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Differential expression of mRNAs for JC virus large and small tumor antigens in brain tissues from progressive multifocal leukoencephalopathy patients with and without AIDS

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ABSTRACT **JC virus (JCV) causes progressive multifocal leukoencephalopathy (PML), the fatal demyelinating infection of oligodendrocytes, in up to 5% of AIDS patients. An intron-differential RNA PCR was developed to study the expression of alternately spliced JCV early mRNAs in brain tissues from PML patients with and without AIDS and in JCV-induced hamster brain tumors. The method utilizes primers that span the large tumor (T) and small tumor (t) antigen introns allowing amplification of specific cDNAs in the presence of contaminating viral genomic DNA. Hybridization with specific junctional probes and DNA sequence analysis confirmed the identity of the PCR products. Sequencing showed that JCV early mRNA is alternatively spliced as previously predicted by analogy to simian virus 40. Large T antigen mRNA was detected in all the brain tissues from PML patients with and without AIDS. The expression of small t antigen mRNA varied depending upon the association of PML with AIDS and upon other unknown factors. Of the 12 PML/AIDS brain tissue samples, 11 (92%) expressed small t antigen mRNA, whereas only 8 of 13 (62%) brain samples from patients with PML alone showed detectable levels of small t antigen mRNA. Human immunodeficiency virus 1 proviral DNA was detected in 10 of 12 PML/AIDS brain samples. The results indicate that alternative splicing of JCV early mRNA is regulated in the human brain and that the production of small t antigen may not be essential for the pathogenesis of PML.**

The neurological manifestations of AIDS may result from human immunodeficiency virus 1 (HIV-1) infection of brain macrophages and glial cells or from opportunistic infections of the central nervous system by DNA viruses (1). The latter include several of the herpesviruses (herpes simplex types 1 and 2, cytomegalovirus, and herpes zoster) and the human polyomavirus JC virus (JCV) (2). JCV is a small (5130 bp) circular DNA virus that is highly homologous to simian virus 40 (SV40) (3, 4). It infects oligodendrocytes and astrocytes, causing the fatal demyelinating disease known as progressive multifocal leukoencephalopathy (PML) in about 5% of AIDS patients (5, 6). In one-fourth of these patients, the neurological symptoms of PML signal the onset of clinical AIDS (2). The mechanisms by which HIV-1 infection may regulate the expression of these DNA viruses are not yet fully delineated. These mechanisms will, of course, include the nonspecific cellular immune defect caused by lysis of $CD4^+$ T lymphocytes. In addition, in the case of JCV, the HIV-1 Tat protein, like the JCV large tumor (T) antigen, may have a transactivating effect on late region (capsid protein) transcription of this divergently transcribed DNA virus. *In vitro* studies have suggested that the Tat protein, which is essential for transactivation of its own long terminal repeat, is an even stronger transactivator of the JCV late promoter in glial-derived cells than is large T antigen itself (7–9).

Low levels of JCV DNA have been detected in normal human brains (10, 11), and it is possible that PML results from reactivation of latent JCV in the brain (12). Any of the events in the chain leading from JCV latency to productive cytolytic infection could provide a site of retroviral interaction. In the first phase of viral reactivation, prior to DNA replication, polyomaviruses express at least two nonstructural regulatory proteins, large T antigen and small tumor (t) antigen. Like SV40 (13), alternative splicing of a single early region JCV transcript yields both large T antigen and small t antigen mRNAs (14, 15). The large T antigen is a complex multifunctional protein with transactivating activity and multiple roles in viral DNA replication (16, 17). The JCV small t antigen, at 172 amino acid residues, is one-fourth the size of large T antigen with which it shares 81 N-terminal amino acid residues (4). Small t antigen seems to enhance viral transformation by SV40 (18) and may have transactivating effects on both the early and late SV40 promoters (19). However, a role for this protein in promoting viral replication has not been defined for any of the polyomaviruses, although the presence of small t antigen increases the burst size of SV40 in permissive cells (20).

