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T′ proteins influence JC virus biology

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The JC virus early mRNA is alternatively spliced to yield five transcripts that encode large T antigen, small t antigen, T'_{135}, T'_{136}, and T'_{165}. The splicing process is regulated differentially in transformed versus lytically infected cells and temporally during the course of a productive infection. The authors have identified a potential exonic splicing enhancer near the 3′ end of the early viral mRNA that, when mutated, results in altered splice site usage. The authors have only recently begun investigating the function of the three T′ proteins using genetic and biochemical approaches. These studies indicate that the T′ proteins enhance viral DNA replication and bind differentially to the pRB family of cellular tumor suppressor proteins *in vitro*. Using a G418 selection scheme, the authors have created cell lines that express either T antigen or each of the T′ proteins individually. Preliminary analyses of these lines suggest that T antigen may induce apoptosis in rodent cells, an activity that may be blocked by other JC virus early proteins. Furthermore, examination of protein-protein interactions within the G418-selected cells reveal differences in binding of the viral proteins to the pRB family members relative to that seen *in vitro*. *Journal of NeuroVirology* (2003) **9(suppl. 1),** 15–20.

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Introduction

With the identification of three bands on a sodium dodecyl sulfate (SDS)-polyacrylamide gel as authentic JC virus (JCV) proteins, Trowbridge and Frisque (1995) concluded that the JCV genome was significantly more complex than originally predicted (Frisque *et al*, 1984). Since this discovery, considerable effort has been invested in determining the structure and function of T'_{135}, T'_{136}, and T'_{165}, JCV early proteins produced from mRNAs alternatively spliced from a single precursor transcript. Initial observations suggested that the T′ proteins contribute to the biology of this human polyomavirus. For example, these proteins are expressed at relatively high levels in infected cells, unlike similar proteins of the related viruses SV40 (17kT; Zerrahn *et al*, 1993) and mouse polyomavirus (tiny T; Riley *et al*, 1997). Furthermore, expression of these proteins appears to be differentially regulated in lytically infected versus transformed cells. Finally, the sequence of T'_{165} completely overlaps that of T antigen (TAg), sharing its N-terminal 132 amino acids and C-terminal 33 amino acids with this multifunctional regulatory protein. T'_{135} and T'_{136} also share their first 132 amino acids with TAg, but encode unique C-termini (three and four amino acids, respectively).

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Genetic and biochemical approaches to the study of the structure and function of JCV T′ proteins

To begin to identify the functions of T′ proteins, genetic and biochemical approaches have been pursued. Initial experiments were guided by information

already available for JCV TAg. Numerous functional domains influencing viral DNA replication and oncogenic behavior are localized within the region of overlap between TAg and the T′ proteins. Using a site-directed mutagenesis approach, the shared donor or the three unique acceptor T′ splice sites were altered to eliminate expression of one, two, or all three T′ proteins. These mutant genomes were transfected into permissive human fetal glial cells, and it was demonstrated that the loss of all three T′ proteins resulted in a 10- to 20-fold reduction in DNA replication activity (Trowbridge and Frisque, 1995; Prins and Frisque, 2001). It was also determined that mutation of the T'_{135} acceptor splice site led to the use of cryptic sites resulting in the appearance of new T′ species.

To assess the transforming potential of the T′ proteins, initial work examined their ability to interact with the tumor suppressor protein, pRB, and its related family members, p107 and p130. TAg, T'_{135}, T'_{136}, and T'_{165}, purified from insect cells infected with recombinant baculoviruses, were mixed with extracts of human MOLT-4 cells containing pRB-related proteins. Each viral protein was found to interact with the hypophosphorylated forms of pRB, p107, and p130 in this *in vitro* assay (Bollag *et al*, 2000). Importantly, the JCV proteins exhibited differential binding to the pRB family members. Recent models suggest that in addition to binding pRB family members through their LXCXE domain, polyomavirus tumor proteins must effect the release of E2F transcription factors found in complex with these family members (Sheng *et al*, 1997; Sullivan *et al*, 2000, 2001). The viral proteins, via their J domain, interact with Hsc70, a molecular chaperone protein, to effect this release. Using extracts of POJ cells expressing all five JCV early proteins and an immunoprecipitation-immunoblotting assay, at least one of the JCV proteins was shown to bind Hsc70 *in vivo*. Recent experiments involving Rat 2 cell lines expressing individual JCV early proteins (see below) revealed that T'_{135} interacts with Hsc70. This result is in contrast to that reported for an N-terminal truncated form of SV40 TAg, called N136, that fails to form a stable complex with the molecular chaperone (Sullivan *et al*, 2001).

