Identification of three new JC virus proteins generated by alternative splicing of the early viral mRNA

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The genome of the human polyomavirus JC Virus (JCV) encodes two regulatory proteins, large and small T antigen which are expressed early in a lytic infection, and three structural proteins, VP1, VP2, VP3, which are produced late in an infection. A fourth late protein, agnoprotein, may contribute to the assembly of the virion. In this study, we demonstrate the presence of three additional early proteins, T'\textsuperscript{139}, T'\textsuperscript{145}, and T'\textsuperscript{161}, which are expressed in lytically-infected cells; T'\textsuperscript{156} is also readily detected in JCV transformants. The three species of T' are phosphoproteins generated via an alternative splicing mechanism. This mechanism involves the excision of a second intron from the large T mRNA using a common donor splice site at JCV nucleotide 4274 and three unique acceptor splice sites at nucleotides 2918, 2777 and 2704 for T'\textsuperscript{139}, T'\textsuperscript{145} and T'\textsuperscript{161}, respectively. The mutant JCV\textsuperscript{T'} was created by converting the G at nucleotide 4274 to an A, thereby disrupting the consensus sequence of the shared donor splice site without altering the amino acid sequence of any early JCV protein. Upon transfection of permissive human brain cells, JCV\textsuperscript{T'} replicated its DNA 10-fold less efficiently than did wild type JCV. Passage of extracts of the infected cells on to fresh human brain cells revealed that the expression of T antigen was greatly reduced and the presence of T' proteins was undetectable in the mutant versus wild type JCV-infected cells.

Keywords: T antigen; T' protein; alternative splicing; DNA replication; viral persistence

Introduction

The importance of improving our understanding of the human virus JCV is underscored by three observations: (1) JCV establishes persistent infections in nearly 90% of the human population, (2) in severely immunocompromised individuals JCV is an opportunistic pathogen which causes progressive multifocal leukoencephalopathy (PML), a fatal demyelinating brain disease that afflicts a significant number of AIDS patients, and (3) evidence indicates that JCV should be considered a potential human tumor virus (reviewed in Frisque and White III, 1992; Major et al, 1992).

JCV belongs to the primate polyomavirus group which includes human BK virus (BKV) and simian virus 40 (SV40), the group's most thoroughly characterized member. The three viruses are closely related genetically, although biologically they exhibit a number of distinct properties (Frisque et al, 1984). JCV, in particular, is severely limited in its lytic and transforming activities in vitro, and a number of studies have implicated both its tissue-specific promoter-enhancer sequences and its major regulatory protein, T antigen (TAg), as contributors to this restricted behavior (Chuke et al, 1986; Feigenbaum et al, 1987; Bollag et al, 1989; Haggerty et al, 1989). The primate polyomavirus TAgS are required for the initiation and maintenance of the transformed state in mammalian cells and are essential for viral DNA replication and productive viral infection (reviewed in Fanning and Knippers, 1992). The JCV protein has not been subjected to the same degree of genetic and biochemical analysis as has the SV40 TAg for which numerous functional domains have been identified (Fanning and...

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Knippers, 1992; Pipas, 1992). SV40 studies indicate that several transformation and replication functions map to the N-terminal region of TAg which includes sequences that (1) specify nuclear localization, (2) contain a cluster of regulatory phosphorylation sites, (3) affect cellular and viral transactivation, and (4) bind the viral replication origin, the cellular DNA polymerase α and cellular tumor suppressor proteins such as pRB and possibly p300 (Privés, 1990; Yačík et al., 1991; Fanning and Knippers, 1992). Based upon sequence comparisons and recent mutational analyses, similar functional domains have been localized within the JCV TAg, although the JCV protein appears to be functionally less robust than its SV40 counterpart (Frisque et al., 1984; Tavis and Frisque, 1991; Tavis et al., 1994).