In an effort to understand the role of alternative T-antigen splicing in regulation of JCV replication in PML brain, we have developed a polymerase chain reaction (PCR) method that amplifies cDNA reverse-transcribed from mRNA. This approach utilizes primers that span the introns and, with junctional probes, specifically detects large T and small t antigen mRNAs in the presence of viral DNA. The results show that small t antigen mRNA is not always detectable in PML tissue and, therefore, appears not to be essential for lytic infection. Further, it appears that HIV-1 may influence JCV small t antigen formation by promoting alternative splicing of early mRNA. Finally, we demonstrate that the cDNAs obtained reflect the splice sites originally predicted based on the DNA sequence homology to SV40 (14).

MATERIALS AND METHODS

Brain Tissues. Frozen human PML brain tissues were obtained at autopsy from 13 patients without AIDS (Table 1) and from 12 AIDS patients (11 males and 1 female) aged 28 to 46 years.

Isolation of mRNA. mRNA was isolated directly from 50 to 100 mg of crude tissue by magnetic separation technology using a PolyATtract system 1000 kit purchased from Promega.

Primers and Probes. Primers (Table 2) were designed to amplify large T and small t cDNAs in the presence of viral DNA that almost always contaminated the mRNA prepara-

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Abbreviations: PML, progressive multifocal leukoencephalopathy; JCV, JC virus; T or t, tumor; SV40, simian virus 40; HIV-1, human immunodeficiency virus 1; PP2A, protein phosphatase 2A.

Table 1. Diseases underlying non-AIDS PML

Coded identifier	Age, years/ sex	Underlying disease	Large T/small t antigen mRNA
SB	6/F	Combined immune deficiency	+/+
HK	42/M	Hodgkin disease	+/+
TB	56/F	Pulmonary lymphoma, immunoblastic sarcoma	+/−
MX	57/M	Lymphocytic lymphoma	+/+
VC	—/F	Hodgkin disease	+/−
SF	61/M	Chronic lymphocytic leukemia	+/+
CB	55/F	Systemic lupus erythematosus	+/+
CT	39/F	Chronic lymphocytic leukemia	+/+
BF	—/F	Lymphoma	+/−
FK	43/M	Hodgkin disease	+/+
RN	57/M	Reticulum cell sarcoma	+/−
FN	45/F	No underlying disease	+/+
LT	51/M	Chronic lymphocytic leukemia	+/−

F, female; M, male; +, present; −, absent.

tions. Both large T and small t antigen genes contain a single intron. Primers were designed such that they span the intron and amplify cDNA fragments that are smaller in size than the PCR products obtained from the contaminating genomic DNA (Fig. 1). Oligonucleotide probes across the splice junctions (Table 2 and Fig. 1) were used to probe the large T and small t antigen cDNAs. These probes were highly specific for cDNAs and did not cross react with the genomic DNA PCR products. The primer pair used for the detection of HIV-1 DNA or RNA was SK38/SK39, which is specific for the HIV-1 *gag* gene and amplifies a 115-bp DNA fragment (21). SK19 was used as an internal oligonucleotide probe to detect the SK38/SK39 PCR products.

Intron-Differential RNA PCR. mRNA (10–15 ng) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of 200 ng of JEX-1 in 20 μl by using an RNA PCR kit from Perkin–Elmer/Cetus. After reverse transcription, the tubes were heated at 95°C for 10 min to inactivate the reverse transcriptase and subjected to PCR in 100 μl. The PCR mixture contained 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, all four dNTPs (each at 0.2 mM), 2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus), and the primer pair JEX-1/JEX-2 (each at 0.25 μM) for the amplification of large T antigen or JEX-1/JEX-3 (each at 0.25 μM) for the amplification of small t antigen cDNA fragments. The hot-start technique was employed and, after an initial denaturation at 94°C for 3 min, PCR was performed with 40 cycles of 94°C (1 min), 55°C (1 min), and 72°C (1 min) with a final extension for 10 min at 72°C.