Functional analyses of T′ proteins expressed in G418-selected cells

To investigate T′ protein function further, we have used a G418 selection protocol to create rat and mouse cell lines expressing one or more JCV early proteins (Kilpatrick, Bollag, McElrath, and Frisque, manuscript in preparation). During the initial isolation of these lines, an observation was made that may be relevant to the question of viral protein function. Rat 2 fibroblasts were transfected with constructs containing either the intact JCV early coding region or individual TAg, T'_{135}, T'_{136} or T'_{165} cDNAs under the control of the cytomegalovirus (CMV) promoter. Cell lines expressing all five JCV proteins or each T′ protein independently were readily established by single-cell cloning following G418 selection. In lines established with the TAg cDNA plasmid, both TAg and the T′ proteins were produced, presumably because the TAg cDNA retains the T′ splice sites. To eliminate expression of T′ mRNAs and proteins by the TAg cDNA, the T′ donor splice site was mutated, and the altered construct was transfected into Rat 2 cells. Upon G418 selection, only a single line expressing TAg in the absence of the other early proteins could be isolated. TAg was expressed at relatively low levels in these cells, and sequence analysis indicated that the integrated TAg gene encoded a wild-type protein. Preliminary experiments utilizing mouse NIH-3T3 cells have yielded similar results. One interpretation of these data is that TAg induces apoptosis in the rodent cells, and that T′ proteins and/or small t protein (tAg) block this activity. It should be noted that SV40 TAg and tAg may have proapoptotic or antiapoptotic activities depending upon experimental conditions (Gjoerup *et al*, 2001 and references therein).

The G418-selected lines expressing different JCV proteins have been assessed for cell-doubling time and for saturation density in medium supplemented with 1% or 10% fetal bovine serum (Kilpatrick *et al*, manuscript in preparation). Growth parameters of cloned, independent lines expressing all five early proteins together or TAg and T′ proteins individually were compared to the parental Rat 2 line and to a JCV-transformed line isolated from a dense focus assay. Two G418-selected lines expressing the intact JCV early region exhibited accelerated growth rates and elevated saturation densities, although these transformation phenotypes did not attain the levels exhibited by the control transformed line. Cells producing a single viral protein (either TAg, T'_{135}, T'_{136}, or T'_{165}) displayed growth parameters only slightly elevated above those of the Rat 2 parental line; by these criteria they did not appear to be transformed. Earlier studies indicated that most G418-selected cells expressing JCV early proteins fail to exhibit an aggressive transformed phenotype, presumably because expression levels of the tumor proteins fail to attain a required threshold necessary for complete transformation (Trowbridge and Frisque, 1993). It might be important to analyze a larger number of independent cell lines and assess a wider range of growth parameters to understand the contribution of T′ proteins to the transformation process.

