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Transcription factor Spi-B binds unique sequences present in the tandem repeat promoter/enhancer of JC virus and supports viral activity

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Progressive multifocal leukoencephalopathy (PML) is an often fatal demyelinating disease caused by lytic infection of oligodendrocytes with JC virus (JCV). The development of PML in non-immunosuppressed individuals is a growing concern with reports of mortality in patients treated with mAb therapies. JCV can persist in the kidneys, lymphoid tissue and bone marrow. JCV gene expression is restricted by non-coding viral regulatory region sequence variation and cellular transcription factors. Because JCV latency has been associated with cells undergoing haematopoietic development, transcription factors previously reported as lymphoid specific may regulate JCV gene expression. This study demonstrates that one such transcription factor, Spi-B, binds to sequences present in the JCV promoter/enhancer and may affect early virus gene expression in cells obtained from human brain tissue. We identified four potential Spi-B-binding sites present in the promoter/enhancer elements of JCV sequences from PML variants and the non-pathogenic archetype. Spi-B sites present in the promoter/enhancers of PML variants alone bound protein expressed in JCV susceptible brain and lymphoid-derived cell lines by electromobility shift assays. Expression of exogenous Spi-B in semi- and non-permissive cells increased early viral gene expression. Strikingly, mutation of the Spi-B core in a binding site unique to the Mad-4 variant was sufficient to abrogate viral activity in progenitor-derived astrocytes. These results suggest that Spi-B could regulate JCV gene expression in susceptible cells, and may play an important role in JCV activity in the immune and nervous systems.

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INTRODUCTION

The human polyomavirus JC is the aetiological agent of the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML), that occurs in patients in states of immune suppression or modulation. PML is an AIDS-defining illness that occurs in approximately 3 % of HIV-infected patients (Major, 2010). Recently, PML has been reported in patients undergoing immuno-modulatory therapies (Major, 2010; Marshall & Major, 2010). PML is caused by lytic multiplication of the JC virus (JCV) in oligodendrocytes, which results in the loss of myelination of neurons and loss of neuronal function. Despite its lytic capability, JCV can persist in a variety of cell types including $CD34^+$ haematopoietic precursors and B cells present in bone marrow (Houff *et al.*, 1988; Marzocchetti *et al.*, 2008; Tan *et al.*, 2009), brain, tonsil and in circulation (Sabath & Major, 2002). Detection of JCV DNA in the absence of viral capsid protein in these cell types from patients prior to the development of PML suggests that such cells may act as a reservoir for latent virus (Houff *et al.*, 1988; Tan *et al.*, 2009). Trafficking of JCV-infected B cells, or haematopoietic precursors, between the bone marrow and brain is a possible method of viral dissemination.

The JCV genome is a closed, circular, supercoiled DNA chromosome that permits temporal expression of early and late genes that are physically separated by the non-coding viral regulatory region (RR) (Frisque *et al.*, 1984). RR is the most highly variable sequence among JCV isolates but always contains the origin of replication, one or more TATA boxes and a variety of enhancer elements (Ault & Stoner, 1993; Frisque, 1983; Frisque *et al.*, 1984; Iida *et al.*, 1993). The RR from the original isolate of JCV, Mad-1, contains a promoter/enhancer that exists as 98 bp tandem repeats containing two TATA boxes and multiple cellular transcription factor-binding sites (Frisque, 1983). TATA boxes in the tandem repeat sequence are essential for transcription of early and late viral genes (Daniel & Frisque, 1993; Kenney *et al.*, 1986a, b; Khalili *et al.*, 1986; Krebs *et al.*, 1995; Vacante *et al.*, 1989). RR variants containing tandem repeats have been isolated from tissues of patients with PML (Martin *et al.*, 1985). The RR of a naturally occurring variant of JCV shed in urine, referred to as 'archetype', contains a single 98 bp unit with internal

23 and 66 bp insertions (Yogo *et al.*, 1990). Archetype is not associated with PML and is replication incompetent in tissue culture (Daniel *et al.*, 1996). Consistent isolation of tandem repeat containing sequences in tissues obtained from PML patients strongly suggests the importance of these sequences in viral pathogenesis (Frisque *et al.*, 1984; Jensen & Major, 1999; Martin *et al.*, 1985; Marzocchetti *et al.*, 2008; Vaz *et al.*, 2000).

Interestingly, JCV multiplication in susceptible cells is restricted by activation of viral gene expression by host transcription factors such as Oct-6/tst-1/SCIP (Wegner *et al.*, 1993), pur α (Chen & Khalili, 1995), YB-1 (Chen *et al.*, 1995c; Kerr *et al.*, 1994) and NF-1 (Amemiya *et al.*, 1989, 1992; Messam *et al.*, 2003; Monaco *et al.*, 2001; Ravichandran & Major, 2008; Shivakumar & Das, 1994; Sumner *et al.*, 1996; Tamura *et al.*, 1988). However, NF-1X is the only factor that has been shown to be important for JCV activity in glial (Kumar *et al.*, 1993, 1996) and immune cells (Monaco *et al.*, 2001). Because JCV latency is associated with cells undergoing haematopoietic development, it is probable that transcription factors previously reported as lymphoid specific regulate JCV gene expression. One such factor, Spi-B, activates gene expression from a lymphotrophic variant of SV40 (Petterson & Schaffner, 1987) and lymphotrophic papovavirus (LPV) (Erselius *et al.*, 1990), both of which share genetic architecture and promoter regulation with JCV. In addition, Spi-B is upregulated in peripheral blood mononuclear cells in response to treatment with Natalizumab (Lindberg *et al.*, 2008), an immuno-modulatory therapy that has been associated with the development of PML (Major, 2010). Spi-B is an Ets transcription factor that is required for normal B-cell receptor signalling and formation of germinal centres in the spleen (Garrett-Sinha *et al.*, 1999). Spi-B binds target sequences containing a 5′-GGAA/T-3′ core (Araki *et al.*, 1988; Dorn *et al.*, 1988; Erselius *et al.*, 1990; Laux *et al.*, 1994; Petterson & Schaffner, 1987; Wasylyk *et al.*, 1993) and can cooperate with the retinoblastoma protein (pRB) to alter expression of proteins involved in B-cell maturation (Hagemeier *et al.*, 1993; Mao *et al.*, 1996; Rao *et al.*, 1999b; Weintraub *et al.*, 1995). Spi-B is involved in differentiation and maturation of B cells and is expressed at high levels in developing and mature B cells (Chen *et al.*, 1995a, b; Ray *et al.*, 1992; Su *et al.*, 1996).

