Expression of human polyomavirus JC T antigen by an adenovirus hybrid vector and its binding to DNA sequences encompassing the JC virus origin of DNA replication

Otto Windl† and Kristina Dörries*

Institut für Virologie und Immunbiologie der Universität Würzburg, Versbacherstrasse 7, D-97078 Würzburg, Germany

In the search for factors that influence the outcome of human polyomavirus JC (JCV) infection, the roles not only of host-related immunological control but also of virus-dependent regulatory steps have to be taken into account. Besides cell-specific control of early expression of the multifunctional virus protein large tumour antigen (T Ag), control mechanisms involve individual steps of the DNA replication process. For the analysis of T Ag DNA binding, the protein was expressed by an adenovirus hybrid vector in the 293 cell line to provide saturating amounts of JCV T Ag. After determination of the size and immunoreactivity, functional activity was analysed by specific DNA binding. To avoid the interference of cellular proteins, T Ag was immuno-precipitated prior to the reaction. Binding to T Ag-binding sites I and II within a 141 bp DNA segment in the control region was analysed using deletion mutants of a JCV subtype from brain tissue of a patient with fatal central nervous system disease. The specificity of the binding was confirmed by recombinant T Ag binding to origin of DNA replication (ori) sequences of wild-type JCV genomes. These data document that recombinant T Ag overexpressed by the adenovirus vector in eukaryotic cells was JCV-specific, had the expected length and exhibited specific ori-binding activity, thus providing the essential tool for future analysis of virus-host interactions at the level of viral DNA replication.

Introduction

Polyomavirus JC (JCV) is endemic in the human population with seroconversion rates between 70 and 80 % in childhood. Infection leads to lifelong subclinical persistence in healthy individuals and causes the demyelinating central nervous system (CNS) disease progressive multifocal leukoencephalopathy (PML) by cytopathic destruction of oligodendroglia cells in severely immunocompromised patients (Walker & Padgett, 1983). Although JCV replicates predominantly in the brain and exhibits stringent cell specificity for CNS cells in vivo and in tissue culture (Walker & Frisque, 1986) the virus can also be isolated from kidney tissue and urine from immunosuppressed individuals (Frisque & White, 1992). In contrast to the CNS, infection in the kidney is attenuated and never leads to disease (Arthur & Shah, 1989). It is believed that this discrepancy might be reflected in the extraordinary genomic heterogeneity of JCV variants (Dörries, 1984; Martin et al., 1985; Loeber & Dörries, 1988; Myers et al., 1989; Yogo et al., 1990).

The structure and function of coding regions appear to be almost identical within JCV variants and other members of the polyomavirus family. The amino acid sequence of early and late proteins is highly conserved, indicating that they are functionally related (Fiers et al., 1978; Frisque et al., 1984; Loeber & Dörries, 1988). The regulatory product of early genes, large tumour antigen (T Ag), is a multifunctional protein involved in the organization of the viral life cycle (Fanning & Knippers, 1992). As one of the first steps, T Ag directs DNA replication by interaction with the origin of DNA replication (ori; Borowiec et al., 1991; Lynch & Frisque, 1991; DePamphilis, 1993). The DNA-binding domain maps within a polypeptide that is almost identical in amino acid sequence in JCV and simian virus 40 (SV40) (Simmons et al., 1990a; Parsons et al., 1990; Tavis & Frisque, 1991). A functional similarity between the T Ags of SV40 and JCV is suggested not only by sequence similarities, but also by the finding that SV40 T Ag interacts productively with the JCV ori in vivo and in vitro (Li & Kelly, 1985; Feigenbaum et al., 1987; Lynch & Frisque, 1991). Whereas protein similarities between virus species appear to be limited to biologically active
regions, variants within a single virus species are almost identical in coding sequence. In contrast, however, significant differences are found within non-coding regions of JCV subtypes isolated from PML brain and persistently infected asymptomatic tissue (Dörries, 1984; Martin et al., 1985; Loeber & Dörries, 1988; Myers et al., 1989; Yogo et al., 1990; Major et al., 1992).