Cellular transformation induced by the primate polyomaviruses requires expression of TAg, and under some conditions TAg, which can be detected by a variety of immunological techniques. In addition, JCV and BKV transformants, but not SV40 transformants, frequently express an abundant 17 kD polypeptide, T' (Bollag et al., 1989; Haggerty et al., 1989; Trowbridge and Frisque, 1993). Because JCV TAg is less stable than its SV40 counterpart (Lynch and Frisque, 1991), T' was initially thought to be a degradation product of TAg (Bollag et al., 1989; Haggerty et al., 1989). If true, proteolytic cleavage could reflect an intracellular event or an artifact of the lysis procedure used to prepare cellular extracts for analysis. It is known that proteolytic treatment of the SV40 TAg in vitro yields a stable, N-terminal 17 kD peptide (Schwyzer et al., 1980). If T' is not generated via proteolytic cleavage, what potential mechanisms could operate to produce this 17 kD viral protein? Possibilities might include altered translational initiation and/or alternative splicing of the early viral mRNA. An altered translational initiation event would require ribosomal recognition of an internal AUG in the JCV early transcript, a process similar to that which occurs during synthesis of the VP3 capsid protein (Buchman et al., 1981). One would predict that a 17 kD polypeptide would contain approximately 155 amino acids; an internal methionine does exist in the TAg sequence at amino acid position 529 (159 amino acids from the C-terminus). It is also possible that T' production results from an alternative splicing event that yields an in-frame termination codon or excises a large second intron from the early message. Aberrant splicing at the 5' splice site of the SV40 TAg mRNA (Huang and Gorman, 1990) and alternative splicing of the SV40 TAg mRNA (Sompayrac and Danna, 1985; Zerrahn et al., 1993) have been demonstrated; in the latter case, production of a 17 kD SV40 T-related protein called 17kT has been observed. In this communication we reopened the question regarding the origin of the JCV T' peptide, especially since T' might encode functional domains that overlap those of TAg, and thereby influence viral transforming and lytic behavior.

Results

The T' protein detected in JCV-transformed Rat2 cells is a phosphoprotein that is also expressed in a lytic infection

Immunoprecipitation of viral proteins from extracts of Rat2 cells transformed by JCV has revealed the presence of a 17 kD polypeptide, called T', in addition to the large and small TAgS expressed by all

![Figure 1](image_url) Immunoprecipitation of viral proteins from lytically-infected PHFG cells. PHFG cells were infected with JCV, and at days 0, 7, 14 and 21 p.i. were labeled for 16 h with 25 μCi ml-1 [35S]methionine (a) or for 7 h with 1 mCi ml-1 [32P]orthophosphate (b). After cell lysis, viral and cellular proteins were incubated without antibody, with polyclonal or monoclonal (PAb416) anti-T antibodies, or with anti-VP1 antibodies. PAb416 recognizes an epitope within the second exon of large T and is therefore, unable to bind small t. PAb416 was found to bind T' more efficiently than our polyclonal antibodies. Immunoprecipitated complexes were electrophoresed through 14% SDS-polyacrylamide gels and subjected to fluorography for 7 days (a) or autoradiography for 12 h (b). Rainbow markers (Amersham) and prestained molecular weight markers (Sigma) were included on each gel to permit size determinations of the viral proteins. Positions of large TAg (T, 94kD), small TAg (t, 21kD), T' (22-23kD doublet and 17kD) and VP1 (42kD) are indicated. As reported previously (Major and Traub, 1986), cellular p35 was not detected in complex with JCV TAg in lytically-infected PHFG cells.
primate polyomaviruses (Bollag et al., 1989; Haggerty et al., 1989; Trowbridge and Frisque, 1993). To determine whether T' is also produced in lytically-infected cells, PHFG cells were infected with JCV for 0, 7, 14 or 21 days and then metabolically-labeled with either 35S-methionine or 32P-orthophosphate. Viral proteins in extracts of these cells were immunoprecipitated with monoclonal (PAB416) or polyclonal anti-T antibodies or with polyclonal anti-VP1 antibodies, separated by SDS-PAGE, and visualized by fluorography (Figure 1a) or autoradiography (Figure 1b). Both the detection of VP1 capsid protein and the increasing levels of early and late proteins over the course of the infection indicated that a productive infection had been established in these cells (Figure 1a). 35S-labeled protein bands representing large T, small t, T', and VP1, were identified on day 7, 14, and 21 post-infection (p.i.) samples, thereby demonstrating for the first time the presence of small t and T' proteins in JCV-infected PHFG cells. In addition to the 17 kD T' protein, two other T' species (22–23 kD) were detected that had not previously been observed in JCV transformed rat cells (Bollag et al., 1989; Haggerty et al., 1989; Trowbridge and Frisque, 1993).

The TAg and all T' species, but not tAg, were shown to be metabolically-labeled with 32P and thus are phosphoproteins (Figure 1b). Phosphorylation of the T' proteins appeared to reach a peak at day 14 p.i. (Figure 1b), although the level of expression of all T' species continued to increase until day 21 p.i. (Figure 1a).