Detection of HIV-1 DNA in Brain Tissues by PCR. PCR was carried out with 5–10 μl of brain tissue extracts. The brain tissue extracts were prepared from 25 to 50 mg of frozen brain tissue. The tissue was digested for 2–4 hr at 56°C in 200 μl of 50 mM Tris·HCl, pH 8.0/1 mM EDTA/0.45% Tween 20/ 0.45% Nonidet P-40/proteinase K (Sigma; 100 μg/ml). Proteinase K was then inactivated by heating at 95°C for 15 min. Samples were then centrifuged and the supernatant was used for PCR. PCR was performed using a GeneAmplimer HIV-1 reagent kit (Perkin–Elmer/Cetus).

Detection of HIV-1 RNA by RNA PCR. mRNA (10–15 ng) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase and 100 ng of SK39 primer in 20 μl by using an RNA PCR kit from Perkin–Elmer/Cetus. The tubes were heated at 95°C for 10 min to inactivate the reverse transcriptase and subjected to PCR with the primer pair SK38/SK39 by using a GeneAmplimer HIV-1 reagent kit (Perkin–Elmer/Cetus).

Cloning and Sequence Analysis. DNA bands were isolated from agarose gels with a Geneclean kit (Bio 101) and cloned in a TA-cloning vector (Invitrogen). Insert-bearing colonies were picked up by junctional probes. Plasmid DNA was prepared by using Insta-Prep tubes (5 Prime → 3 Prime, Inc.) and sequenced using the Sequenase DNA sequencing kit (United States Biochemical). Some PCR products were also directly sequenced using an AmpliTaq cycle sequencing kit from Perkin–Elmer/Cetus.

RESULTS

Viral mRNA preparations from tissues heavily infected with DNA viruses are not suitable for the amplification of cDNAs by standard RNA PCR methods due to coamplification of contaminating viral genomic DNA. An intron-differential reverse transcription–PCR was, therefore, developed for the detection of large T and small t antigen mRNAs. After reverse transcription of the alternatively spliced mRNAs into cDNA, PCR was performed with a single downstream primer JEX-1 combined with an upstream primer JEX-2 for large T antigen cDNA or JEX-3 for small t antigen cDNA. The JEX-3 primer binds a region deleted in the large T antigen mRNA and, thus, is specific for small t antigen mRNA. Fig. 2*A* shows the gel bands amplified from large T and small t antigen cDNAs after reverse transcription of mRNAs. The amplification of large T antigen cDNA using primer pair JEX-1/ JEX-2 yielded a fragment of 246 bp that was clearly differentiated from the 590-bp genomic DNA band. The amplification of small t antigen cDNA with the primer pair JEX-1/ JEX-3 yielded an 85-bp fragment that was easily differentiated from the 152-bp genomic DNA fragment. The presence of these putative cDNA bands was absolutely dependent on the reverse transcription step. To identify the amplified cDNA bands, they were hybridized with oligonucleotide probes that spanned the splice site for the large T antigen (LTA-1.1) or small t antigen (STA-1.1) (Table 2) and were thus specific for one or the other amplified cDNA band. These junctional (exon–exon) probes confirmed that the bands of the expected size represented cDNA amplified from spliced large T and small t antigen mRNA (Fig. 2*B Left* and *Right*, respectively). The bands were excised from the gel and cloned with a TA-cloning vector or sequenced directly by cycle sequencing. DNA sequences obtained from small t (Fig. 3) and large T (data not shown) antigen cDNA bands from both PML and hamster brain tissues confirmed that the

Table 2. Oligonucleotide primers and probes

Designation	Sequence (5′–3′)	Positions
JEX-1	TTCTACTAGTATGTATTCCACCAGGATTCC	4371–4400
JEX-2	CCTTCTCGAGTCTGCATGGGGGAACATTCC	4960–4931
JEX-3	CACCCCCTACAGGGATCTAAAG	4522–4501
LTA-1.1	**TTGGCACCTCTGAACT**	**4420–4426**/4771–4779
STA-1.1	**GTTGGCACCTTAAAGCT**	**4419–4426**/4494–4502

Nucleotide positions in the JCV genome are from ref. 3. JEX-1 and JEX-2 were altered to introduce *Spe* I and *Xho* I restriction sites, respectively, on their 5′ ends. Nucleotides in boldface type represent the sequences contributed from the common 3′ acceptor site.