The control region of JCV subtypes can be subdivided into a conserved segment carrying the putative DNA ori and overlapping, highly rearranged enhancer/promoter sequences (Lynch & Frisque, 1991). In the SV40 genome the ori region consists of a core element and auxiliary domains. The core has a palindromic region on the early side, a central GC-rich region containing the consensus T Ag-binding site II, and an AT-rich auxiliary segment with overlapping enhancer/promoter elements on the late side (Borowiec et al., 1991; Parsons et al., 1990). The architecture of JCV ori regions appear to be similar but, in contrast to SV40, the JCV core ori has a stringent requirement for auxiliary sequences as the core alone is essentially inactive (Lynch & Frisque, 1990; Sock et al., 1991). This suggests a significant role for auxiliary sequences in the efficiency of JCV DNA replication. The binding elements for transcription factors on the late side of the ori are highly rearranged, variable in number and contain heterogeneous DNA stretches in JCV subtypes (Dörries, 1984; Martin et al., 1985; Loeber & Dörries, 1988; Yogo et al., 1990). It has been repeatedly discussed that these differences might not only influence early transcriptional activity, but also the DNA replication efficiency of JCV subtypes (Amirhaeri et al., 1988; Lynch & Frisque, 1990, 1991; Sock et al., 1991, 1993; Tavis & Frisque, 1991).

However, evaluation of virus control mechanisms on the level of DNA replication are hampered by the stringent cell specificity of JCV. Tissue culture cells provide cellular proteins required for JCV DNA replication only to a limited extent and attenuated virus expression in tissue culture limits the amount of T Ag available. To establish a system for the analysis of essential steps in JCV-specific DNA replication we cloned the JCV early coding genomic region in the high expression, helper-independent adenovirus type 5 (Ad5) hybrid vector system (Berkner, 1992). Specificity and functional activity of recombinant protein was verified by immunoreaction to a T Ag-specific monoclonal antibody and by specific binding to both JCV DNA sequences spanning the putative core ori in deletion mutants and the rearranged control regions of naturally occurring JCV subtypes.

**Methods**

**Cells and viruses.** The Ad5-transformed human embryonic kidney cell line 293 (Graham et al., 1977) was grown in Dulbecco's modified Eagle's MEM (DMEM) supplemented with 10% fetal calf serum (FCS). The SV40-transformed mouse cell line VLM was kindly provided by Dr G. Brandner (Institut für medizinische Mikrobiologie und Hygiene der Universität Freiburg, Germany). The VLM line and the JCV-transformed hamster cell line HJC-15 (Walker et al., 1973) were grown in MEM supplemented with 10% FCS. The Ad5 deletion mutant 309 (Ad5dl309; Jones & Shenk, 1979) was propagated in 293 Cells and viruses. The Ad5-transformed human embryonic kidney cell line 293 (Graham et al., 1977) was grown in Dulbecco's modified Eagle's MEM (DMEM) supplemented with 10% fetal calf serum (FCS). The SV40-transformed mouse cell line VLM was kindly provided by Dr G. Brandner (Institut für medizinische Mikrobiologie und Hygiene der Universität Freiburg, Germany). The VLM line and the JCV-transformed hamster cell line HJC-15 (Walker et al., 1973) were grown in MEM supplemented with 10% FCS. The Ad5 deletion mutant 309 (Ad5dl309; Jones & Shenk, 1979) was propagated in 293 cells, purified by CsCl gradient centrifugation and virus titres were determined by a plaque assay (Precious & Russell, 1985).

**Plasmids and construction of recombinant virus.** JCV strains GS/B and GS/K were cloned in pBR322 at the BamHI site within the early gene region (Loebner & Dörries, 1988). To obtain an intact T Ag gene, the genome of JCV GS/B was redoned in the phagemid pGEM-3Z (Promega) at the EcoRI site located within the late coding region to
give pJCV(GS/B)-GEM. The adenovirus-derived shuttle vector pSKAG and the SV40 T Ag shuttle vector pSKAT (Simmons et al., 1990a) were kindly provided by Dr D. Simmons (University of Delaware, Del., USA). Standard recombinant DNA procedures were performed as described by Sambrook et al. (1989) and enzymes were used as recommended by the supplier.

