664 has been mutated to an Ala and a Ser, respectively. pJCV-E666A and pJCV-E666S contain JCV T Ag coding sequences in which the Glu residue at position 666 has been mutated to an Ala and a Ser, respectively. pJCV-N316K specifies a JCV T Ag in which the Asn residue at position 316 has been mutated to a Lys; pJCV-H317Y specifies a JCV T Ag in which the His residue at position 317 has been mutated to a Tyr. pJCV-NHKY produces a JCV T Ag having a Lys residue at position 316 and the Tyr residue at position 317 in place of an Asn and His, respectively. Replacement of the JCV regulatory sequences with those of SV40 in the constructs pJCV-N316K, pJCV-H317Y and pJCV-NHKY yields pSRJCV-N316K, pSRJCV-H317Y and pSRJCV-NHKY, respectively. Table 1 summarizes the structures of the JCV T Ag mutants.

Mutagenesis

Site-directed *in vitro* mutagenesis was performed according to the method described by Kunkel (1985) as modified in the Muta-Gene Phagemid *In Vitro* Mutagenesis kit (Bio-Rad Laboratories). The following T Ag mutants were generated by altering nucleotides (nts) at the indicated positions: Asn316 to Lys mutant (pJCV-N316K), C to G, nt#3722; His317 to Tyr mutant (pJCV-H317Y), C to T, nt#3721; Asn316 to Lys and His317 to Tyr double mutant (pJCV-NHKY), C to G, nt#3722 and C to T, nt#3721; Thr664 to Ala mutant (pJCV-T664A), A to G, nt#2680; Thr664 to Ser mutant (pJCV-T664S), A to T, nt#2680; Glu666 to Ala mutant (pJCV-E666A), A to C, nt#2673; Glu666 to Ser mutant (pJCV-E666S), A to C, nt#2673 and G to T, nt#2674. The Thr125 mutant (pJCV-T125A) was generated by cassette mutagenesis using a complementary pair of synthetic oligonucleotides spanning nt#4243 to 4311 and containing the desired mutation (A to G, nt#4297).

All recombinant DNAs were subjected to Sanger dideoxy sequencing (Sanger *et al*, 1977) to confirm the expected mutation(s). While conducting this analysis, an error was found in the published sequence of the prototype Mad1 strain of JCV (Frisque *et al*, 1984). The corrected sequence has a C in place of a T at nt#3824 (Genbank Accession #J02226); this change does not alter the T Ag a.a. sequence.

DNA transfection

DNA transfections of PHFG cells were performed using a modified DEAE-dextran procedure (Sompayrac and Danna, 1981). Viral DNA was removed from plasmid vectors, purified and self-ligated prior to transfection. PHFG cells in 60 mm plates were incubated with 1 ml DEAE-dextran solution (DMEM supplemented with 0.20 or 0.25 mg/ml DEAE-dextran; 50 mM Tris, pH 7.5) and viral DNA (0.1 μg) at 37°C for 90 to 120 min. Cells were washed with DMEM and refed with DMEM containing 10% BCS.

Viral DNA replication assay

Viral DNA was isolated at various times post-transfection (day 0, 3, 7, 14, 21 and 28) according to the method of Hirt (1967). Extracts were treated with DNAse-free Proteinase K prior to extraction, ethanol precipitation and suspension in water or 1 × TE (10 mM Tris, pH 7.5; 1 mM EDTA, pH 8.0). Viral DNA was digested with *EcoRI* and *DpnI*; *DpnI* specifically cleaves the fully-methylated input DNA produced in bacterial cells, while *EcoRI* linearizes viral DNA replicated in mammalian cells. Digested samples were separated by electrophoresis on a 0.8% TBE agarose gel. DNA was transferred to a nitrocellulose membrane according to Southern (1975) or to a GeneScreen Plus membrane (NEN Research Products) according to the manufacturer's instructions. The immobilized DNA was detected by an oligo-labeled (Pharmacia) linearized

pM1TCR1A DNA probe and visualized by autoradiography on Kodak X-Omat film. Bands were quantitated on a βetascope 603 Blot Analyzer.

Viral infectivity

Extracts were prepared from transfected PHFG cells at 21 or 28 days post-transfection (Myers *et al*, 1989) and added to fresh cultures of PHFG cells. Low molecular weight DNA was isolated at 0, 7, and 14 days post-infection and analyzed by the *DpnI* assay. Accumulation of replicating DNA in transfected cells, in addition to the presence of replicated DNA in the infected cells, was taken as evidence that infectious virions were produced following the initial transfection.

Acknowledgements

We thank Karen Miller for the construction of JCV T Ag Zn finger mutants. This work was supported by Public Health Service Grant CA44970 from the National Cancer Institute and by the American Cancer Society Grant VM-133.