RESULTS

Spi-B-binding sites are present in JCV promoter/enhancer sequences

The Spi-B-binding site consensus sequence was identified as 5′-WWWRR**RGAA**SNDR-3′ where the internal **RGAA** core is conserved (Laux *et al.*, 1994). Promoter/enhancer sequences from PML-associated Mad-1 (Frisque *et al.*, 1984), Mad-4 (Martin *et al.*, 1985) and the non-pathogenic archetype variants (Yogo *et al.*, 1990) were analysed for Spi-B-binding sites using the criteria of a 5′-**NGAA**-3′ core. Four potential Spi-B-binding sites (Fig. 1) were identified in the JCV promoter/enhancer sequences and are listed in Table 1. Mad-1 (Fig. 1a) and Mad-4 (Fig. 1b) promoter/enhancers contain a binding site, labelled L5, adjacent to the TATA box in the first repeat. Mad-1 contains a second L5 site in the second repeat. A naturally occurring 19 bp deletion in the Mad-4 promoter/enhancer results in the loss of the second TATA box and a unique Spi-B-binding site labelled L4. The archetype promoter/enhancer (Fig. 1c) contains 23 and 66 bp insertions within a single 98 bp unit, resulting in the loss of the L5/L4-binding sites. The 23 bp insertion results in a single base pair change, 5′-AAAAGGGAAGGGA-3′, at the 3′ end of the L5 site to 5′-AAAAGGGAAGGTA-3′, labelled L3. The 66 bp insertion resulted in a unique site labelled L14.

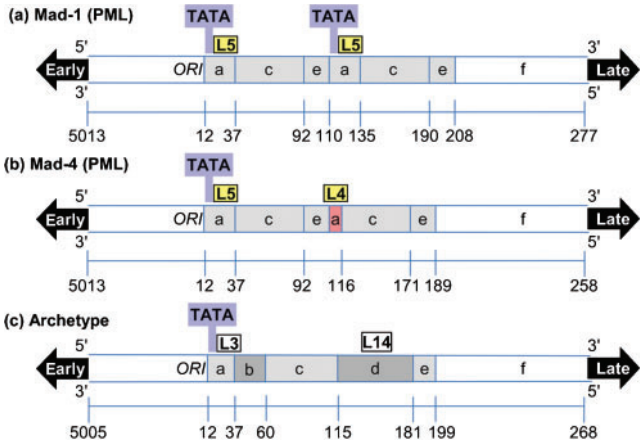

Fig. 1. Diagram of potential Spi-B-binding sites in JCV promoter/enhancer sequences. The non-coding viral RRs from the PML-associated Mad-1 (a), Mad-4 (b) and the non-pathogenic archetype (c) JCV variants are represented. The PML-associated variants contain 98 bp tandem repeats (light grey). A 19 bp deletion in the second repeat of Mad-4 (red) results in the loss of the second TATA box (blue). The archetype sequence contains two inserts (dark grey) in a single 98 bp unit. Sites that bind Spi-B protein in EMSA assays are shaded in yellow and sites that did not bind protein are white.

Spi-B is expressed in JCV susceptible cells

JCV infects a variety of different cell types including $CD34^+$ haematopoietic precursors, B cells, and astrocytes in culture. However, Spi-B gene expression has been described exclusively in the B cell lineage (Dahl *et al.*, 2002; Erselius *et al.*, 1990; Garrett-Sinha *et al.*, 2005; Kim *et al.*, 2003; Ray *et al.*, 1992; Schmidlin *et al.*, 2008; Schweitzer & DeKoter, 2004; Su *et al.*, 1996, 1997). Northern blot analysis of Spi-B mRNA in brain tissue from mice showed no message present (Ray *et al.*, 1992; Su *et al.*, 1996). Using a sensitive method of quantitative real-time RT-PCR (qRT-PCR), Spi-B mRNA expression was measured in the various cell types susceptible to JCV (Table 2). Spi-B mRNA was detected above the non-template control for

Table 1. Potential Spi-B-binding site sequences present in the promoter/enhancers of JC virus variants

Spi-B-binding site	Viral variant	Sequence (5′–3′)
SV40	SV40	CTGAAAGAGGAACTTG
SV40 mutant	–	CTGAAAGACCAACTTG
L4	Mad-4	CAAGGGGAAGGGA
L4 mutant	–	CAAGGCCAAGGGA
L5	Mad-1/Mad-4	AAAAGGGAAGGGA
L5 mutant	–	AAAAGCCAAGGGA
L3	Archetype	AAAAGGGAAGGTA
L14	Archetype	TATAGTGAAACCC