For the construction of the JCV T Ag shuttle vector pSKAG-JCT, the vector pJCV(GS/B)-GEM was cleaved by restriction endonucleases MscI and KpnI followed by treatment with exonuclease III at 21 °C (Erase-a-Base; Promega; Henikoff, 1984) to remove elements at the unprotected 5' end of the MscI–KpnI DNA fragment (at about 60 bp/min). Reaction products were treated with T4 nuclease and the Klenow fragment of DNA polymerase I, religated and transformed in Escherichia coli strain 71/18 (Sambrook et al., 1989). The extent of the deletions was determined by agarose gel electrophoresis and further nucleotide sequencing (Tabor & Richardson, 1987). The JCV Sall–EcoRI fragment from clone JCV(GS)D15 was eluted from a 1% agarose gel and inserted into the Sall–Clal site of shuttle vector pSKAG by the sequential steps of ligation, T4 polymerase treatment and blunt-end ligation. This resulted in the JCV T Ag sequences positioned downstream of the major late promoter (MLP) of Ad2. For recombination, Ad5dl309 DNA was cleaved by Xbal and Clal, and 2 μg of subfragment A [Ad5 map units (m.u.) 4–100] were cotransfected with 10 gg JCV TAg shuttle vector DNA, linearized by KpnI, and 178 μg salmon sperm carrier DNA onto a subconfluent layer of 293 cells using the calcium phosphate technique (Graham & van der Eb, 1973). Cells were incubated under a layer of 1% agar with DMEM and 1–4% FCS at 37 °C for 8–9 days. Virus plaques were isolated by aspiration of agar plates. After addition of 0.5 ml DMEM and two rounds of freezing and thawing, recombinant virus was amplified by two passages in 293 cells. JCV T Ag recombinants were identified by T Ag-specific immunofluorescence with the monoclonal antibody PAb 416, PAb 419 or a monoclonal antibody at a concentration of 1.1 gg/ml. The substrate was 4-chloro[(POD)-conjugated goat anti-mouse IgG was used as secondary antibodies at a concentration of 10 gg/ml. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was used as secondary antibodies at a concentration of 14 μg/ml.

Analysis of recombinant JCV T Ag. For protein analysis, 2 x 10⁶ confluent 293 cells were infected with Ad5dl309 or Ad5-derived recombinant virus at a multiplicity of 10 p.f.u. per cell. Cells were harvested 20 h post-infection (p.i.) or as described in the text. The presence of JCV T Ag was demonstrated in situ by immunofluorescence. Cells were fixed with acetone for 10 min at −20 °C. T Ag-specific monoclonal antibody PAb 416, PAb 419 or a monoclonal antibody directed against the S protein of murine hepatitis virus (MHV) strain JHM, PAb 873 (kindly provided by Dr H. Wege, Institut für Virologie und Immunobiologie, Würzburg, Germany), were used as primary antibodies at a concentration of 10 μg/ml. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was used as secondary antibody at a concentration of 14 μg/ml.

Western blotting was performed on lysates of 2 x 10⁶ cells in 0.25 ml RIPA buffer (Harlow & Lane, 1988). After separation of cell lysates by PAGE in a 8% denaturing gel, proteins were transferred to nitrocellulose filters. Non-specific binding was blocked by 10% BSA. Filters strips (3 mm wide) were incubated successively with monoclonal antibodies at a concentration of 1 μg/ml. Horseradish peroxidase (POD)-conjugated goat anti-mouse IgG was used as secondary antibody at a concentration of 1:1 μg/ml. The substrate was 4-chloro-1-naphthol. Western blotting and immunofluorescence were carried out according to standard protocols (Harlow & Lane, 1988). Molecular masses of proteins were calculated from regression curves of marker proteins (Sigma).

DNA-binding assay. DNA-binding reactions were performed by a modified T Ag–DNA binding assay (Hinzpeter et al., 1986). Protein lysate (250 μl) was immunoprecipitated with an excess of monoclonal antibody PAb 416 and Protein A–Sepharose (Pharmacia). After

Results

Construction of the viral JCV T Ag expression hybrid vector Ad5JCVTAg

Analysis of the JCV early protein T Ag required higher amounts of protein than were available in transformed or transfected cell lines. Therefore the early coding region was cloned in a helper-independent Ad5 high level expression vector under the control of the Ad2 MLP (Berkner, 1992; Fig. 1). As a first step, the gene segment was isolated from JCV GS/B genomic DNA cloned in the bacterial plasmid pGEM-3Z. Restriction endonuclease cleavage with the enzymes MscI (site located within the enhancer/promoter region) and KpnI (within the multiple cloning site of the vector) was followed by exonuclease III digestion. Regulatory sequences upstream of the early genes, including putative T Ag-binding sites I and II, were deleted to exclude the possibility of the protein down-regulating its own expression (Rigby & Lane, 1983). Blunt-end ligation resulted in a JCV T Ag EcoRI–SalI segment (Lieber & Dörries, 1988) containing 24 bp upstream from the initiation codon (as determined by sequence analysis), the complete early genomic segment with its transcriptional signal sequences and an additional 882 bp downstream from the stop codon of T Ag.

