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Structure and function of JC virus T′ proteins

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Introduction

The initial sequence analyses of the primate polyomavirus genomes confirmed the identity of 2 proteins, the large and small tumor antigens (TAg and tAg), encoded by the viral early regions. These multifunctional regulatory proteins are translated from transcripts generated by alternative splicing of the viral early mRNA. TAg, and to a lesser extent tAg, mediates viral DNA replication and oncogenic activities. Over a decade after the publication of the simian virus 40 (SV40), JC virus (JCV), and BK virus (BKV) genetic maps, additional early proteins were discovered that are products of transcripts that undergo further processing of each virus' precursor early mRNAs (Figure 1). There are good reasons to predict that these "truncated" versions of TAg contribute to the pathogenic and oncogenic potential of the viruses that produce them. For example, these proteins are conserved in polyomaviruses of mice, monkeys, and humans, expressed in significant amounts in cells infected by JCV and BKV, and encoded by sequences overlapping the amino-terminal functional domains of T/tAgs. This paper focuses on the functional analysis of three of these proteins, T'_{135}, T'_{136}, and T'_{165}, produced by JCV. Also included are a summary of their discovery, structure, and interactions with cellular tumor suppressor and chaperone proteins.

Initial discovery of JCV T′ proteins

The first report of a JCV T′ protein resulted from the analysis of viral proteins in JCV-transformed rodent cells (Bollag *et al*, 1989; Haggerty *et al*, 1989). Upon SDS-polyacrylamide gel electrophoresis of proteins immunoprecipitated with anti-TAg antibodies, a 17-kD band was detected in addition to bands representing the large and small TAgs. Initially, this single band was thought to represent a proteolytic cleavage product of TAg and was called T′; however, pulse-chase experiments did not support this suggestion (Trowbridge and Frisque, 1995). Furthermore, similar experiments performed in JCV-infected primary human fetal glial (PHFG) cells revealed the presence of 3 or 4 T′-like proteins ranging in size from 16–23 kD. All of these T′ proteins were recognized by monoclonal antibodies directed against the amino terminus of TAg; the two largest T′ species were also recognized by a monoclonal antibody that bound a carboxy-terminal TAg epitope.

Given the preceding information and the knowledge that TAg and tAg were produced from alternatively spliced transcripts, we predicted that T′ proteins were also generated by posttranscriptional processing of the early mRNA. Reverse transcription polymerase chain reaction (RT-PCR) confirmed this prediction; 1 T′ transcript (T'_{136}) was amplified from JCV-transformed rat fibroblasts and 3 T′ transcripts (T'_{135}, T'_{136}, and T'_{165}) were detected in JCV-infected human glial cells. Each transcript is generated by the removal of 2 introns from the immature early mRNA. One of these introns is the same as that removed from the TAg mRNA. The second splicing reaction utilizes a new 5′-donor splice site shared by all three T′ transcripts that is joined to a different 3′-acceptor splice site for each message. These transcripts encode proteins of 135, 136, and 165 amino acids, with the first 132 residues overlapping those of TAg (Figures 1 and 2). The carboxy terminus of T'_{165} is also shared with that of TAg, whereas the carboxy-termini of T'_{135} and T'_{136} are translated in a different reading frame and therefore are unique.

Once the origin of the T′ proteins was determined, a question arose concerning the detection of 4 T′ bands on SDS-polyacrylamide gels but only 3 T′ transcripts in RT-PCR experiments; had a fourth T′ mRNA been missed or did one of the gel bands represent a modified form of a T′ protein? Treatment of lysates of infected human cell with lambda protein phosphatase indicated that the slowest migrating T′ band was a