T' shares N-terminal sequences with JCV TAg

Immunoprecipitation of T' with anti-T antibodies indicated either that T' is a T-related protein or that it complexes with TAg and is co-precipitated by the antibodies. The former possibility was demonstrated by detecting similarities in the V8 protease digestion patterns of 35S- and 32P-labeled TAg and 17 kD T' and by the ability of anti-T antibodies to recognize T' via Western blot analysis (data not shown). To more precisely localize the sequences shared by T antigen and 17 kD T', 35S-labeled proteins in Neo-ER, M1(SV40)-RR and SV40-JR cell extracts were immunoprecipitated with several anti-T monoclonal antibodies, separated by gel electrophoresis and visualized by fluorography (Figure 2). These antibodies included PAB2000 which binds an epitope within amino acids 1–81 (Bollag and Frisque, 1992), PAB416 which recognizes an epitope within amino acids 82–131, and therefore does not detect small t (Arthur et al., 1988), PAB1614 which recognizes an N-terminal sequence (Ball et al., 1984), and PAB901 which is directed against the C-terminal 24 amino acids (Deckhut et al., 1991). As previously shown, PAB2000 was specific for the JCV TAg, whereas the other monoclonal antibodies cross-reacted with both the SV40 and the JCV T proteins (Figure 2; Bollag and Frisque, 1992). PAB416 and PAB1614, but not PAB901 or PAB2000, recognized the JCV 17 kD T' species expressed in M1 (SV40)-RR cells, indicating that this polypeptide shares sequences within the N-terminal region of the T protein. PAB2000 recognizes only a subset of the JCV TAg species in a cell, and it is sensitive to the conformation of the protein (Bollag and Frisque, 1992), possibly explaining why it failed to precipitate any detectable 17 kD T' in these experiments.

Comparison of the relative half-lives of JCV TAg and T'

Previous studies suggested that T' was a degradation product of TAg. If true, one might ask whether proteolysis occurred within the cells or during preparation of the cell extracts. To determine if protein degradation occurred during the extraction procedure, cell lysis conditions were varied to enhance potential protease activity. Protease, phosphatase, and kinase inhibitors (Yaciuk and Shalloway, 1986) were routinely included in our 32P-labeled protein extracts (see materials and methods). Omission of these inhibitors did not lead to the accumulation of T' with a concomitant loss of TAg (data not shown), suggesting that if JCV TAg is degraded to T', this event occurs within the cells. To pursue this second possibility, pulse-labeling experiments were con-
ducted with M1(SV40)-RR cells, in which a brief pulse label with $^{35}$S-methionine was followed by a cold chase of increasing duration. Even after very short pulse-labeling times (5 and 15 min), 17 kD T' was detected in the extracts, indicating that it is rapidly produced within the cells (Figure 3a). In addition, the band intensities for both TAg and T' decreased at approximately the same rate over time (Figure 3a). These results did not support the hypothesis that T' represents a TAg degradation product. A time-course experiment was also done using $^{32}$P-orthophosphate to determine the relative phosphate turnover rates of T and T' in M1(SV40)-RR transformed cells. Initially, 17 kD T', which contains the N-terminal phosphorylation sites, loses phosphates more rapidly than TAg, which has both the N- and C-terminal phosphorylation domains. After 3 h, however, the phosphate turnover rates were equivalent for the two proteins (Figure 3b).

![Figure 3](image-url)  
**Figure 3** Pulse-chase analysis of TAg and T'. (a) $^{35}$S-Met labeling: M1(SV40)-RR cells were starved in methionine-free medium containing 5% dialyzed FBS and then pulse-labeled with $^{35}$S-methionine (600 $\mu$Ci ml$^{-1}$ for 5 and 15 min samples; 250 $\mu$Ci ml$^{-1}$ for 60 min samples). (b) $^{32}$P labeling: M1(SV40)-RR cells were starved in phosphate-free medium containing 10% dialyzed FBS and then pulse-labeled for 60 min with 2 mCi ml$^{-1}$ $^{32}$P-orthophosphate. All plates were rinsed with DMEM and incubated in DMEM containing 5% FBS for the appropriate time. After cell lysis, viral proteins were immunoprecipitated with PAB416, electrophoresed on 12.5% SDS-polyacrylamide gels and subjected to fluorography for 7 days (a) or autoradiography for 3 days (b). Signals were quantitated using a Betascope 603 blot analyzer (Betagen).

These results suggest that T' is recognized by at least one cellular phosphatase that also dephosphorylates JCV TAg.