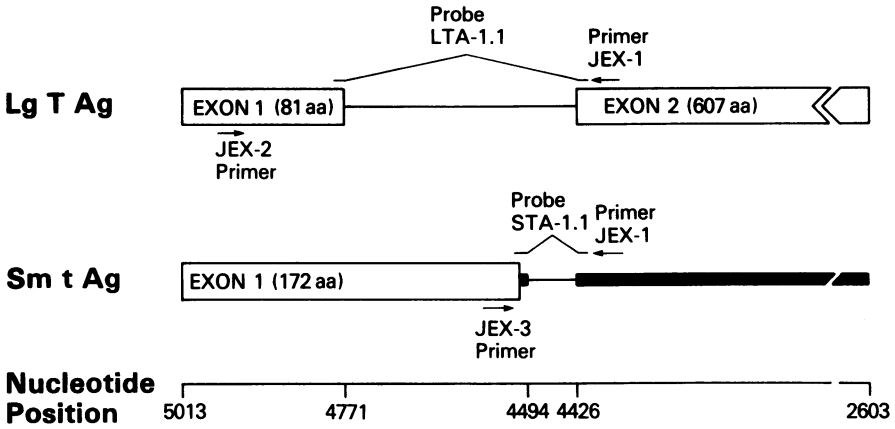

FIG. 1. Schematic diagram showing the location of PCR primers and probes. Open box, translated exons; solid box, 3′ untranslated region of the small t mRNA; solid lines, introns. Lg, large; Ag, antigen; Sm, small.

amplified bands represent mRNAs with splice sites identical to those previously predicted (3, 14).

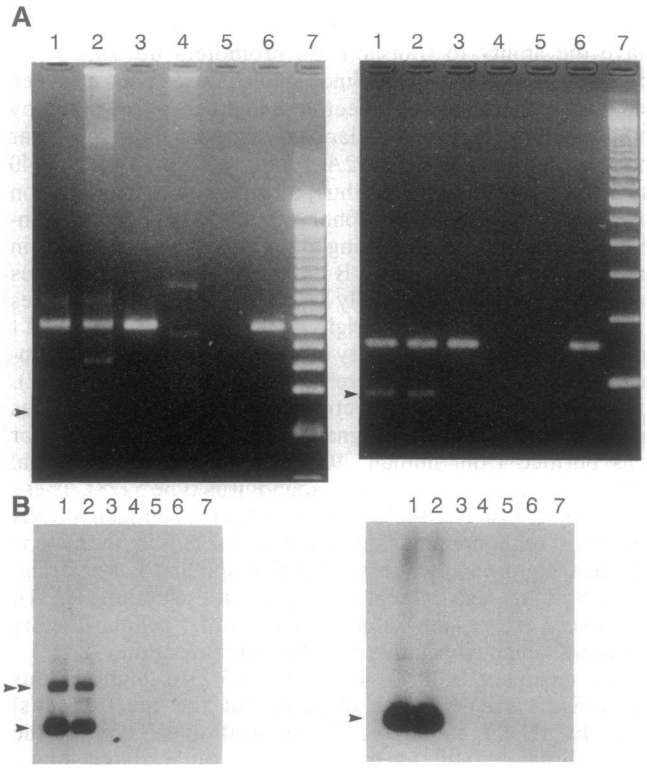

FIG. 2. Agarose gel electrophoresis and Southern blot analysis of the RNA PCR products. (*A*) PCR products (20 μl) were electrophoresed on 1.5% (for large T) (*Left*) or 2% (for small t) (*Right*) agarose gels. The heavy bands at 590 bp (large T) and 152 bp (small t) represent amplified fragments of viral DNA contaminating the mRNA preparations. The arrowheads identify the position of cDNA bands. (*B*) Gels were transferred to nylon and hybridized at 52°C with radiolabeled junctional probes specific for large T (LTA-1.1) (*Left*) or small t (STA-1.1) (*Right*). The filters were washed with 2× SSC/0.1% SDS at 52°C before exposing to the x-ray film. Lanes: 1, PML/AIDS brain mRNA; 2, mRNA from a JCV-induced hamster brain tumor; 3, RNA PCR without reverse transcriptase using mRNA from JCV-induced hamster brain tumor; 4, normal human brain mRNA; 5, reagent blank; 6, PCR with pBR322-cloned JCV DNA (10 fg); 7, 100-bp DNA ladder (BRL). The arrowheads identify the expected cDNA bands of 246 bp (large T) (*Left*) and 85 bp (small t) (*Right*). The double arrowheads indicate the heteroduplex consisting of cDNA and genomic DNA PCR fragments formed during the amplification of large T cDNA.