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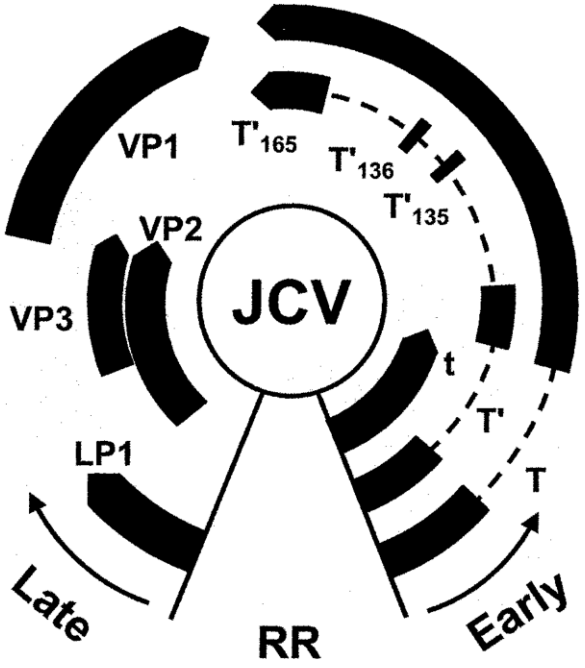

Figure 1 The JCV genome. The circular, double-stranded genomes of most JCV isolates are approximately 5100 base pairs in length, and each is divided into an early and late coding region plus a regulatory region (RR) that contains the origin of viral DNA replication and the promoter and enhancer elements for transcription. The early region was originally proposed to encode 2 regulatory proteins, TAg and tAg, translated from alternatively spliced mRNAs (Frisque *et al*, 1984). Later work (Trowbridge and Frisque, 1995) identified 3 additional early proteins, T'_{135}, T'_{136}, or T'_{165}, translated from mRNAs that have had a second intron excised (denoted by dashed lines). The 3 T′ proteins share their amino-terminal 81 amino acids with tAg and 132 amino acids with TAg (coding sequences represented by shaded arcs). The T′ proteins have different carboxy-termini of either 3 (T'_{135}), 4 (T'_{136}), or 33 (T'_{165}) amino acids (coding sequences for all 3 T′ proteins are denoted by the same arc). The late region encodes at least 3 capsid proteins, VP1, VP2, and VP3, and a fourth protein, LP1.

hyperphosphorylated form of T'_{165} (Prins and Frisque, 2001). Interestingly, examination of lines of rat fibroblasts producing only T'_{165} revealed that both forms of the protein were present in proliferating cells, but only the hypophosphorylated form was detectable in quiescent cells (Bollag, Kilpatrick, and Frisque, unpublished data). Unlike T'_{135} and T'_{136}, T'_{165} has an additional potential phosphorylation site in its carboxy-terminus that might yield the hyperphosphorylated form (Figure 2).

Our early studies raised a second question that was related to the detection of 3 T′ proteins in productively infected human glial cells but only a single T′ protein in transformed rat cells. Was differential T′ expression a consequence of the species or tissue origin of the cell or of a difference in transformed versus lytically infected cells? Upon RT-PCR analysis of a collection of JCV transformed and tumor cells, including glial cells and fibroblasts derived from human, hamster, rat, and mouse tissues, we detected 1 prominent transcript encoding T'_{136}. On the other hand, infected PHFG cells were again found to produce approximately equal amounts of all 3 T′ transcripts, indicating that alterations in splicing resulted from differences between a JCV-transformed and JCV-infected cell (Jones and Frisque, unpublished data). These experiments also revealed for the first time that low levels of T'_{135} and T'_{165} mRNAs were generated in all of the transformed lines, in contrast to our original study that only detected the T'_{136} transcript (Trowbridge and Frisque, 1995).

T′ protein structure suggests roles in viral transformation and replication functions