**T' is produced via alternative splicing of the JCV early mRNA**

Based upon the immunoprecipitation and pulse-chase experiments, we concluded that 17 kD T' is a phosphoprotein that shares N-terminal sequences with JCV TAg. Because T' does not appear to be a proteolytic fragment of TAg, some mechanism must operate to introduce a termination codon into the early mRNA such that a 17 kD protein could be translated. Recently Zerrahn et al (1993) reported that a 17 kD protein detected in SV40 transformants was produced by alternative splicing of the early mRNA. Utilization of a second splice donor/acceptor site (SV40 nt 4425/3679) within the early viral transcript resulted in a protein that shared the first 131 amino acids with TAg and terminated with a unique set of four amino acids encoded after the second intron in an alternate reading frame. Sequence comparisons of the SV40 donor and acceptor splice sites within the corresponding regions in JCV revealed 100% and 20% sequence identities, respectively. Using the consensus (Mount, 1982; Figure 4a) and pertinent known splicing sequences (Buchman et al, 1981; Frisque et al, 1984; Zerrahn et al, 1993; Figure 4b), we searched the JCV early region to identify potential
donor and acceptor sites. As a result, one donor and six acceptor splice sites were located which, if used, would generate transcripts encoding proteins ranging in size from 135–165 amino acids (Figure 4c). To determine whether such messages were produced in transformed or lytically-infected cells, RT-PCR was performed on RNA extracted from JCV transformed [M1(SV40)-RR, M11-CR, POJ-19] or infected (PHFG) cells. The results of this analysis are shown in Figure 5. In Rat2 cells transformed by the TAg of the Mad1 [M1(SV40)-RR cell line] or the Mad11 (M11-CR cell line) strains of JCV, one prominent (293 bp) and three minor (1789, 220, 148 bp) reaction products were amplified. In the transformed (POJ-19) cells and lytically-infected PHFG cells, five RT-PCR products (1789, 434, 293, 220, 148 bp) were observed.

To identify the JCV mRNAs represented by the RT-PCR products, the reaction products were first cloned into a TA Cloning™ vector (Invitrogen). Plasmid DNAs isolated from selected bacterial colonies were screened by digestion with EcoRI, which cleaves within the vector sequences positioned 10 nucleotides (nt) to either side of the insert. Digestion of a representative set of these DNAs yielded four different inserts that matched, in size, the four smaller RT-PCR products amplified from the infected glial cells (Figure 6); the largest product (1789 bp) was cloned in subsequent experiments. Once multiple clones representing each species present in the RT-PCR reaction mixtures had been obtained, the inserts were sequenced. The 148 bp RT-PCR product was shown by this analysis to be an artifact that arose when T'primer-1 annealed improperly to a sequence at nt 4243. The three other small RT-PCR product inserts (434, 293, 220 bp) were found to represent mRNAs that were spliced using the predicted donor site at nt 4274 and acceptor sites at nt 2918, 2777 or 2704, respectively (Figure 4c). The donor site, but none of the acceptor sites, is equivalent to those used to generate the SV40 17kT mRNA. The insert representing the largest RT-PCR product (1789 bp) proved to be the cDNA derived from the shared, unspliced region of the large T and small t transcripts.

The results from the RT-PCR analysis are illustrated schematically in Figure 7. The early JCV mRNA is differentially spliced one time to yield the large T and small t transcripts. The three T' transcripts would be produced by the excision of a second intron from the large T transcript. Upon translation, the five mRNAs would encode the JCV large T and small t proteins, and three newly-defined T' proteins (T'_165, T'_136 and T'_135) of 165, 136, and 135 amino acids. The first 132 amino acids of all three
transfect PHFG cells, and at various times post-transfection (p.t.), low molecular weight DNAs were extracted from the cells and DNA replication activity was measured by the DpnI assay (Peden et al., 1980; Figure 8). At day 14 p.t., JCVΔT' DNA replication activity was 10-fold lower than that of wild type JCV. To confirm that infectious virus was produced in the JCVΔT'-transfected cultures, cell lysates from day 21 p.t. were used to infect fresh PHFG cells. Mutant and wild type JCV-infected cells were metabolically-labeled with 35S-methionine at day 21 p.t., and viral proteins were immunoprecipitated with anti-T and anti-VP1 antibodies and analyzed by SDS-PAGE (Figure 9). The results indicated that JCVΔT' was viable, albeit less active than wild type JCV. In addition, this experiment allowed us to confirm that the donor splice site mutation did indeed prevent the expression of the T' proteins. Unexpectedly, we found that TAg expression was greatly reduced in the mutant-versus wild type-infected cells when examined with polyclonal and monoclonal anti-T antibodies. On the other hand, TAg levels were nearly equivalent in cells infected with the two viruses. It should also be noted that the monoclonal antibody PAb901, which recognizes an epitope in the C-terminus of TAg, only immunoprecipitated the larger molecular weight T' species produced during the wild type infection; the 17 kD species was not detected with this antibody.