Several other slower migrating bands also hybridized with the specific probe. These PCR products appear to represent heterologous duplexes consisting of cDNAs and viral genomic DNA of variable structure that migrate between the expected cDNA duplex and the coamplified genomic DNA duplex.

We employed intron-differential RNA PCR to study the expression of large T and small t antigen mRNAs in brain tissues from PML patients with and without AIDS. Large T antigen mRNA was readily detectable in all the brain tissues from PML patients with and without AIDS (Figs. 4 and 5 *Upper*). The expression of small t antigen mRNA varied depending upon the association of PML with AIDS. Of the 12 PML/AIDS brain tissues studied, 11 (92%) expressed small t antigen mRNA, whereas only 8 of 13 tissues (62%) from patients with PML but without AIDS (Table 1) showed detectable levels of small t antigen mRNA (Figs. 4 and 5 *Lower*). This difference does not achieve statistical signifi-

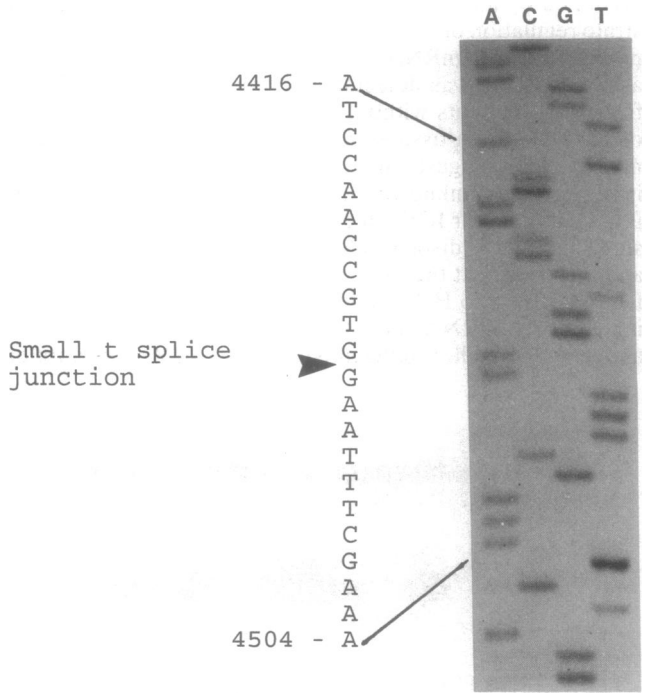

FIG. 3. Sequence analysis of PCR-amplified small t antigen cDNA. The cDNA fragment was cloned and sequenced. The sequence spanning the splice junction is shown. JCV genome nucleotide positions are from ref. 3.

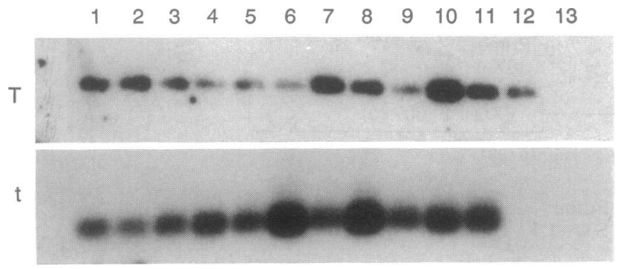

FIG. 4. RNA PCR amplification of large T (*Upper*) and small t (*Lower*) antigen mRNAs from brain tissues from PML patients with AIDS. PCR products (10 μl) were electrophoresed on agarose gels and hybridized with radiolabeled large T- and small t-specific oligonucleotide probes after transfer to nylon as in Fig. 2. Lanes: 1–12, 12 PML/AIDS brain mRNAs; 13, reagent blank.

cance ($P = 0.16$, Fisher's exact test). The samples found to be negative for small t antigen mRNA were tested repeatedly and remained negative. When the tissues from the PML/AIDS brains were amplified using SK38/SK39 primers, 10 of 12 (83%) were positive for HIV-1 *gag* DNA (Fig. 6), indicating HIV-1 infection in these same tissues. Of the two samples negative for HIV-1 DNA, one was found to be positive for HIV-1 RNA by reverse transcription–PCR. The only brain tissue from a PML/AIDS patient that did not express detectable small t antigen mRNA did, however, contain HIV-1 DNA.