The shared amino-terminal 132 amino acids of TAg, T'_{135}, T'_{136}, and T'_{165} contain important functional domains that influence the ability of JCV to replicate its genome and to exhibit oncogenic activity (Figure 2). By employing a genetic approach that involved mutating the common T′ donor splice site or the three unique T′ acceptor sites, the T′ proteins have been shown to make significant contributions to JCV-DNA replication (Trowbridge and Frisque, 1995; Prins and Frisque, 2001). Additional evidence supporting a biological role for T′ proteins was provided in early studies that suggested high levels of T'_{136} expression in a Rat2 cell line correlated with a more transformed phenotype (Trowbridge and Frisque, 1993). The TAgs of primate polyomaviruses contain at least 3 domains that influence oncogenic potential, an amino-terminal J domain, the LXCXE domain, and the p53 binding domain (Figure 2). The latter sequences are lost during removal of the second intron from the T′ mRNAs; however, the J and LXCXE domains are preserved in the JCV T′ proteins. These 2 domains have been hypothesized to collaborate to induce S-phase progression of infected cells and thereby promote viral DNA replication in permissive human cells or unregulated proliferation in nonpermissive rodent cells (reviewed in Brodsky and Pipas, 1998). A model proposed by Sheng and coworkers (1997) and supported by more recent studies (Sullivan *et al*, 2000a, 2001) predicts that TAg, through its LXCXE motif, binds to underphosphorylated forms of the pRB family of tumor suppressor proteins (pRB, p107, p130). These latter proteins may repress cellular transcription via their interaction with the E2F-DP family of transcription factors, and in doing so prevent exit from the G_0/G_1 phase of the cell cycle. Once bound to the complex, TAg recruits Hsc70, a member of the DnaK family of molecular chaperones, and activates its intrinsic ATPase activity. By inducing a conformational change in the complex, Hsc70 is thought to effect the release of E2F-DP from pRB, p107, or p130, leading to the activation of genes involved in S-phase progression. It has been directly demonstrated that JCV TAg binds pRB family members (Dyson *et al*, 1989, 1990; Tavis *et al*, 1994; Howard *et al*, 1998); interaction with Hsc70 has