To confirm that T' transcripts were absent in the JCVΔT'-infected cells, RNA was extracted from the day 21 p.t. PHFG cells and subjected to RT-PCR analysis. The mutant-infected cells did not yield bands representing cDNAs for T'136, T'136, nor T'165 (Figure 10), although the T/t cDNA band was detected and was of approximately the same intensity. The two small bands (148, 263 bp) that do

\[ \text{Figure 7} \] Schematic diagram illustrating transcription and translation products encoded by the JCV early region. The top line represents immature JCV early mRNA, which is processed to yield the T, t and T' mRNAs. Solid lines depict the exons; dotted lines represent the introns. Nucleotide numbers below the lines indicate the exon–intron boundaries for the five processed mRNAs. Proteins translated from these mRNAs are diagramed in the lower portion of the figure. Solid boxes represent the reading frame from which the Tag and N-terminal regions of the Tag and T' species are read. Hatched boxes represent sequences in a second reading frame which specify the second exon of Tag and portions of all 3 T' proteins. Boxes with horizontal lines denote a third open reading frame which encodes the C-termini of T'136 and T'135.

\[ \text{Figure 8} \] DNA replication activity of JCVΔT'. PHFG cells were transfected with JCVΔT' and wild type JCV DNA, and low molecular weight DNAs were extracted at the indicated times. Viral DNAs were subjected to digestion with DpnI and EcoRI, separated on a 0.8% agarose gel, transferred to GeneScreen Plus and probed with 32P-labeled JCV DNA. CT represents calf thymus DNA which was transfected into PHFG cells in parallel as a negative control. Dried membranes were subjected to autoradiography, and relative viral DNA replication activity was determined from counts obtained on a Betascope 603 blot analyzer (Betagen).
appear in the JCVΔT' lane have been cloned and sequenced. Both bands represent artifacts of the PCR amplification; the 148 bp band was identical to that described above, while the 263 bp band arose because of partial complementarity of T' primer#1 to sequences surrounding JCV nt 4128.

Discussion
The relatively abundant JCV 17 kD T' protein was first described in virally transformed rat and hamster fibroblasts; the corresponding protein was also detected readily in BKV transformants (Bollag et al., 1989; Haggerty et al., 1989; Trowbridge and Frisque, 1993; Zorrah et al., 1993). Although T' was initially considered to be a degradation product of JCV TAg, the present study demonstrates that at least three T' species are produced during a productive JCV infection via an alternative splicing mechanism. Based upon genetic and biochemical analyses presented here, we conclude that T' contributes significantly to the in vitro and in vivo activity of this persistent virus.

RT-PCR analysis revealed the presence of three differentially-spliced early mRNAs in infected and transformed PHFG cells that use a shared donor site and three different acceptor sites. These splicing signals were first predicted on the basis of their identity with consensus sequences. The three mRNAs are expected to give rise to proteins containing 135 (T'135), 136 (T'136) or 165 (T'165) amino acid residues. Based upon this information and the immunoprecipitation data, we predict that the T'135 and T'136 species represent the single 17 kD band seen on our gels. In support of this prediction, we recently resolved this band into two closely migrating species on 18% SDS-polyacrylamide gels (data not shown). We also conclude that the T'165 protein represents either one or both of the 22–23 kD doublet bands that are specifically immunoprecipitated by PAb901. Among the three T' species identified, only T'165 would contain the epitope recognized by this antibody. It is possible that one band of the doublet is a differentially-phosphorylated form of T'165. Alternatively, the mRNA encoding this second species may not yet have been cloned during our RT-PCR analysis. In this latter regard it is important to note that one of the six predicted acceptor splice sites in Figure 4c would yield a T' that shares the C-terminal 23 amino acids with TAg, and thus might be recognized by PAb901. We are currently generating acceptor-site mutants to confirm these assignments and to permit functional analysis of the individual T' species.