DISCUSSION

The splicing of JCV early mRNA, like that of BK virus and SV40, involves the alternative selection of one of two 5′ donor sites for joining to a common 3′ acceptor site. Utilization of the first 5′ donor site yields mRNA for large T antigen, whereas utilization of the second site located 277 bases downstream yields the truncated small t antigen with shared N-terminal amino acid sequence. We have demonstrated that the JCV early mRNA is spliced as previously predicted by analogy to SV40. Further, our results demonstrate regulation of alternative 5′ splice site selection for JCV precursor early mRNA in the human brain. JCV small t antigen mRNA was detectable in only 8 of 13 brain tissues from PML patients without AIDS. In PML/AIDS brain, 11 of 12 (92%) brain tissues showed detectable small t antigen mRNA. This suggests that the production of small t antigen is regulated by unknown factors in the non-AIDS brain and is not essential for JCV infection of the brain. It is known that small t antigen is dispensable for the lytic growth of BK virus and SV40 (22). At the same time, it is possible that unknown factors related to HIV-1 infection promote alternative splicing of early mRNA and, thus, the production of small t antigen. If small t antigen stimulates JCV transcription

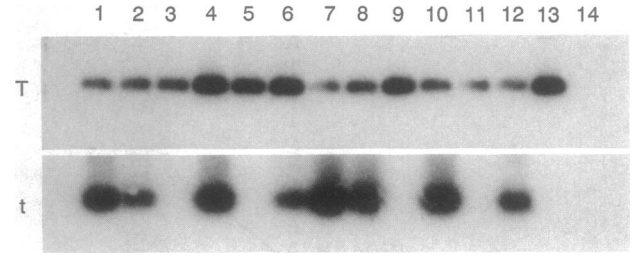

FIG. 5. RNA PCR amplification of large T (*Upper*) and small t (*Lower*) antigen mRNAs from brain tissues of PML patients without AIDS. PCR products (10 μl) were electrophoresed on agarose gels and hybridized with radiolabeled large T- and small t-specific oligonucleotide probes after transfer to nylon as in Fig. 2. Lanes: 1–13, 13 PML brain mRNAs; 14, reagent blank.

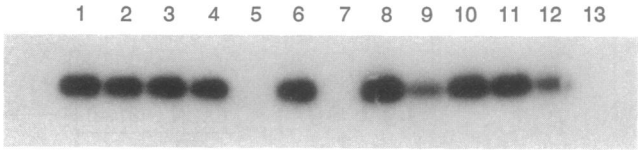

FIG. 6. PCR amplification of HIV-1 proviral DNA (*gag* gene) with primer pair SK38/SK39 in brain tissues from PML patients with AIDS. PCR products (10 μl) were electrophoresed on 2% agarose gel and hybridized with radiolabeled SK19 oligonucleotide probe after transfer to nylon. The filters were hybridized at 57°C and washed with 2× SSC/0.1% SDS at 57°C before exposing to x-ray film. Lanes: 1–12, 12 PML/AIDS brain tissue extracts; 13, reagent blank.

and/or replication, an additional mechanism whereby HIV-1 could promote JCV replication in the brain is suggested. Detailed sampling of the PML brain will be required to determine whether small t antigen expression varies from lesion to lesion and whether there is any correlation with the local presence or absence of HIV and its regulatory proteins.