References

Arthur AK, Höss A, Fanning E (1988). Expression of simian virus 40 T antigen in Escherichia coli: localization of T-antigen origin DNA-binding domain to within 129 amino acids. *J Virol* **62:** 1999–2006.

Arthur RR, Shah KV (1989). Occurrence and significance of papovaviruses BK and JC in the urine. *Prog Med Virol* **36:** 42–61.

Berg JM (1986). Potential metal binding domains in nucleic acid binding proteins. *Science* **232:** 485–487.

Berger J, Concha M (1995). Progressive multifocal leukoencephalopathy: the evolution of a disease once considered rare. *JNV* **1:** 5–18.

Bollag B, Chuke WF, Frisque RJ (1989). Hybrid genomes of the polyomaviruses JC virus, BK virus, and simian virus 40: identification of sequences important for efficient transformation. *J Virol* **63:** 863–872.

Borowiec JA, Dean FB, Bullock PA, Hurwitz J (1990). Binding and unwinding – how T antigen engages the SV40 origin of DNA replication. *Cell* **60:** 181–184.

Cegielska A, Virshup DM (1993). Control of simian virus 40 DNA replication by the HeLa cell nuclear kinase casein kinase-I. *Mol Cell Biol* **13:** 1202–1211.

Cegielska A, Shaffer S, Derua R, Goris J, Virshup DM (1994). Different oligomeric forms of protein phosphatase 2A activate and inhibit simian virus 40 DNA replication. *Mol Cell Biol* **14:** 4616–4623.

Chuke WF, Walker DL, Peitzman LB, Frisque RJ (1986). Construction and characterization of hybrid polyomavirus genomes. *J Virol* **60:** 960–971.

DePamphilis ML, Bradley MK (1986). Replication of SV40 and polyoma virus chromosomes. In: Salzman NP (ed). *The Papovaviridae, The Polyomaviruses.* Vol 1. Plenum Press: New York, pp. 99–246.

Fanning E (1992). Simian virus-40 large T-antigen – The puzzle, the pieces, and the emerging picture. *J Virol* **66:** 1289–1293.

Fanning E, Knippers R (1992). Structure and function of simian virus 40 large tumor antigen. *Annu Rev Biochem* **61:** 55–85.

Feigenbaum L, Khalili K, Major E, Khoury G (1987). Regulation of the host range of human papovavirus JCV. *Proc Natl Acad Sci USA* **84:** 3695–3698.

Frankel A, Pabo C (1988). Fingering too many proteins. *Cell* **53:** 675.

Frisque RJ, White FA (1992). The molecular biology of JC virus, causative agent of progressive multifocal leukoencephalopathy. In: Roos RP (ed). *Molecular Neurovirology.* Humana Press: Totowa, NJ, pp. 25–158.

Frisque RJ, Bream GL, Cannella MT (1984). Human polyomavirus JC virus genome. *J Virol* **51:** 458–469.

Haggerty S, Walker DL, Frisque RJ (1989). JC virus-simian virus 40 genomes containing heterologous regulatory signals and chimeric early regions: identification of regions restricting transformation by JC virus. *J Virol* **63:** 2180–2190.

Hirt B (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol Biol* **26:** 365–369.

Höss A, Moarefi IF, Fanning E, Arthur AK (1990). The finger domain of simian virus 40 large T antigen controls DNA-binding specificity. *J Virol* **64:** 6291–6296.

Kadonaga JT, Carner KR, Masiarz FR, Tjian R (1987). Isolation of a cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51:** 1079–1090.

Kunkel TA (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc Natl Acad Sci USA* **82:** 488–492.

Loeber G, Parsons R, Tegtmeyer P (1989). The zinc finger region of simian virus 40 large T antigen. *J Virol* **63:** 94–100.

Loeber G, Stenger JE, Ray S, Parsons RE, Anderson ME, Tegtmeyer P (1991). The zinc finger region of simian virus-40 large T-antigen is needed for hexamer assembly and origin melting. *J Virol* **65:** 3167–3174.

Lynch KJ, Frisque RJ (1990). Identification of critical elements within the JC virus DNA replication origin. *J Virol* **64:** 5812–5822.

Lynch KJ, Frisque RJ (1991). Factors contributing to the restricted DNA replicating activity of JC virus. *Virology* **180:** 306–317.

Lynch KJ, Haggerty S, Frisque RJ (1994). DNA replication of chimeric JC virus-simian virus 40 genomes. *Virology* **204:** 819–822.

McVey D, Brizuela L, Mohr I, Marshak DR, Gluzman Y, Beach D (1989). Phosphorylation of large tumor antigen by cdc2 stimulates SV40 DNA replication. *Nature* **341:** 503–507.