Availability of the G418-selected Rat 2 lines has permitted us to examine *in vivo* interactions between individual JCV early proteins and the pRB family of proteins (Kilpatrick *et al*, manuscript in preparation). Lysates of these cells were immunoprecipitated with antibodies specific either for pRB, p107, and p130 or for JCV early proteins. Complexes were electrophoresed on polyacrylamide gels and subjected to

Western blot analysis using anti-pRB, anti-p107, or anti-p130 antibodies. p107 from the parental Rat 2 cells migrated as a doublet; but in cells expressing all five JCV early proteins or cells containing T'_{135} or T'_{165} alone, only the band with the greater mobility was detected. It was this hypophosphorylated form of p107 to which the viral proteins bound. In cells expressing TAg or T'_{136}, we failed to observe an interaction between the viral proteins and p107. In addition, p107 from these cells included the hyperphosphorylated form. The second pRB family member, p130, was found to exist as multiple phosphorylated forms in the parental cells and in the line expressing TAg only. In the latter cells, TAg did not form a complex with p130. In cells expressing all five JCV early proteins or cells producing T'_{135}, T'_{136}, or T'_{165}, hypophosphorylated p130 was the most prominent form. Again, it was this fastest migrating band with which the viral proteins interacted. Because pRB was present at low levels in these cell lines, we were unable to convincingly demonstrate an *in vivo* interaction between it and the viral proteins.

Our *in vivo* and *in vitro* binding assays yielded different results in regard to the ability of TAg and T'_{136} to bind p107 and p130. At this time we are unable to explain the discrepancy involving the ability of T'_{136} to form a complex with p107. Two different cell lines were examined, and T'_{136} failed to bind p107 in both instances. It is possible that T'_{136} recognizes differences between human p107 in the MOLT-4 extracts and rat p107 in the Rat 2 cells. Alternatively, the two opposing outcomes may reflect differences in the concentrations of proteins used in the *in vitro* and *in vivo* experiments. In the case of TAg, its inability to bind p107 and p130 *in vivo* may reflect its reduced level of expression in the only cell line available for examination. Alternatively, this result could signal the possibility that a nonfunctional TAg is expressed in the G418-selected Rat 2 cells (although the integrated gene is not mutated). If, as we have suggested, TAg induces programmed cell death in Rat 2 cells, then one might speculate that our G418 approach selected for a cell expressing a TAg defective in this, and perhaps other (e.g., p107 and p130 binding), activities.

Regulation of alternative splicing of the JCV early mRNA

The process of alternative splicing that yields the transcripts encoding the five JCV early proteins is regulated by both *cis*-acting signals and *trans*-acting factors. The SR family of cellular proteins plays important roles in splice site selection. Regulation of their expression and function is influenced by cell type and post-translational modification, respectively (reviewed in Elliot, 2000; Lopez, 1998; Xiao and Manley, 1997). SR proteins bind to short sequences in the RNA called exonic splicing enhancers (ESEs) and may antagonize the activity of other splicing factors by competing for binding to overlapping recognition sites (reviewed in Lopez, 1998). Interactions between SR proteins and ESEs may lead to the recruitment of additional splicing factors that enhance splicing at suboptimal 3′ acceptor sites (reviewed in Blencowe, 2000; Zheng *et al*, 1997). Mutations introduced into ESEs have a wide range of effects on splicing patterns, suggesting that sequence context and position contribute to ESE function (Liu *et al*, 2000). A number of viruses have exploited the process of alternative splicing to enhance viral multiplication. For example, during the course of an infection, adenovirus regulates the activity of one or more SR proteins (Kanopka *et al*, 1998; Molin and Akusjärvi, 2000) to alter its own splicing program, and thus the proteins it produces. In the case of SV40, TAg alters the relative proportions of splice variants of TEF-1, a cellular transcription factor that influences SV40 promoter activity (Zuzarte *et al*, 2000).