The EcoRI–SalI fragment was then inserted in the multiple cloning site (Sall–Clal) of the adenovirus shuttle vector pSKAG (Simmons et al., 1990a) containing Ad5 DNA from m.u. 0 to 14 with the 5' ori, the Ad2 MLP and Ad5 sequences from m.u. 9–1–15–5 for recombination. In the resulting vector, pSKAG-JCT, the JCV T Ag gene was positioned downstream of the Ad2 MLP. For rescue of the hybrid transcription unit into infectious Ad5 virus, the E1A and E1B early regulatory gene region and the 5' ori (Ad5 m.u. 0–4) was removed from the genome of the viral deletion mutant Ad5dl309. The
Fig. 2. Ad5-JCVTAg-expressed T Ag in 293 cells at different times after infection. Indirect immunofluorescence staining of Ad5-JCVTAg-infected 293 cells (m.o.i. 10 p.f.u./cell) at 0 h (a), 12 h (b) and 20 h (c) p.i.; (d), the JCV-transformed hamster cell line HJC. (e), Cultured 293 cells transfected with 10 μg pJCV(GS/B)-GEM DNA by the calcium phosphate technique. (f), Ad5-SV40TAg-infected 293 cells at a multiplicity of 10 p.f.u./cell at 12 h p.i. Cells were stained with the T Ag-specific monoclonal antibody PAb 416 followed by FITC-labelled goat anti-mouse secondary antibody.

replication-inactive Ad5d1309 subfragment (m.u. 4–100; Simmons et al., 1990a) was cotransfected with KpnI-linearized pSKAG-JCT vector DNA in Ad5-transformed 293 cells. These cells express the early Ad5 E1A and E1B proteins constitutively and therefore allowed a recombinant Ad5-JCVTAg hybrid virus to grow carrying the JCV genome segment under the control of the Ad2 MLP between Ad5 m.u. 1–4–9–1. Analysis of plaque isolates revealed the presence of the JCV early gene segment in seven out of 46 plaques, as detected by T Ag-specific immunofluorescence in infected 293 cells (Fig. 2). The activity was identical among all isolates and expression was stable upon further rounds of plaque purification. Recombinant virus grew routinely to high titres (10⁸–10⁹ p.f.u./ml) in 293 cells.

Analysis of recombinant JCV T Ag synthesis in 293 cells after infection with the hybrid virus Ad5-JCVTAg

To study the course of infection and recombinant JCV T Ag synthesis, 293 cells were infected with the hybrid virus Ad5-JCVTAg or the adenovirus deletion mutant Ad5d1309 at a multiplicity of 10 p.f.u./cell. Morphological alterations resembling adenovirus-induced CPE were detected at 12 h p.i. with the hybrid virus (Precious & Russell, 1985). From 20 h p.i., rounded cells were released into the medium and by 28 h p.i. almost all cells were in suspension.

T Ag expression was demonstrated by indirect immunofluorescence with the monoclonal antibody PAb 416, which reacts with both JCV and SV40 T Ag (Harlow et al., 1981; Stoner et al., 1988), and FITC-conjugated goat anti-mouse Ig as a secondary antibody (Fig. 2a–c). In contrast, PAb 419, which binds to SV40 T Ag and small t antigen, did not bind to Ad5-JCVTAg-infected cells. Neither antibody reacted with uninfected or Ad5d1309-infected 293 cells. Similarly, the MHV-specific PAb 873 gave no signal in T Ag-expressing cells (data not shown). Isolated nuclear staining appeared as early as 8 h p.i. (1/10⁴ cells) and between 12 and 16 h p.i. about 20% of the cells exhibited positive signals (Fig. 2b). The amount of JCV T Ag increased considerably and by 20 h p.i. almost all cells expressed T Ag (Fig. 2c). Thereafter, expression appeared to reach a plateau, although between 24 and 28 h the total number of cells decreased. Comparison with the JCV-transformed cell line HJC (Fig. 2d) and 293 cells transfected with the T Ag-expressing plasmid pJCV(GS/B)-GEM (Fig. 2e) revealed that the expression of recombinant JCV T Ag in Ad5-JCV hybrid virus-infected cells was higher than in
the other cells and comparable to the expression of SV40 T Ag by the recombinant Ad5-SV40TAg hybrid virus (Fig. 2f).