A function of small t antigen in virus replication has not yet been defined for JCV, BK virus, or SV40. Recently, SV40 small t antigen was suggested to transactivate both early and late SV40 promoters, especially when the large T antigen levels were low (19). In addition, SV40 small t antigen may have the ability to transactivate promoters utilizing RNA polymerase II and III (23). Since small t antigen has not been shown to bind DNA, its effect is thought to be mediated by modification of viral or cellular transcription factors. Cellular protein phosphatase 2A (PP2A) forms complexes with SV40 small t antigen (24) and is, thus, a candidate for modulation of phosphorylation/dephosphorylation. Small t antigen inhibits PP2A activity by binding to the AC enzyme complex in place of regulatory subunit B (25). PP2A dephosphorylates large T antigen preferentially at Ser-120 and Ser-123, sites that affect binding to the origin of SV40 DNA (26). Small t antigen also inhibits the ability of PP2A to inactivate mitogen-activated protein kinase, thereby stimulating cell growth (27).

The factors regulating selection of alternative 5′ donor sites are unknown. A factor designated alternative splicing factor was purified from human 293 cells, a human embryonal kidney cell line transformed by adenovirus type 5 (28). These cells produce 10- to 20-fold more SV40 small t antigen mRNA than most other mammalian cell lines (29). This factor may be identical to an independently purified splicing factor designated SF2 that also influences 5′ splice site selection (30, 31). It is likely that regulation of these and other cellular factors could influence alternative 5′ splice site selection (31).

The incidence of PML in AIDS (≈5%) is high for this otherwise extremely rare disease, but the mechanism(s) whereby HIV-1 stimulates the replication of JCV in the human brain are still uncertain. *In vitro* studies have shown that HIV-1 Tat protein transactivates the JCV late promoter (7–9), and a putative TAR-like target element in the JCV promoter has been identified (9). Although there is little evidence that Tat substantially increases transcription from the JCV early promoter in glial cells, Tat has been reported to transactivate the SV40 early promoter in HeLa cells (32). Our results raise an additional possibility. An HIV-1 factor might promote selection of the alternative 5′ donor splice site, boosting expression of small t antigen. Most of the PML/AIDS brains studied here had demonstrable HIV-1 DNA sequences, and HIV-1 has many potential splice sites arranged to allow for the differential expression of various mRNAs, including the regulatory proteins Tat and Rev (33, 34). The influence of HIV-1 infection and its protein factors on the level and activity of splicing factors remains to be investigated.

Whatever the effect of HIV-1 regulatory proteins, if any, on JCV early mRNA splicing, it is clear that still other factors

are important in regulating this splicing in the non-AIDS brain. There are several possibilities to be considered: (*i*) The JCV regulatory region is hypervariable and is unique in each PML brain (4, 35). (*ii*) Two genotypes of JCV have been identified (36) that could differ in their level of alternatively spliced early mRNA. (*iii*) It is known that heat shock can influence splice site selection (37), and we have detected Hsp70 in JCV-infected glial cells in PML brains (G.L.S. and C. F. Ryschkewitsch, unpublished data). Local expression of inducible cellular proteins in JCV-infected glial cells might influence alternative splicing.

Previous methods for the detection of JCV large T and small t antigen mRNAs have been based on Northern blot and S1 nuclease analyses of the total RNA isolated from the tissues or cell lines (15). The PCR-based method for the identification of large T and small t antigen mRNAs described in this paper is specific and highly sensitive and can be used to process simultaneously a large number of samples. The method will also be useful for studying the regulation of latent JCV infections of the brain and other tissues. A study of nontumor brain tissue from 6-month-old hamsters infected at birth demonstrated abundant mRNA for both large T and small t antigen despite the absence of detectable JCV T antigen protein expression in glial cells (M.I. and H. G. Ressetar, unpublished data). Recently, a third T antigen (17kT) has been described in SV40-infected and SV40-transformed cells (38). Methods similar to those described here might be used to detect a comparable third T antigen mRNA in cells infected or transformed by JCV.

The picture that emerges for the role of JCV small t antigen in PML pathogenesis is that of a nonessential protein that serves to enhance viral replication (or cellular transformation) when present. A fuller understanding of the regulation of alternate early mRNA splicing and of the functions of small t antigen in viral replication may aid efforts to interrupt viral replication in this fatal brain infection.

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