Splicing of the JCV early precursor mRNA is regulated. Early observations revealed that T'_{136} is the only T′ protein produced in significant amounts in transformed rat fibroblasts, whereas all three T′ proteins are readily detected in JCV-infected human fetal glial cells (Trowbridge and Frisque, 1995). Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of T′ transcripts produced in a lytic infection versus a variety of transformed and tumor cells indicates that the differential splicing patterns are not due to the tissue or species origin of the cells, but rather to differences in the lytic versus transformed state (Jones and Frisque, unpublished data). In a second set of experiments, we observed that splicing is temporally regulated during the course of a lytic infection (Prins, Jones, and Frisque, unpublished data). At day 3 post infection (p.i.), the pattern of T′ mRNA splicing resembled that seen in the transformed cells in the first experiment (i.e., the T'_{136} transcript was the most abundant mRNA detected). By day 7 p.i., however, the lytic pattern of splicing, as seen in the initial RT-PCR experiment, became apparent (i.e., all three T′ transcripts were produced at similar levels). One could hypothesize that these two examples of regulated splicing are related. Late gene expression does not occur in JCV-transformed cells or in lytically infected cells before approximately day 5 p.i. It is possible that one or more late viral proteins alter the initial splicing program.

Several years ago our group, while examining cells transformed by a series of JCV TAg mutants, made an observation that could not be explained by information then available. Mutations had been introduced near the C-terminus of TAg in an attempt to identify potential phosphorylation sites (Swenson and Frisque, 1995; JJ Swenson, PhD thesis, The Pennsylvania State University, 1995). Cells expressing the mutant TAgs were labeled with [^{35}S]-methionine, lysates were prepared and immunoprecipitated with an anti-T antibody, and complexes