The identification of three new early JCV proteins leads one to ask about their influence upon viral transforming and lytic behavior and persistence of...
the virus in its host. In an earlier study (Trowbridge and Frisque, 1993), increased expression of the 17 kD T' species was found to correlate with the ability of JCV transformants to exhibit enhanced anchor-age-independent growth. The T' proteins and Tag share their N-terminal 132 amino acid residues, a region that specifies binding to the tumor suppressor protein pRB and directs nuclear localization (reviewed in Lynch et al, 1994). These sequences would also encode a transformation domain, identified in the SV40 Tag, which might associate with a cellular protein related to p300 (Montano et al., 1990; Marsilio et al., 1991; Yaciuk et al., 1991). The prediction that T' influences JCV's transforming activity is further supported by work involving the SV40 Tag-related proteins 17kT and T147D (a truncated form of SV40 Tag containing the N-terminal 147 amino acids) which are capable of transforming rat fibroblasts (Sompayrac and Danna, 1992; Zerahn et al., 1993).

Data presented in Figures 8 and 9 suggest that at least one of the T' species regulates the DNA replicating activity of JCV. All three T' species encode a Tag domain which participates in the binding of DNA α polymerase, the cellular enzyme recruited to the viral origin to initiate DNA replication (reviewed in Fanning and Knippers, 1992). In addition, T' 158 and T' 185 contain N-terminal phosphorylation sites, while T' 105 contains both the N- and C-terminal domains. The phosphorylation state of the SV40 Tag has been shown to influence viral DNA replication and transforming potential, and it is regulated by the interaction of the C-terminal sites with the N-terminal region (Schneider and Fanning, 1988, Prives, 1990; Scheidtmann et al., 1991). One might speculate that relative to Tag, phosphorylation of T' would be regulated differently, since the C-terminal domain in T' 158 and T' 185 is absent and the spacing between the two domains in T' 185 is greatly reduced. The immunoprecipitation and pulse-chase data support this suggestion; relative to the JCV Tag, 17 kD T' is labeled to a greater extent with 32P and its initial phosphate turnover is higher (Figures 1b, 3b).

Direct evidence for the influence of T' upon viral DNA replication is provided by characterization of the mutant JCVΔT'. Mutation of JCV nt 4274 from the G to an A disrupted the donor splice site used in processing the three T' mRNAs, but left the protein coding sequences intact. Immunoprecipitation experiments confirmed the absence of T' proteins in JCVΔT'-infected cells, and the DpnI assay results indicated that at least one T' species is required to promote efficient viral DNA replication. We were surprised to find that expression of large Tag was significantly reduced in mutant-versus wild type-infected cells. This observation might suggest that the T' proteins are required to stabilize large T protein. Alternatively, it is possible that utilization of the second donor splice site in the early mRNA might affect processing of the T/t messages.

The means by which JCV persists in its human host is unknown; however, the discovery of previously unidentified early JCV proteins requires that their potential role in this process be considered. The mechanism of alternative splicing by which T' proteins are produced allows for the translation of multiple proteins from a limited amount of genetic material and is employed as a way to regulate gene expression (reviewed in Leff et al., 1986; Breitbart et al., 1987). Several factors have been shown to regulate splice site usage including the length of the intron (Fu and Manley, 1987; Robberson et al., 1990), the sequences used to form the lariat structure (Noble et al., 1987, 1989; Fu et al., 1988), the secondary RNA structure (Solnick, 1985), the presence of cellular and viral proteins (e.g. SF2, ASF and SV40 Tag; Delsert et al., 1989; Ge and Manley, 1990; Krainer et al., 1990) and the cell type in which the genes are being transcribed (Fu and Manley, 1987; Noble et al., 1987; Fu et al., 1988; Latchman, 1990; Maniatis, 1991). In the present study, cell-specific effects on splicing were demonstrated in rat fibroblasts versus human glial cells; infected or transformed glial (POJ) cells contained three T' transcripts whereas transformed Rat2 cells produced only one predominant T' message. This observation may be related to the findings of Ishaq and Stoner (1994) who recently reported differences in the expression of the JCV large T and small t mRNAs in the brain tissues from PML patients with or without AIDS.

An example in which alternative splicing may influence viral latency has been reported for Epstein-Barr virus (Furnari et al., 1994). In this viral system, latency is disrupted following the production of the transactivating Z protein (the BZLF1 gene product). When the BZLF1 mRNA is differentially spliced to yield the chimeric protein RAZ, reactivation of the viral lytic cycle is prevented. This is thought to result from inactivation of Z via complex formation with RAZ. Because Tag is a transactivating protein that functions as an oligomer, one could speculate that T' might act as a transdominant repressor similar to RAZ. An alternative means by which the regulation of mRNA processing could affect the balance between viral persistence and productive infection is suggested by the analysis of the JCVΔT' mutant. In the absence of T' production, greatly reduced Tag levels are observed in infected cells. We are now investigating the mechanism by which T' influences the expression of this key viral regulatory protein.