background detection in all cell types tested. Spi-B mRNA expression was measured at low levels in two cell types, HeLa cells and primary $CD3^+$ T lymphocytes, in which Spi-B protein has not been detected. Immortalized B cells (Raji and BJAB) and primary $CD19^+$ B cells expressed 100-fold and greater levels of Spi-B mRNA in comparison. A $CD34^+$ cell line (KG-1a) and primary $CD34^+$ haematopoietic precursors expressed less Spi-B mRNA than B cells, which is consistent with published reports (Su *et al.*, 1996). The human fetal brain-derived SVG cell line, which supports robust JCV multiplication, expressed Spi-B mRNA comparable to $CD34^+$ haematopoietic precursors. Human fetal brain-derived cells (progenitors, astrocytes and neurons) expressed Spi-B mRNA at levels similar to HeLa and primary T lymphocytes. Spi-B protein expression was measured by protein blotting using an antibody that detects a single 43 kDa band for Spi-B (Arguello *et al.*, 2003). Spi-B protein was detected in BJAB cells and slightly lower in KG-1a cells (Fig. 2a) consistent with mRNA levels. Spi-B protein was detected in SVG cells and in progenitor-derived astrocytes (PDA), but not progenitor cells or progenitor-derived neurons (PDN) (Fig. 2b). Spi-B protein expression in the absence of high levels of mRNA in PDAs may be due to instability and/or turnover of the mRNA. Stability of the highly related Spi-1 mRNA has been demonstrated to be extensively regulated at the post-transcriptional level (Hensold *et al.*, 1996), which may also be the case for Spi-B.

Spi-B binds unique sites on JCV enhancers

Because potential Spi-B-binding sites are present in JCV promoter/enhancer sequences and cells susceptible to JCV infection express Spi-B, the association of Spi-B with potential binding sites was measured by electromobility shift assay (EMSA) using whole-cell extracts from KG-1a cells, BJAB cells and PDAs (Fig. 3). Cell extracts were incubated with double-stranded oligonucleotide probes encoding the Spi-B-binding sites described in Table 1, or the SV40 Spi-B site described previously (Petterson & Schaffner, 1987) as a positive control. Fig. 3(a) illustrates that protein present in BJAB cell extract binds the SV40 probe and causes a shift that was competed by a 5-, 100- and 400-fold excess of unlabelled probe. Alteration of the Spi-B site core from 5′-GGAA-3′ to 5′-CCAA-3′ abrogated protein binding to the mutant probe, indicating that complex formation was specific for the Spi-B core. Addition of Spi-B antiserum, suitable for distinguishing Spi-B from other related proteins in an EMSA assay (Laux *et al.*, 1994), caused a supershift in the original complex,

Table 2. Spi-B mRNA expression in immune- and brain-derived cells types

Category	Cell type	Spi-B mRNA, relative level	Standard deviation
Control	HeLa	0.01	0.0002
	$CD3^+$ lymphocytes	0.01*	0.0061
B cells	Raji	2.17	0.1262
	BJAB	1.66	0.0014
	$CD19^+$ lymphocytes	1.16*	0.1616
Haematopoietic precursor	KG-1a	0.31	0.0082
	$CD34^+$ lymphocytes	0.75*	0.3661
Brain-derived cells	SVG	0.30	0.0273
	Human fetal brain progenitor cells	0.02	0.0001
	PDA	0.01	0.0013
	PDN	0.10	0.0036

*Spi-B mRNA value is a mean of cell purified from multiple blood donors.

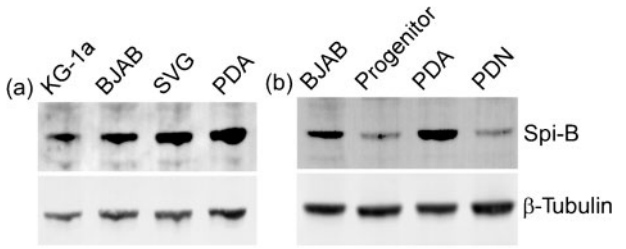

Fig. 2. Spi-B is expressed in JCV susceptible cells. Spi-B protein was detected in JCV susceptible KG-1a, BJAB, SVG and PDA (a) and cells derived from human fetal brain progenitors, PDA and PDN (b) by protein blotting. β-Tubulin protein levels were detected to demonstrate equal loading.

which indicates that protein bound to the SV40 probe in the shifted complex is Spi-B.

The same EMSAs were carried out for the potential JCV Spi-B-binding sites. Probes for Spi-B sites present in archetype promoter/enhancers, L3 and L14, did not bind protein in any cell type tested (Fig. 3b). Probes for Spi-B sites present in PML-associated promoter/enhancers, L5 and L4, bound protein expressed in KG-1a, BJAB and PDAs shown in Fig. 3(c) and (d), respectively. Incubation of both the L5 and L4 probes with cellular extracts resulted in the formation of multiple protein–DNA complexes labelled shifts C1–C3 that were consistent between cell types. Formation of multiple protein–DNA complexes on Spi-B-binding sites has been described using EMSA analysis for many promoters in published reports (Dekoninck *et al.*, 2003; Erselius *et al.*, 1990; Garrett-Sinha *et al.*, 2005; Laux *et al.*, 1994; Petterson & Schaffner, 1987; Zhao & Sample, 2000). In the case of JCV L5 and L4, each complex was competed by excess of unlabelled oligonucleotide probe and alteration of the Spi-B site core to 5′-CCAA-3′ resulted in abrogation of formation of all complexes. Addition of Spi-B antiserum caused supershifts of the original complexes, similar to that observed for SV40. The C2 shift for the L5 site and C3 shift for the L4 site required higher excess unlabelled probe for competition and were not completely supershifted upon addition of Spi-B antiserum, suggesting an abundance of these complexes over others. These results suggest that Spi-B is a component of multiple complexes at these locations on the viral promoter/enhancer. Spi-B is known to bind multiple cofactors that cooperate to affect gene expression such as cellular proteins TBP and pRB (Rao *et al.*, 1999b), OBF-1 (Bartholdy *et al.*, 2006) and CBP (Yamamoto *et al.*, 2002), and the viral proteins EBNA-2 and EBNA-3C for Epstein–Barr virus (Zhao & Sample, 2000). It is possible that the multiple shifts represent separate complexes that may include cofactors necessary for activity of Spi-B. Importantly, competition with varying levels of unlabelled probe and Spi-B antiserum supershifts demonstrate that each of the complexes formed for both L5 and L4 are Spi-B specific.