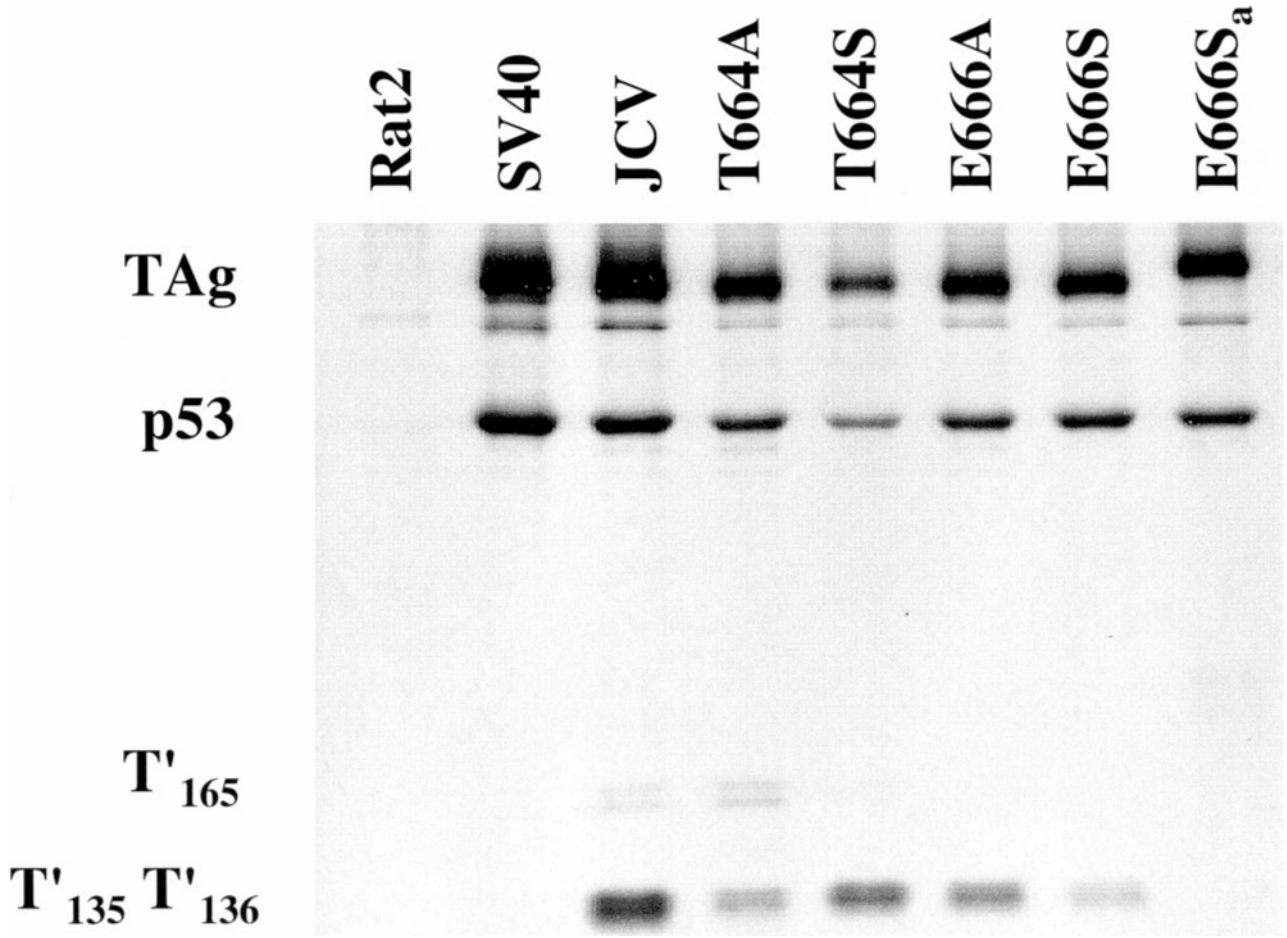

Figure 1 Altered T′ protein expression in cells expressing mutant JCV TAgs. Extracts of cells expressing JCV or SV40 early proteins labeled with [^{35}S]-methionine were immunoprecipitated with PAb 416, a monoclonal antibody that recognizes an epitope shared by TAg and the three T′ proteins. The immune complexes were electrophoresed on an SDS-polyacrylamide gel and protein bands were visualized by fluorography. The positions of the JCV and SV40 TAgs, the three JCV T′ proteins, and the cellular tumor suppressor protein, p53, to which each TAg binds, are noted. Lanes labeled T664A, T664S, E666A, and E666S represent independent cell lines containing four different JCV TAgs mutated at amino acid position 664 or 666 (Swenson and Frisque, 1995). Note that only the lines expressing the wild-type (lane JCV) or T664A TAg produce detectable levels of T'_{165} (T'_{165} migrates as a doublet due to differential phosphorylation). Furthermore, in the T664A line, T'_{165} appears to be expressed at higher levels relative to the other JCV early proteins (compare the relative amounts of T'_{165} in lane 4 versus 3). Finally, lane E666S$_a$ represents a second cell line containing the E666S TAg mutant, but in these cells JCV early protein expression is altered more extensively; all three T′proteins are missing and the mobility of TAg is shifted.

were electrophoresed on an SDS-polyacrylamide gel. The observed pattern of early protein expression was unexpected; in most lines, T'_{165} was not readily detected, and in some cloned lines expressing mutant E666S, all three T′ proteins were absent and the mobility of TAg was shifted (Figure 1; data not shown). Upon reexamination of these results recently, we noted that the mutated sequence resembles a consensus binding site for the alternative splicing factor, ASF/SF2 (Figure 2; Tacke and Manley, 1995). Of the four mutants examined, only T664A contains an alteration that converts the JCV sequence to one more closely resembling an ASF/SF2 site. A cell line expressing this mutant produces all three T′ proteins, and T'_{165} expression appears to be elevated relative to the other viral proteins. Changes in the other three mutants all result in JCV sequences that diverge from the potential ESE, and cells expressing these mutants fail to produce T'_{165}. Two of 11 lines expressing E666S, which alters the predicted ASF/SF2 binding site at two positions, fail to produce any T′ proteins and the mobility of TAg is altered. ASF/SF2 acts upon proximal 3′ splice sites that include a suboptimal polypyrimidine tract. The potential ESE discussed here is located adjacent to the T'_{165} 3′ splice site that is associated with a weak polypyrimidine tract. We have now constructed two mutants in this potential ESE, transfected them into Rat 2 cells, isolated G418-resistant cell lines, and examined early protein expression patterns (Tyagarajan and Frisque, unpublished data). The alteration in the consensus mutant changes the relevant JCV sequence to a