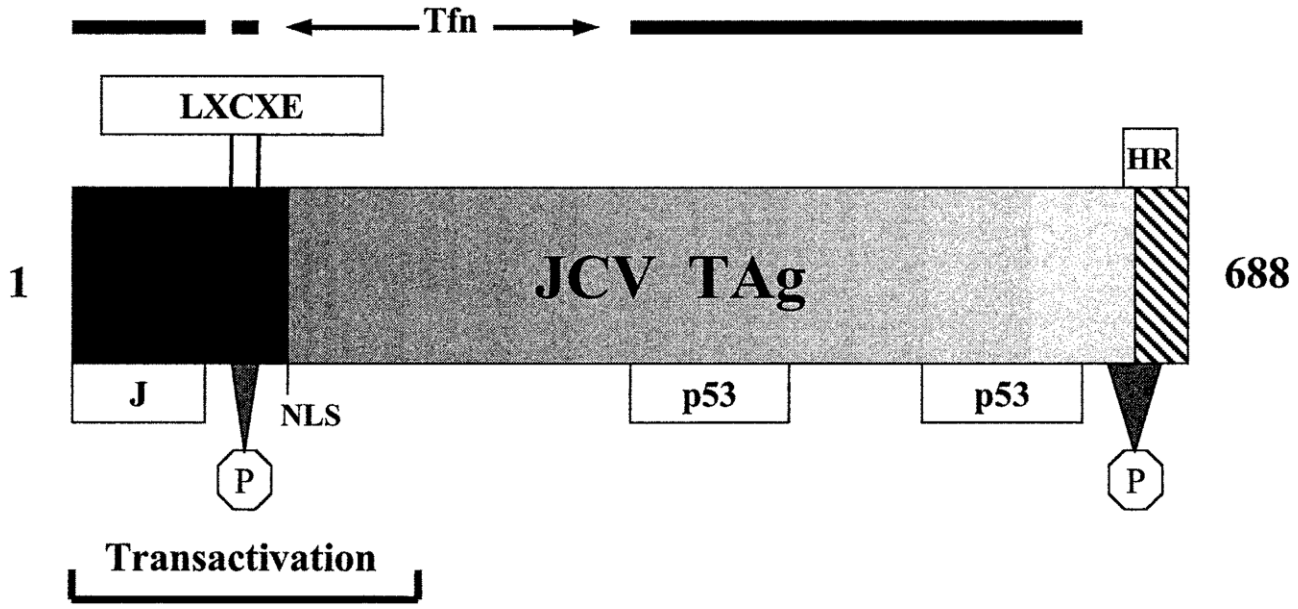

Figure 2 The multifunctional JCV TAg. The amino-terminal 132 amino acids of the 3 T′ proteins are shared with those of the 688 amino acid JCV-TAg (dark shading). The carboxy-terminal 33 amino acids of T'_{165} overlap with the carboxy terminus of TAg (diagonal lines), whereas the carboxy termini of T'_{135} and T'_{136} are specified by sequences in a different reading frame relative to that used for TAg and T'_{165}. Three regions of TAg, the J, LXCXE, and p53-binding domains, contribute to viral transformation (Tfn); the T′ proteins include the first 2. The J domain includes sequences that interact with the molecular chaperone Hsc70, the cellular DNA polymerase α, and the transcription factor Tst-1 found in myelin-producing cells. The LXCXE domain binds members of the pRB family of tumor suppressors (pRB, p107, and p130). All three T′ proteins include a group of serine and threonine phosphorylation sites (P) that are present in the amino terminus of TAg; T'_{165} also contains a subset of the carboxy-terminal TAg phosphorylation sites. In addition, T′ proteins contain a nuclear localization signal (NLS) and a host range (HR) domain (T'_{165}); they may also possess transactivation potential.

only been shown using a chimeric JCV-SV40 TAg (Sullivan *et al*, 2000b).

Interactions between JCV early proteins and the cellular proteins Hsc70, pRB, p107, and p130

Prior to investigating potential interactions between viral and cellular proteins, JCV TAg and the 3 T′ proteins were produced in a baculovirus expression system and purified by immunoaffinity chromatography (Bollag *et al*, 1996, 2000). Attempts to demonstrate binding of these viral proteins to purified Hsc70 by mixing the proteins and performing co-immunoprecipitation experiments failed. However, such an interaction could be demonstrated *in vivo* using POJ cells as a source of all 5 JCV early proteins and Hsc70 (Bollag and Frisque, unpublished data). Results from the co-immunoprecipitation assay indicated that at least 1 of the early viral proteins bound to the chaperone protein. Lysates from newly created cell lines expressing individual JCV early proteins (Kilpatrick and Frisque, unpublished results) are now being examined to define individual viral proteins with binding activity. Although each T′ protein contains the J domain, recent work with a truncated SV40-TAg (N136) indicated that sequences in addition to the J domain may be required to establish a stable association with Hsc70 (Sullivan *et al*, 2001).

JCV TAg, like its SV40 counterpart, has been shown to bind the pRB family of proteins and to influence their phosphorylation status and stability (Dyson *et al*, 1989, 1990; Tavis *et al*, 1994; Howard *et al*, 1998). The LXCXE domain is present in the carboxy-terminal region of the JCV T′ proteins, and one would predict that binding to pRB, p107, and p130 should occur. However, these "truncated" versions of TAg are structurally distinct from the full-length T protein, so it was not clear how differences in folding and phosphorylation status would influence interactions with these cell-cycle regulators. Purified TAg, T'_{135}, T'_{136}, and T'_{165} were incubated with extracts of MOLT-4 cells, a human T-cell line containing pRB, p107, and p130 (Bollag *et al*, 2000). Complexes were then co-immunoprecipitated with antibodies directed against either the viral proteins or the individual pRB family members and electrophoresed on SDS-polyacrylamide gels. Immunoblotting was performed with the appropriate anti-pRB, -p107, or -p130 antibodies. The 4 viral proteins preferentially bound hypophosphorylated species of the cellular proteins and exhibited highest binding affinity to p107 and lowest affinity to pRB. Importantly, these data indicated that the T′ proteins exhibit unique binding properties and are not simply defective or less active forms of TAg. For example, TAg and T'_{165} bound more pRB and less p107 than did T'_{135} and

T'_{136}; T'_{165} also bound less p130 than the other three early proteins.