Expression of exogenous Spi-B in semi- and non-permissive cells increases T-antigen expression

Because Spi-B is expressed in cells that are susceptible to JCV infection, we sought to determine if Spi-B overexpression in non-permissive cells could support viral activity. Previous studies showed that exogenous expression of NF-1X in non-permissive cells by transfection prior to JCV exposure increased T-antigen expression (Messam *et al.*, 2003). NF-1X and Spi-B were expressed by transient transfection in semi-permissive progenitor cells and non-permissive PDNs for 24 h followed by exposure to Mad-4 JCV. Five days after JCV exposure, cells were analysed for expression of T antigen, as well as the cell-specific marker for progenitor cells (nestin) or neurons (β III tubulin). Double-positive cells for T antigen and the appropriate cell-specific marker were quantified from three separate experiments. Pre-expression of NF-1X and Spi-B resulted in a statistically significant increase in T-antigen expression in progenitors (Fig. 4a) and PDNs (Fig. 4b). NF-1X protein expression was confirmed 3 days post-transfection by immunofluorescence (progenitors: 5–10 %; PDN: 1 %). An increase in Spi-B mRNA expression was confirmed 3 days post-transfection by qRT-PCR. Spi-B gene expression was increased from 0.0005 to 1.7 in progenitor cells and from 0.05 to 1.7 in PDNs. These results demonstrate that expression of Spi-B, like NF-1X, supports early viral gene expression.

Mutation of the L4 Spi-B-binding site in the Mad-4 promoter/enhancer is sufficient to abrogate JCV activity in PDAs

To determine if Spi-B sites in the JCV promoter/enhancer that bind Spi-B are important for viral activity, site-directed mutagenesis was used to generate plasmids encoding mutations in the Spi-B site core of the L5 and L4 sites in Mad-4 (Table 1). Plasmids encoding archetype, Mad-1, Mad-4, L5 mutant Mad-4, L4 mutant Mad-4 or L5/L4 double-mutant Mad-4 were introduced into PDAs via nucleofection. T-antigen expression was quantified on a per cell basis by immunofluorescence, 6 days after nucleofection. Fig. 5 demonstrates that T-antigen expression occurred at similar levels from the Mad-1 and Mad-4 plasmids, while expression from the archetype plasmid was rarely detected. Mutation of the L5 site resulted in a slight, but not statistically significant, increase in T-antigen expression comparable to Mad-1. Mutation of both the L5 and the L4 sites resulted in abrogation of T-antigen expression. Importantly, mutation of the L4 site alone also resulted in abrogation of T-antigen expression. These results demonstrate that abrogation of viral activity due to the L4 site mutation is dominant in the presence of a wild-type L5 site.

DISCUSSION

The promoter/enhancer of JCV is considered to be the portion of the RR that confers specific tissue tropism and supports development of PML. PML-associated variants