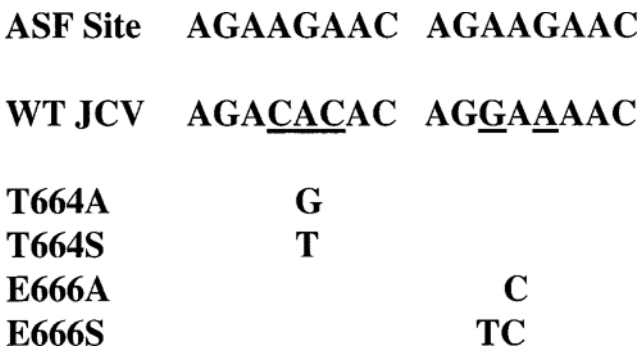

Figure 2 The 3′ end of the JCV early mRNA contains a potential ESE. A tandem duplication of a consensus ASF/SF2 binding site (Tacke and Manley, 1995) is shown at the top of the figure. Such sites might function as exonic splicing enhancers or ESEs. The JCV sequence from nucleotides 2684 to 2669 (the T'_{165} acceptor site is at nucleotide 2704) encodes a portion of the C-terminal region of TAg and is shown below the consensus ASF/SF2 binding site. Differences between the two sequences are underlined. One- or two-nucleotide changes were introduced into the JCV sequence to create the four TAg mutants identified in Figure 1. Note that the change in T664A (A to G) creates a JCV sequence more similar to that of the AFS/SF2 site, whereas the other three mutations create sequences that are less similar (T664S, A to T; E666A, A to C; E666S, GA to TC).

duplicated ASF/SF2 consensus site; preliminary results indicate this mutant yields a wild-type expression pattern. Changes in the nonconsensus mutant create a JCV sequence predicted to further disrupt the ASF/SF2 binding site; it does not alter protein-coding information. Preliminary results indicate that cells containing this mutant express reduced levels of T′ proteins.

In summary, we have shown that the JCV T′ proteins interact with the pRB family of proteins both *in vitro* and *in vivo*, and that JCV TAg, T'_{135}, T'_{136}, and T'_{165} exhibit differential binding activities. In addition, T'_{135} binds to the DnaK protein, Hsc70, *in vivo*. These interactions indicate the J and LXCXE domains in TAg and T′ proteins are functional, and that each of these proteins might influence cell-cycle progression and transforming potential through the release of the E2F family of transcription factors. Furthermore, interactions between JCV T′ proteins and Hsc70 could be responsible for the positive effects T′ proteins have upon JCV DNA replication. This suggestion is supported by earlier studies with SV40 TAg and this molecular chaperone (Campbell *et al*, 1997).

We suggest that T′ proteins and/or tAg might also influence JCV biology by interfering with an apoptotic function of TAg. This speculation rests upon our inability to readily isolate rodent cell lines only expressing TAg and upon the documented effects SV40 early proteins have on apoptosis. Experiments now in progress will attempt to confirm that our observations involve programmed cell death. Furthermore, because our findings have been made in nonpermissive rodent cells, it will be important to determine whether this activity is also displayed in permissive human glial cells.

Alternative splicing of the JCV immature early mRNA is regulated temporally during the course of an infection. In addition, JCV early splicing patterns in transformed cells resemble those seen early in a lytic infection, but differ from patterns seen late in infected cells. It is possible that the function and/or expression levels of splicing factors remain stable in the transformed cell, but change in the lytically infected cell as late viral proteins are produced. These cellular factors bind to ESEs, *cis*-acting regulatory signals in the mRNA, to influence splicing patterns. We believe we have identified one such ESE near the 3′ end of the JCV early mRNA, and we predict that the SR protein, ASF/SF2, binds this sequence and regulates the selection of the T′ acceptor splice sites.

References

Blencowe BJ (2000). Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *Trends Biochem Sci* **25:** 106–110.