Materials and methods

Cell culture

Primary human fetal glial (PHFG) cell cultures were prepared and propagated as described previously (Padgett et al., 1977). The transformed Rat2 cell
lines, Neo-ER, M1(SV40)-RR, SV40-JR and M11-CR (Trowbridge and Frisque, 1993), and the transformed PHFG cell line POJ-19 (Mandl et al., 1987) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 5 to 10% fetal bovine serum (FBS), penicillin (99 U ml⁻¹), and streptomycin (73 U ml⁻¹) in a humidified 37°C atmosphere containing 10% CO₂.

**Immunoprecipitation of ³⁵S-labeled viral proteins**

PHFG cells were infected with JCV at weekly time intervals, beginning 21 days prior to metabolic labeling of the cultures with ³⁵S-methionine. This infection schedule permitted the simultaneous labeling of viral proteins expressed on day 0, 7, 14, and 21 p.i. cultures. Following a 15 h incubation period in which cells were grown in ³⁵S-labeling medium (84% DMEM without methionine [GIBCO], 10% complete DMEM, 5% FBS, 1% L-glutamine [200mM L-glutamine]) containing 100 μCi [³⁵S] methionine (Trans³⁵S Label, >1100 Ci mmole⁻¹; ICN), the cells were lysed by the addition of 1.8 ml RIPA buffer (Coussens et al., 1985). Lysates preabsorbed with Pansorbin Staph A cells (Calbiochem) were divided into four 450 μl aliquots and their viral proteins were immunoprecipitated with monoclonal (PAb416; Harlow et al., 1981) and hamster polyclonal antibodies that recognize both the JCV and SV40 TAg or with rabbit polyclonal antibodies (Lee Biomolecular) that react with the JCV and SV40 VP1 proteins. Immunoprecipitated proteins were separated by electrophoresis through 14% SDS-polyacrylamide gels, and gels were fixed, treated with Enhance to permit detection by fluorography (NEN, Dupont), dried under vacuum, and exposed to film (Trowbridge and Frisque, 1993).

Transformed Rat2 cells were seeded at a density of 1 x 10⁶ per 100 mm plate, allowed to grow for 20–24 h, and then metabolically-labeled for 16 h as described above. The M1(SV40)-RR and SV40-JR cell lines express JCV and SV40 early proteins, respectively; the Neo-ER line does not express viral proteins (Trowbridge and Frisque, 1993). Following their extraction from these transformed cells, viral proteins were immunoprecipitated with excess polyclonal anti-T serum or one of four monoclonal anti-T antibodies (PAb416, Harlow et al., 1981; PAb1614, Ball et al., 1984; PAb901, Deckhut et al., 1991; PAb2000, Bollag and Frisque, 1992) as described previously (Trowbridge and Frisque, 1993). The protocol was modified to permit incubation of rabbit anti-mouse IgG antibody (Sigma) with activated Pansorbin Staph A cells (Calbiochem) prior to their addition to the immune complexes containing monoclonal antibody. Immunoprecipitated proteins were analyzed on 12.5% SDS-polyacrylamide gels as noted above.

**Immunoprecipitation of ³²P-labeled viral proteins**

PHFG cells, infected using the time intervals outlined above, were metabolically-labeled in phosphate-free DMEM (Gibco) supplemented with 10% dialyzed FBS (Sigma) and 5 mCi of ³²P-orthophosphate (ICN). After a 7 h labeling period, cells were lysed with 1.8 ml RIPA buffer containing inhibitors of protease (100 KIU ml⁻¹ aprotinin, 1mM phenylmethylsulfonlfyl chloride, PMSF) and phosphatase and kinase (0.2mM sodium vanadate, 14mM 2-mercaptoethanol, 1mM sodium molybdate) activities. Viral proteins were immunoprecipitated as described above with the following modifications. Clarified lysates were immediately incubated with excess polyclonal or monoclonal (PAb416) antibodies, and immune complexes were precipitated by incubation with activated Pansorbin Staph A cells. Samples were electrophoresed through 14% SDS-polyacrylamide gels, and the gels were dried under vacuum without fixation. The ³²P-labeled proteins were detected by autoradiography (gels were not treated with Enhance).