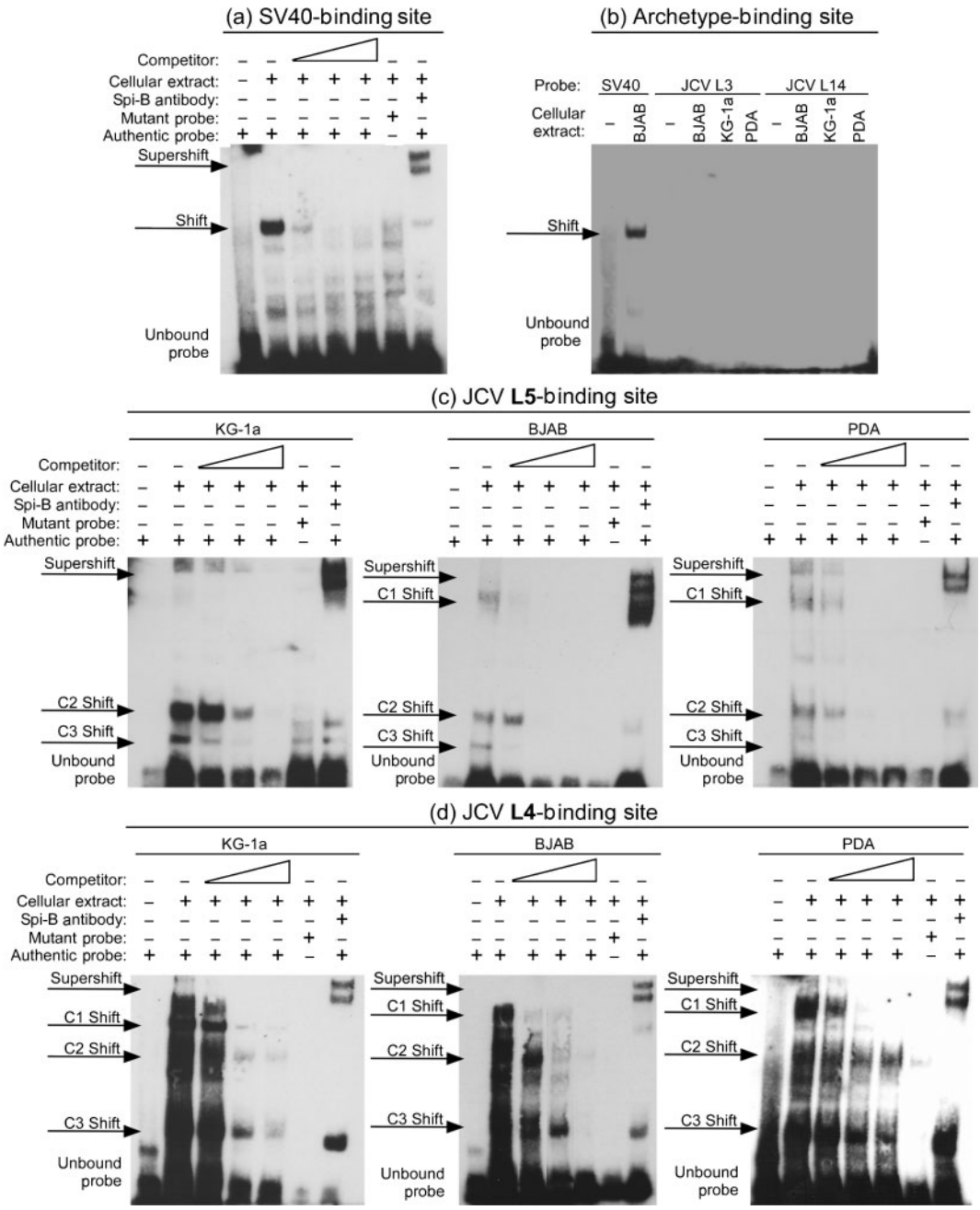

Fig. 3. Spi-B binds unique sites present on JCV promoter/enhancers. An EMSA was performed using BJAB cell extract and biotin-labelled oligonucleotides for the SV40 Spi-B site as a positive control for Spi-B-DNA complex formation (a). Authentic-binding site probe was incubated with cell extract alone or in combination with 5-, 100- or 400-fold excess unlabelled oligonucleotide competitor, or Spi-B antiserum. Mutant probe was incubated with cell extract to demonstrate specificity for the Spi-B-binding site core. Identical EMSAs were performed using BJAB, KG-1a, and/or PDA cell extract and biotin-labelled oligonucleotides for the archetype JCV L3 and L14 (b) and JCV L5 (c) and L4 (d) Spi-B sites as probes.

Mad-1 and Mad-4 contain promoter/enhancers that exist as tandem repeats, which contain TATA boxes and duplications of essential transcription factor-binding sites including Spi-B. In this study, Spi-B-binding sites that actively bound Spi-B protein expressed in JCV susceptible cell types are present in promoter/enhancer sequences from Mad-1 and Mad-4, but not the non-pathogenic archetype (Table 2, Figs 2 and 3). Mad-1 contains two identical Spi-B-binding sites (L5) within each of the 98 bp repeats, while Mad-4 contains the same site as Mad-1 (L5) in the first repeat followed by a unique site (L4) in the second repeat that results from a naturally occurring 19 bp deletion.

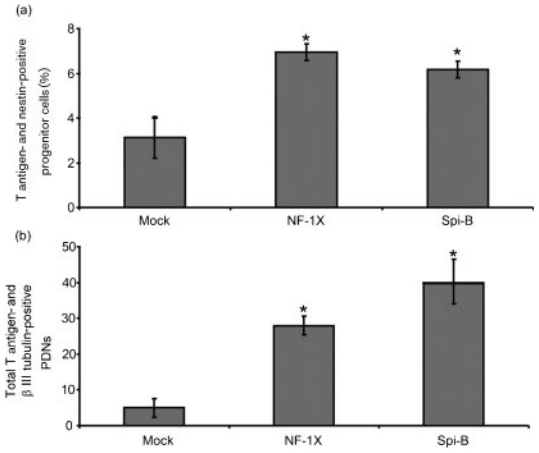

Fig. 4. Expression of exogenous Spi-B in semi- and non-permissive cells increases T-antigen expression. The fraction of T antigen and nestin double-positive progenitor cells (a) or β III tubulin double-positive PDN (b) is represented along with the SD. An asterisk denotes the following statistically significant change in value. Progenitors: NF-1X $P=0.0006$, Spi-B $P=0.0063$; PDN: NF-1X $P=0.0004$, Spi-B $P=0.0012$.

Importantly, mutation of the unique Mad-4 L4 Spi-B site and not L5 resulted in abrogation of T-antigen expression in PDAs. The locations of the L5- and L4-binding sites are of particular interest in relation to JCV activity.

Abrogation of T-antigen gene expression in response to mutation of the L4 site within the viral promoter/enhancer suggests that an Spi-B site in the second tandem repeat is important for early viral gene expression (Fig. 5). Strikingly, the presence of a functional Spi-B site in the first repeat (L5) is incapable of compensating for the loss of the Spi-B site in the second repeat (L4). Mutation of the L5 site alone did not significantly alter the ability of the virus

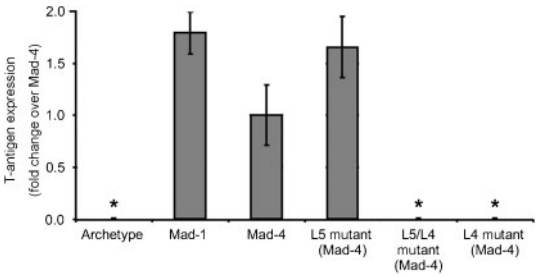

Fig. 5. Mutation of the L4 Spi-B-binding site in the Mad-4 promoter/enhancer is sufficient to abrogate JCV activity in PDA. The fraction of T antigen-positive cells as a fold change over the wild-type Mad-4 plasmid for archetype, Mad-1, Mad-4, L5 mutant Mad-4, L4 mutant Mad-4 or L5/L4 mutant Mad-4 plasmids is represented along with SD. An asterisk denotes the following statistically significant change in value. Archetype, $P=0.039$; L5/L4 mutant, $P=0.039$; L4 mutant, $P=0.039$.

to express T antigen; however, this could be due to compensation by the L4 site. The L4 site may be dominant over an initial L5 site in the context of the tandem repeat. Due to its deletion in a variety of RR sequences from PML patients including Mad-4, Her-1, Mad-7, Mad-8, Mad-9 and Mad-11 (Major *et al.*, 1987; Martin *et al.*, 1985; Matsuda *et al.*, 1987) the second TATA box was not thought to be necessary for virus multiplication (Lynch & Frisque, 1990; Martin *et al.*, 1985). In fact, molecular studies on the function of the Mad-1 RR often utilize JCV sequences that only contain a single 98 bp unit and, therefore, a single TATA box and Spi-B site (Kerr *et al.*, 1994; Sunden *et al.*, 2007). However, the maintenance of tandem repeat nucleotide sequences throughout PML-associated variants illustrates their importance in the pathogenesis of JCV (Gosert *et al.*, 2010). Utilizing RRs with a single 98 bp unit may miss the importance of measuring the relationship between tandem repeats. Future studies should include full-length RR to include the effects of a functional second repeat in the context of JCV multiplication and pathogenesis.