After establishing that purified JCV early proteins bound the pRB family of tumor suppressors expressed in MOLT-4 cells, these interactions were tested *in vivo* in cells co-expressing the viral and cellular proteins. Recently, we generated Rat2 cell lines that stably express individual JCV T′ proteins (Kilpatrick and Frisque, unpublished data). Using extracts from cells producing T'_{135}, interactions between this protein and the hypophosphorylated forms of pRB, p107, and p130 were again demonstrated (Bollag, Kilpatrick, and Frisque, unpublished data). Furthermore, experiments were performed to compare the levels and phosphorylation status of pRB, p107, or p130 in the parental Rat2 line versus the Rat2 line expressing T'_{135}. Reduced levels of hyperphosphorylated p107 and p130 were observed in this latter cell line relative to that seen in the parental cells. In addition, the overall levels of p130 appeared to be lower in the T'_{135}-expressing line. This latter finding was also observed when the status of p130 was compared in transformed POJ cells and the parental PHFG cells; levels of p130 were reduced and modified forms of p130 were absent in the cells expressing early JCV proteins. Taken together, these data suggest that JCV T′ proteins, like SV40 and JCV TAgs, inhibit phosphorylation of p107 and p130 and promote p130 degradation (Stubdal *et al*, 1997; Howard *et al*, 1998).

Alternative splicing of the BK virus (BKV) early mRNA generates multiple viral proteins

Previously, we demonstrated that BKV, like JCV, produces significant amounts of a T′-like protein(s) in transformed cells (Bollag *et al*, 1989). Recently we conducted RT-PCR analysis of RNA extracted from BKV-transformed rat and infected human lung cells and discovered that one prominent BKV cDNA band was amplified from the rat transformants and three bands were amplified from the human cells. The viral cDNA from rat cells was cloned and sequenced and found to represent an alternatively spliced BKV transcript encoding a 136-amino acid protein that we call miniT$_{136}$ (Prins and Frisque, unpublished data). As in the case of JCV, this BKV transcript appears to be one of the three miniT mRNAs found in the infected cells; their identities are now being confirmed. As with JCV, we expect that the BKV miniT transcripts will utilize a common 5′ donor splice site and unique 3′ acceptor splice sites, and that the BKV miniT proteins will exhibit a subset of TAg functions important to viral replication and oncogenic behavior.

Biological consequences of observed JCV T′ activities

We propose that the JCV T′ and BKV miniT proteins influence important biological activities of these viruses through their ability to complement, and in some instances, to antagonize TAg-mediated functions. We have shown that each JCV T′ protein interacts with members of the pRB tumor suppressor family of proteins and is likely to contribute to TAg's ability to disrupt cell-cycle regulation. The ability to stimulate entry into S-phase would, in turn, influence viral replication or transforming efficiency, depending on the cell type infected. In addition, the ability of the 3 T′ proteins to differentially bind pRB, p107, and p130 and affect their expression not only suggests that overlapping and unique functions of these 3 cell cycle regulators might be altered, but that the activities of other cellular factors involved in cell-cycle progression and in cellular and viral DNA replication (e.g., DNA polymerase α) and transcription (e.g., Tst-1) might also be influenced differently by each T′ protein. Given that these T-related proteins differ in their activities and in their abundance in different cell types, we suggest that the T′ proteins not only contribute to the lytic and transforming behavior of JCV *in vitro*, but that they have the potential to influence the tissue tropism and the pathogenic and oncogenic potential of this virus *in vivo*.

Finally, it should be noted that T′ proteins might block certain effects that TAg imposes on cells. We have successfully established cell lines that express individual JCV early proteins by transfecting Rat2 cells with cDNA constructs encoding these proteins and by then employing a G418 selection scheme. Although we have readily isolated lines expressing T'_{135}, T'_{136}, or T'_{165} as well as lines expressing TAg plus one or more of the T′ proteins, we have been unable to obtain a line that produces only TAg. One interpretation of this result is that TAg induces apoptosis of these rat fibroblasts and that T′ proteins prevent this cellular response. SV40 TAg has been reported to be capable of either inducing or preventing apoptosis depending on the cell type examined. In the case of rat embryo fibroblasts, SV40 TAg appears to induce apoptosis via a mechanism that requires a functional LXCXE domain (Kolzau *et al*, 1999). Further, tAg counteracts this effect by a mechanism that differs from that of known anti-apoptotic proteins. Such an activity might be relevant to the observation that tAg contributes to SV40-induced transformation. These findings suggest that the JCV T′ and BKV miniT proteins might play a similar role in modulating TAg functions, and it will be important to determine how T′ activities influence lytic and transforming behavior of these human opportunistic pathogens.

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