**Pulse–Chase analysis**

M1(SV40)-RR cells (7 x 10⁵) were grown on 60 mm plates for 24 h before metabolic labeling. Prior to labeling, cells were rinsed with 1 ml of phosphate-free or methionine-free DMEM and then incubated in 2 ml of ³²P- or ³⁵S-labeling medium containing dialyzed FBS. After 1 h, the medium was removed and replaced with 1.5 ml of ³²P- or ³⁵S-labeling medium containing 3 mCi ³²P-orthophosphate or 375 μCi ³⁵S-methionine, respectively. Cells were pulse-labeled for 1 h, rinsed with DMEM, and incubated for the desired chase time in 4 ml complete DMEM supplemented with 5% FBS. In addition, some cells were labeled for only 5 or 15 min in the presence of 900 μCi ³⁵S-methionine. Labeled cells were lysed at several time points with 500 μl RIPA buffer containing sodium molybdate and the protease inhibitors. The clarified supernatants were kept frozen until all samples were collected. Viral proteins in these lysates were immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography or fluorography.

**Total RNA isolation**

Total RNA from transformed cells or lytically-infected cells was isolated as described previously (Daniel and Frisque, 1993) or by the GlassMAX™ RNA Microisolation Spin Cartridge System (Gibco-BRL). Concentrations of RNA samples were determined using a DU-50 Series Spectrophotometer (Beckman) and samples were subjected to DNase treatment in accordance with the GlassMAX™ system protocol.

**RT-PCR**

Reverse transcription was performed with 200 ng of the appropriate total RNA and the SuperScript™ Preamplification System for first strand cDNA synthesis (Gibco-BRL). Random hexamers provided
with the kit were used as the primers. The cDNAs were then subjected to PCR amplification using the buffer provided in the kit, 10 pmoles T′primer#1 (JCV nt 2580–2602; 5′CCAGCTTACTAAAGCTTTGCA3′), 10 pmoles T′primer#3 (JCV nt 4368–4345; 5′GGATGAAGACTGTGTGGCATG3′), and 5 U Taq DNA polymerase (Promega) in a total volume of 100 μl. The reaction was performed on a Perkin-Elmer Cetus DNA thermal cycler and the cycling parameters included a 5 min incubation at 94°C to denature the RNA/cDNA followed by 30 cycles of 1 min at 94°C, 1 min at 61°C, and 2 min at 72°C (9 min for cycle 30). A portion (20%) of the products from the RT-PCR reaction was electrophoresed on a 7% polyacrylamide gel and visualized by ethidium bromide staining. Using the TA Cloning™ kit (Invitrogen), 1 μl of each RT-PCR reaction mixture was ligated to the TA Cloning™ vector (pCR™ II Vector) and transfected into bacterial cells [TA Cloning One Shot™] provided with the kit. Plasmid DNA purified from isolated colonies using the Wizard™ Miniprep kit (Promega) was screened by digestion with EcoRI which removes the insert along with 20 bp of the vector. Inserts of various sizes were sequenced by the dideoxy method (Sanger et al, 1977) using primers (Vec Primer#1; pCR™ II nt 295–309, 5′GATCCACTAGTAAAG3′ and/or Vec Primer#2; pCR™ II nt 346–332, 5′GTGATGGATATCCTG3′) which anneal to the pCR™ II Vector just beyond the EcoRI site on either side of the insert.

**Cassette mutagenesis**

The mutant recombinant DNA pJCVA T was generated by replacing the G (JCV nt 4274) with an A. Briefly, the 2610 bp BamHI-ClaI fragment of pM1TCR1B (prototype JCV DNA cloned into pBR322 at the EcoRI site; Frisque et al, 1984) was inserted into the Bluescript KS cloning vector (Stratagene). A BglII-BamHI DNA fragment (JCV nt 4242–4307) was removed from this latter construct and replaced with a cassette formed by four synthetic oligonucleotides spanning this region ([JCV oligo#199; nt 4243–4264, 5′GATCTACAGGAAATCTTTCAGG3′, [JCV oligo#200; nt 4270–4247, 5′GAAAGCTTAAAGAGCTCTTCTGTA3′], [ΔT oligo#207; nt 4311–4271, 5′GATCCCAACACTCTACCGCACTATGAAAAAAAGGTA3′], [ΔT oligo#208; nt 4265–4307, 5′GTCTCTTTTCTTTTCTAGGTGGAATGTTGG3′]). Synthetic oligonucleotides (Penn State University Biotechnology Institute) were phospho-

**References**


Virology 201: 223–239.