Spi-B-binding sites within the first repeat of the promoter/enhancers of PML-associated JCV variants are situated directly downstream of the viral origin and TATA box elements, which are essential for T-antigen binding and activation of viral gene expression (Daniel & Frisque, 1993; Kenney *et al.*, 1986a, b; Khalili *et al.*, 1986; Krebs *et al.*, 1995; Vacante *et al.*, 1989). Activation of gene expression from the basal promoter is an essential event for JCV multiplication and reactivation from latency. SV40 T antigen interacts with proteins that recruit the basal transcriptional apparatus (TFIID) to the viral promoter/enhancer including TATA-binding protein (TBP) and transcription-enhancing factor 1 (Damania & Alwine, 1996; Gruda *et al.*, 1993; Zhai *et al.*, 1997) and some evidence suggests similar interactions for JCV T antigen (Rekvig, 1997). Importantly, initial events of JCV infection occur in the absence of T-antigen protein, suggesting that other factors regulate this process. The amino-terminal portion of Spi-B binds TBP and is capable of recruiting the TFIID complex to promoters (Rao *et al.*, 1999b). Therefore, a potential mechanism for activation of JCV early gene expression in the absence of T protein could involve Spi-B binding to TBP on the JCV promoter/enhancer and recruitment of the TFIID complex. It has been demonstrated that TBP containing transcription factor complexes form on TATA-less promoters and are required for transcription (Pugh & Tjian, 1991). Therefore, Spi-B bound to the L4 site in the TATA-less second repeat of Mad-4 could be capable of recruiting TBP and TFIID to initiate transcriptional activation in the absence of a TATA box. In this case the L4 site is positioned to compensate for the lack of the TATA box in the Mad-4 variant.

In addition to TATA boxes, these areas are bound heavily by other host factors that regulate viral activity. Oct-6/tst-1/SCIP-binding sites are located adjacent to both the L5 and L4 Spi-B sites on their 5′ ends and NF-1-binding sites

are located adjacent to both Spi-B sites on their 3′ ends. Importantly, neither the Oct-6/tst-1/SCIP nor the NF-1-binding sites directly overlap the Spi-B-binding sites. Therefore, these factors would not affect Spi-B binding in the EMSA analysis presented in Fig. 3 and would not be affected by the mutations introduced in the Mad-4 L5, L4 or L5/L4 double mutants presented in Fig. 5. Pur α and YB-1 bind opposite strands of the single-stranded JCV promoter/enhancer sequence, which overlap the L5 Spi-B-binding site, and regulate early and late viral gene expression, respectively, in cooperation with the large T antigen (Chen *et al.*, 1995c; Chen & Khalili, 1995; Kerr *et al.*, 1994). Nucleotide changes equivalent to the mutations in the Mad-4 L5, L4 or L5/L4 double mutants were introduced into the YB-1/pur α-binding site reported by Chang *et al.* (1996) and did not have any significant effect on the ability of protein from BJAB cells or PDAs to bind the authentic probe in competition experiments (data not shown). Importantly, studies on YB-1/pur α-binding sites focus on sequences present in the first tandem repeat and emphasize the importance of T-antigen binding to sites present in the origin of replication (Chen *et al.*, 1995c; Chen & Khalili, 1995; Kerr *et al.*, 1994). In the context of a Mad-4 JCV promoter/enhancer, the naturally occurring 19 bp deletion results in alteration of the YB-1/pur α-binding site in the second tandem repeat. In addition, YB-1/pur α-binding site in the second tandem repeat of both Mad-1 and Mad-4 would not contain the important T-antigen binding sites in proximity because they occur directly after the first repeat. Taken together these points suggest that the YB-1/pur α-binding site would not play a role in the abrogation of early gene expression demonstrated in Fig. 5 for the L4 mutant plasmid.

Spi-B is largely considered a lymphotrophic transcriptional activator (Rao *et al.*, 1999a; Yamamoto *et al.*, 2002) and is known to transactivate the λ2–4 enhancer (Su *et al.*, 1996), the LMP/TP2 promoter of Epstein–Barr virus (Laux *et al.*, 1994), the SV40 enhancer (Petterson & Schaffner, 1987) and the LPV promoter (Erselius *et al.*, 1990). JCV is capable of infecting a variety of cells of the lymphoid system. Detection of tandem repeat containing JCV DNA sequence in the absence of viral capsid protein in the bone marrow in patients prior to the development of PML suggests that these cells may act as a reservoir for latent virus (Houff *et al.*, 1988; Tan *et al.*, 2009). Spi-B expression in developing B cells correlates with reactivation of JCV in immune cells. Studies in mice demonstrate that Spi-B is expressed at a low level in pro-B cells, increases in pre-B cells and is highest in mature B cells (Su *et al.*, 1996). Spi-B expression during B-cell development was confirmed in human using prototypic cell lines (Ray *et al.*, 1992). If Spi-B is indeed a transcriptional activator of JCV gene expression, a minimal level of Spi-B expression in haematopoietic progenitors and pro-B cells may support latency of JCV, while upregulation of Spi-B expression in pre-B and mature B cells may lead to reactivation of JCV gene expression and production of viral progeny.