Bollag B, Prins C, Snyder EL, Frisque RJ (2000). Purified JC virus T and T′ proteins differentially interact with the retinoblastoma family of tumor suppressor proteins. *Virology* **274:** 165–178.

Campbell KS, Mullane KP, Aksoy IA, Stubdal H, Zalvide J, Pipas JM, Silver PA, Roberts TM, Schaffhausen BS, DeCaprio JA (1997). DnaJ/hsp40 chaperone domain of SV40 large T antigen promotes efficient viral DNA replication. *Genes Dev* **11**: 1098–1110.

Elliot DJ (2000). Splicing and the single cell. *Histol Histopathol* **15:** 239–249.

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

Gjoerup O, Zaveri D, Roberts TM (2001). Induction of p53-independent apoptosis by simian virus 40 small t antigen. *J Virol* **75:** 9142–9155.

Kanopka A, Muhlemann O, Petersen-Mahrt S, Estmer C, Ohrmalm C, Akusjärvi G (1998). Regulation of adenovirus alternative RNA splicing by dephosphorylation of SR proteins. *Nature* **393:** 185–187.

Liu HX, Chew SL, Cartegni L, Zhang MQ, Krainer AR (2000). Exonic splicing enhancer motif recognized by human SC35 under splicing conditions. *Mol Cell Biol* **20:** 1063–1071.

Lopez AJ (1998). Alternative splicing of pre-mRNA: developmental consequences and mechanisms of regulation. *Annu Rev Genet* **32:** 279–305.

Molin M, Akusjärvi G (2000). Overexpression of essential splicing factor ASF/SF2 blocks the temporal shift in adenovirus pre-mRNA splicing and reduces virus progeny formation. *J Virol* **74:** 9002–9009.

Prins C, Frisque RJ (2001). JC virus T′ proteins encoded by alternatively spliced early mRNAs enhance T antigen–mediated viral DNA replication in human cells. *J NeuroVirol* **7:** 250–264.

Riley MI, Yoo W, Mda NY, Folk WR (1997). Tiny T antigen: An autonomous polyomavirus T antigen amino-terminal domain. *J Virol* **71:** 6068–6074.

Sheng Q, Denis D, Ratnofsky M, Roberts TM, DeCaprio JA, Schaffhausen B (1997). The DnaJ domain of polyomavirus large T is required to regulate RB family tumor suppressor function. *J Virol* **71:** 9410–9416.

Sullivan CS, Cantalupo P, Pipas JM (2000). The molecular chaperone activity of simian virus 40 large T antigen is required to disrupt Rb-E2F family complexes by an ATP-dependent mechanism. *Mol Cell Biol* **20:** 6233–6243.

Sullivan CS, Gilbert SP, Pipas JM (2001). ATP-dependent simian virus 40 T-antigen-Hsc70 complex formation. *J Virol* **75:** 1601–1610.

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

Tacke R, Manley JL (1995). The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. *EMBO J* **14:** 3540–3551.

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.

Trowbridge PW, Frisque RJ (1995). Identification of three new JC virus proteins generated by alternative splicing of the early viral mRNA. *J NeuroVirol* **1:** 195–206.

Xiao SH, Manley JL (1997). Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. *Genes Dev* **11:** 334–344.

Zerrahn J, Knippschild U, Winkler T, Deppert W (1993). Independent expression of the transforming amino-terminal domain of SV40 large T-antigen from an alternatively spliced third SV40 early messenger RNA. *EMBO J* **12:** 4739–4746.

Zheng ZM, He PJ, Baker CC (1997). Structural, functional, and protein binding analyses of bovine papillomavirus type 1 exonic splicing enhancers. *J Virol* **71:** 9096–9107.

Zuzarte PC, Farrance IK, Simpson PC, Wildeman AG (2000). Tumor cell splice variants of the transcription factor TEF-1 induced by SV40 T-antigen transformation. *Biochim Biophys Acta* **1517:** 82–90.