McVey D, Ray S, Gluzman Y, Berger L, Wildeman AG, Marshak DR, Tegtmeyer P (1993). cdc2 phosphorylation of threonine 124 activates the origin-unwinding functions of simian virus 40 T antigen. *J Virol* **67:** 5206–5215.

Miller J, McLachlan AD, Klug A (1985). Repetitive zinc binding domains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO J* **4:** 1609–1614.

Moarefi IF, Small D, Gilbert I, Hopfner M, Randall SK, Schneider C, Russo AAR, Ramsperger U, Arthur AK, Stahl H, Kelly TJ, Fanning E (1993). Mutation of the cyclin-dependent kinase phosphorylation site in simian virus 40 (SV40) large T-antigen specifically blocks SV40 origin DNA unwinding. *J Virol* **67:** 4992–5002.

Myers C, Frisque RJ, Arthur RR (1989). Direct isolation and characterization of JC virus from urine samples of renal and bone marrow transplant patients. *J Virol* **63:** 4445–4449.

Padgett BL, Rogers CM, Walker DL (1977a). JC virus, a human polyoma virus associated with progressive multifocal leukoencephalopathy: additional biological characteristics and antigenic relationships. *Infect Immun* **15:** 656–662.

Padgett BL, Walker DL, Zu Rhein GM, Varakis JN (1977b). Differential neurooncogenicity of strains of JC virus, a human polyoma virus, in newborn Syrian hamsters. *Cancer Res* **37:** 718–720.

Pipas JM (1992). Common and unique features of T-antigens encoded by the polyomavirus group. *J Virol* **66:** 3979–3985.

Prives C (1990). The replication functions of SV40 T antigen are regulated by phosphorylation. *Cell* **61:** 735–738.

Rose P, Schaffhausen B (1995). Zinc-binding and protein-protein interactions mediated by the polyomavirus large T antigen zinc finger. *J Virol* **69:** 2842–2849.

Sanger F, Nicklen S, Coulson AR (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74:** 5463–5467.

Scheidtmann K, Harding M, Echle B, Walter G (1984) DNA binding activity of simian virus 40 large T antigen correlates with a distinct phosphorylation state. *J Virol* **50:** 1–12.

Scheidtmann KH, Buck M, Schneider J, Kalderon D, Fanning E, Smith AE (1991). Biochemical characterization of phosphorylation site mutants of simian virus 40 large T-antigen: evidence for interaction between amino- and carboxy-terminal domains. *J Virol* **65,** 1479–1490.

Schneider J, Fanning E (1988). Mutations in the phosphorylation sites of simian virus 40 (SV40) T antigen alter its origin DNA-binding specificity for site I or II and affect SV40 DNA replication activity. *J Virol* **62:** 1598–1605.

Sompayrac LM, Danna KJ (1981). Efficient infection of monkey cells with DNA of simian virus 40. *Proc Natl Acad Sci USA* **78:** 7575–7578.

Southern EM (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98:** 503–517.

Stillman B (1994). Smart machines at the replication fork. *Cell* **78:** 725–728.

Swenson JJ, Frisque RJ (1995). Biochemical characterization and localization of JC virus large T antigen phosphorylation domains. *Virology* **212:** 295–308.

Tavis JE, Frisque RJ (1991). Altered DNA binding and replication activities of JC virus T-antigen mutants. *Virology* **183:** 239–250.

Tavis JE, Trowbridge PW, Frisque RJ (1994). Converting the JCV T antigen Rb binding domain to that of SV40 does not alter JCV's limited transforming activity but does eliminate viral viability. *Virology* **199:** 384–392.

Tooze J (1981). Molecular biology of tumor viruses, part 2: DNA tumor viruses. (ed). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Trowbridge PW, Frisque RJ (1993). Analysis of G418-selected Rat2 cells containing prototype, variant, mutant, and chimeric JC-virus and SV40 genomes. *Virology* **196:** 458–474.

Van Roy F, Fransen L, Fiers W (1983). Improved localization of phosphorylation sites in simian virus 40 large T antigen. *J Virol* **45:** 315–331.

Virshup D, Cegielska A, Russo A, Kelly T, Shaffer S (1993). The initiation of SV40 DNA replication is controlled by a phosphorylation-dephosphorylation cycle. *Advances Prot Phos* **7:** 271–293.

Walker DL, Frisque RJ (1986). The biology and molecular biology of JC virus. In: Vinken PJ, Bruyn GW, Klawans HL (eds).. *The Papovaviridae, The Polyomaviruses,* Vol I. Plenum Publishing Corp: New York, pp. 327–377.