Because the number of patients undergoing immunomodulatory therapies that develop PML continues to rise (Major, 2009), understanding the role of Spi-B during JCV latency and reactivation is increasingly important. These therapies cause mobilization and expansion of cells that have the potential to harbour latent JCV infection. Natalizumab has also been shown to upregulate genes involved in B-cell differentiation, including Spi-B (Lindberg *et al.*, 2008). If Spi-B is an activator of JCV gene expression it could be a contributing factor in the development of PML in patients undergoing these immuno-modulatory therapies. In addition, PML-associated Mad-4 contains a duplication of the sites due to the tandem repeat nature of its promoter/enhancer, one of which (L4) was essential for T-antigen expression demonstrated by mutational analysis in Fig. 5. Accumulation of Spi-B sequence motifs in promoters can confer tissue specificity (Gerster *et al.*, 1987; Ondek *et al.*, 1987; Schirm *et al.*, 1987) as illustrated in the case of a lymphotrophic SV40 variant (Petterson & Schaffner, 1987). Accumulation of additional, or unique, Spi-B-binding sites in the promoter/enhancers of JCV RRs may contribute to higher levels of replication in lymphoid tissues that in turn lead to development of PML. Further investigation into the role of Spi-B during JCV infection and the presence of duplicated, and or unique, Spi-B-binding sites in the RR sequences derived from PML patients will offer insight into the molecular mechanism of JCV reactivation and the development of PML.

METHODS

Cells, plasmids and viruses. Human growth factors for cell culture were obtained from Peprotech. KG-1a cells were maintained in RPMI 1640 medium (Cellgro) supplemented with 20 % FBS (Atlanta Biologics) and 2 mM L-glutamine (Quality Biologics). BJAB and Raji cells were maintained in RPMI 1640 medium supplemented with 10 % FBS and 2 mM L-glutamine. SVG cells were maintained in minimal essential medium (MEM; Cellgro) supplemented with 10 % FBS and 2 mM L-glutamine. Human central nervous system progenitors were isolated and differentiated into astrocytes and neurons as described previously (Messam *et al.*, 2003).

A plasmid encoding the full-length human Spi-B cDNA, pΔEB-Spi-B, was obtained from Francoise Moreau-Gauchelin at the Institut Curie in Paris, France (Ray *et al.*, 1992). A plasmid encoding the full-length human NF-1X cDNA with an amino-terminal haemagglutinin (HA) tag, pCHA-NF1X, was described previously (Monaco *et al.*, 2001). A plasmid encoding the full-length JCV Mad-4 genome, pMad4(586), was described previously (Martin *et al.*, 1985). A plasmid encoding the full-length JCV Mad-4 genome, $pM1_{TC}$, was described previously (Frisque *et al.*, 1984). A plasmid encoding the full-length JCV archetype genome, CY, was described previously (Yogo *et al.*, 1990).

Isolation of primary lymphocytes. Mononuclear cells were isolated from the peripheral blood of normal donors provided by the NIH clinical centre blood bank by centrifugation on Ficoll-Hypaque gradients. $CD34^+$ haematopoietic precursors, $CD19^+$ B cell and $CD3^+$ T-cell populations were purified using the RoboSep brand of immunomagnetic cell separation (StemCell Technologies).

Antibodies. A mouse mAb for SV40 T antigen, which cross-reacts with JCV T antigen (EMD Calbiochem) was used at 5 μg ml^{-1}. The

mouse mAb GA5 for human glial fibrillary acid protein (GFAP) (Cell Signaling Technology) was used at a 1 : 300 dilution. The rabbit polyclonal antibody for GFAP (Covance) was used at a 1 : 1000 dilution. The rabbit polyclonal antibody TUJ1 for neuronal class III β-tubulin (Covance) was used at a 1 : 2000 dilution. HA-tagged human NF-1X was visualized with the rat mAb 3F10 for HA (Roche Diagnostics) at 200 ng ml^{-1}. The rabbit polyclonal antibody 331B for human nestin was used at a 1 : 200 dilution (Messam *et al.*, 2000). The goat polyclonal antibody for human Spi-B (Santa Cruz Biotechnology Inc) was used at a 1 : 200 dilution. The mouse mAbs for β actin AC-15 and for β-tubulin TUB 2.1 (Sigma) were used at 1 : 1000 dilutions. Fluorescent-labelled antibodies qualified for multiple labelling experiments were obtained from Invitrogen and used at 1–2 μg ml^{-1}.

Preparation of Spi-B-binding site mutants in a Mad-4 background. Site-directed mutagenesis was performed on the pMad4(586) plasmid to alter the L5 and L4 Spi-B site cores from 5′-GGAA-3′ to 5′-CCAA-3′ using the QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Sense oligonucleotides (L4: 5′-GTAAACAAAGCACAAGGCCAAGGGATGGCTGCCAGC-3′; L5: 5′-TCCTGTATATATAAAAAAAAGCCAAGGGATGGCTGCCAGCCA-3′) and antisense oligonucleotides (L4: 5′-GCTGGCAGCCATCCCTTGGCCTTGTGCTTTGTTTAC-3′; L5: 5′-TGGCTGGCAGCCATCCCTTGGCTTTTTTTTATATATACAGGA-3′) containing the 5′-GG-3′ to 5′-CC-3′ mutations were synthesized by Integrated DNA Technologies (IDT). To create the L5/L4 double-mutant plasmid, site-directed mutagenesis was performed on the L4 mutant plasmid using the L5 sense and antisense oligonucleotides. DNA sequencing was performed to confirm the presence of each desired mutation as well as the fidelity of Mad-4 by the NINDS DNA sequencing facility.

Isolation of total RNA. Total RNA was isolated from cell lines and primary cells using the RNeasy plus mini kit (Qiagen) according to the manufacturer's instructions. RNA was eluted from the column in nuclease-free water and quantified using a Nanodrop 8000 (Thermo Scientific).

Preparation of whole-cell extracts. Whole-cell extracts were prepared by using a modification of the method of Andrews & Faller (1991) as described previously (Monaco *et al.*, 2001).

Protein blotting. Fifteen micrograms of protein was separated by electrophoresis in a 4–12 % Bis-Tris gel followed by transfer to PVDF membrane. Membranes were blocked for 1 h in 5 % non-fat dry milk in Tris-buffered saline containing 50 mg BSA ml^{-1}, 10 mg glycine ml^{-1}, 0.05 % Tween-20 (TBS-BGT). Membranes were incubated with primary antibodies diluted in TBS-BGT for 3 h. Unbound antibody was removed by washing in TBS-BGT followed by 1 h incubation in secondary fluorescent-conjugated antibodies (Invitrogen). Unbound antibody was removed by washing in TBS-BGT. The antibody–antigen complex was visualized using a FluorChem Q imager (Alpha Innotech).

Immunofluorescence. Immunofluorescence was conducted on Lipofectamine 2000 transfected, nucleofected and JCV-infected cell cultures. Cells were fixed with 4 % paraformaldehyde and permeabilized with 0.2 % Triton X-100 before indirect antibody labelling. Samples were mounted with a glycerol-based mounting medium containing the DNA dye, 4′,6-diamidino-2-phenylindole (DAPI) and analysed by fluorescence microscopy using a Zeiss Axiovert 200M microscope fitted with filters appropriate for DAPI, Alexa Fluor 488 and Alexa Fluor 568 excitation.

qRT-PCR. Spi-B mRNA present in cells was measured by reverse transcription followed by qRT-PCR using the TaqMan gene expression assay (Applied Biosystems) for human Spi-B (Hs00162150_m1), human β actin (Hs99999903_m1) and human PUM1 (Hs00206469_m1). Reverse transcription (RT) of 1 μg of total RNA was performed using qScript cDNA supermix (Quanta Biosciences) according to the manufacturer's instructions. Six 1 : 10 serial dilutions of the resultant BJAB RT reaction were prepared to generate a relative standard curve to determine Spi-B mRNA levels. Singleplex qRT PCRs were assembled using each RT reaction with 2 × TaqMan universal PCR master mix and 20 × TaqMan human Spi-B gene assay or 20 × TaqMan human β actin or PUM1 endogenous control assay according to the manufacturer's instructions (Applied Biosystems). Relative quantification of Spi-B gene expression was determined using the relative standard curve method described on the Applied Biosystems website (Biosystems, 2008). Spi-B mRNA levels were normalized to input template based on the endogenous control. The BJAB standard is assigned a value of one and the other cell types are reported as values relative to BJAB.

EMSA. Oligonucleotides with the sequence of the SV40 Spi-B site (5′-CTGAAAGAGGAACTTG-3′), or the JCV Spi-B site L3 (5′-AAAAGGGAAGGTA-3′), L4 (5′-CAAGGGGAAGGGA-3′), L5 (5′-AAAGGGAAGGGA-3′) and L14 (5′-TATAGTGAAACCC-3′) were synthesized with and without 5′ biotinylation by IDT. Mutated versions of the SV40 Spi-B site (5′-CTGAAAGACCAACTTG-3′), and JCV Spi-B L4 (5′-CAAGGCCAAGGGA-3′) and L5 (5′-AAAGCCAAGGGA-3′) were synthesized with 5′ biotinylation. Oligonucleotides with sequences complementary to those listed above were also synthesized with or without 5′ biotinylation as indicated above. The oligonucleotides for the authentic- or mutated-binding sites were annealed to form double-stranded probe at a concentration of 100 ng μl^{-1}. The biotin labelled probes were diluted 1 : 200 in water. Biotin labelled authentic probe or mutant probe was incubated with 5–25 μg nuclear extract from KG-1a cells, BJAB cells or PDA in the presence or absence of a 5-, 100- and 400-fold excess of unlabelled authentic probe. Supershifts were carried out by incubation of the cellular extracts with 2 μl Spi-B antiserum for 30 min on ice before the addition of probe as described previously (Laux *et al.*, 1994). The reactions were incubated at room temperature for 20 min and electrophoresed in a 6 % polyacrylamide–TBE DNA retardation gel (Invitrogen). The complexes were transferred to a positively charged nylon membrane and detected using the LightShift chemiluminescent EMSA kit (Thermo Scientific/Pierce).

Transfection and infection. Exogenous Spi-B and NF-1X were pre-expressed in human fetal brain-derived progenitor cells or PDN followed by exposure to Mad-4 JCV in a modification of the method described by Messam *et al.* (2003). Cells were allowed to attach to the wells of PDL-coated six-well dishes at a density of 2–4 × 10^5 cells per well for 2 days. Each well of cells was transfected with 1 μg pΔEB-Spi-B DNA or pCHA-NF1X using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Cells were exposed to medium alone for the mock condition. Approximately 48 h after transfection each well of cells was exposed to 333 haemagglutinaton units (HAU) of Mad-4 JCV or medium alone from the mock condition. Cells were fed with half new medium every 2–3 days. Immunofluorescence was performed on days 5 and 8 post-JCV exposure and T-antigen expression was quantified versus the cell-specific marker expression of nestin for progenitors or β III tubulin for PDN.

Nucleofection. Archetype, Mad-1, Mad-4, L5 mutant Mad-4, L4 mutant Mad-4 and L5/L4 double-mutant Mad-4 plasmids were introduced into PDAs by nucleofection using the Amaxa basic kit for primary neurons (Amaxa) according to the manufacturer's instructions. Briefly, 1 × 10^6 cells were nucleofected with 2 μg DNA using program C13. Cells were allowed to attach for 4 h, followed by replacement of culture medium. Cells were fed with half new medium every 2–3 days. Nucleofection using the pmaxGFP (Amaxa) reporter plasmid was included to determine nucleofection success and

efficiency. Immunofluorescence was performed on day 6 post-nucleofection to measure T-antigen expression in GFAP-positive PDAs.

Generation of figures. Digital Western blot images were detected using fluorescent filters in a FluorChem Q imager (Alpha Innotech). Digital EMSA images were obtained by scanning the exposed film using an hp Scanjet 8250 (Hewlett Packard Development Company). Figures were generated using Canvas II (ACD Systems International Inc.) and Adobe photoshop CS2 (Adobe).

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