**Characterization of recombinant JCV T Ag expressed in human kidney cells**

The molecular mass of recombinant JCV T Ag was determined by Western blotting of protein lysates from hybrid virus Ad5-JCVTAg-infected cells at 20 h p.i., followed by immunohistological staining with the T Ag-specific monoclonal antibody PAB 416 and POD-conjugated goat anti-mouse Ig. Recombinant Ad5-JCVTAg virus expressed JCV-specific protein with a molecular mass of about 85 kDa (Fig. 3, lane 1). This was not detected either in uninfected 293 cells (Fig. 3, lane 5) or by analysis of any lysates with PAB 419 specific for SV40 early proteins or MHV-specific PAB 873 (Fig. 3, lane 2, 4, 6, 8 and 10). POD-conjugated goat anti-mouse IgG was used as the secondary antibody and 4-chloro-1-naphthol as a substrate. The positions of marker proteins in kDa are given and the bands corresponding to T Ag are indicated.

![Western blot analysis of JCV T Ag](image)

**Fig. 3.** Characterization of recombinant JCV T Ag by Western blot analysis. JCV T Ag was characterized in lysates of 293 cells that were infected with hybrid virus Ad5-JCVTAg (lanes 1, 2) or Ad5-SV40TAg (lanes 3, 4) and were analysed by the Western blot procedure compared to lysates of uninfected 293 cells (lanes 3, 6), HJC cells (lanes 7, 8) and VLM cells (lanes 9, 10). Proteins were separated by 8% PAGE, blotted to nitrocellulose and incubated with PAB 416 as primary antibody in lanes 1, 3, 5, 7 and 9 and with PAB 873 in lanes 2, 4, 6, 8 and 10. POD-conjugated goat anti-mouse Ig was used as the secondary antibody and 4-chloro-1-naphthol as a substrate. The positions of marker proteins in kDa are given and the bands corresponding to T Ag are indicated.

DNA binding of recombinant JCV T Ag to JCV DNA ori sequences

The DNA-binding activity of recombinant JCV T Ag was analysed by a modified McKay assay performed under almost physiological salt conditions (McKay, 1981; Hinzpeter et al., 1986). For T Ag binding, JCV DNA [pJCV(GS/B)-GEM] was cleaved by the restriction endonuclease BglIII into four fragments (A–D) of lengths 3.7 to 0.7 kb (Fig. 4a, b). Fragment C (1.6 kb) contains the non-coding region with the putative DNA orf. DNA fragments were incubated with recombinant JCV T Ag after immunoprecipitation with monoclonal antibody PAb 416 from a lysate of Ad5-JCVTAg-infected 293 cells. T Ag-binding DNA fragments were eluted from the protein, radioactively labelled and autoradiographically visualized after electrophoretic separation. As shown in Fig. 4(a), JCV fragment C was heavily labelled, indicating binding to the T Ag-specific consensus binding sites within the non-coding control region (Fig. 4a, lane 2). In almost all experiments, weak binding of fragment A, B and D was observed. This is believed to be a non-specific reaction of T Ag with any DNA sequence (Simmons et al., 1990a). No reaction was found after immunoprecipitation of T Ag with a non-specific antibody (PAB 873; Fig. 4a, lane 3, 5) or with lysates from uninfected 293 cells (Fig. 4a, lane 4, 5), thus confirming the specificity of the signal. This demonstrates that recombinant JCV T Ag has DNA-binding activity and binds predominantly to the fragment containing the control region of the JCV genome.

The control region of JCV subtype GS/B was truncated in an attempt to localize T Ag-binding DNA segments further. The putative ori and duplicated promoter/enhancer elements (nucleotides 3315–3773; Loeb & Dörrries, 1988) were deleted successively by exonuclease III digestion from the late side of the control region (Fig. 5). T Ag-binding activity was analysed with BglII- and Scal-cleaved DNA of deletion mutants JCV(GS)d120, 19, 18 and 15 (Fig. 6). The cleavage resulted in four fragments A to D (2.1 kb–0.7 kb) with fragment C containing the truncated control region (1.5–1.3 kb). Mutant JCV(GS)d120 contains the putative ori and one of the enhancer/promoter elements (TR1; nucleotides 3315–3519; Fig. 6, lane 4). The binding assay with recombinant JCV T Ag revealed a strong binding
signal at the position of DNA fragment C (1.55 kb). In mutant JCV(GS)d119, tandemly repeated transcriptional control elements were deleted (nucleotides 3315–3456) leaving an intact core ori sequence and the TATA box within the AT-rich subdomain (Borowiec et al., 1991; Lynch & Frisque, 1990, 1991). No significant difference in the binding of T Ag to fragment C (1.49 kb) was observed (Fig. 6, lane 3). The core ori in mutant JCV(GS)d118 was truncated (nucleotides 3315–3379). T Ag-binding site I (Lynch & Frisque, 1990, 1991) was left intact and 77 bp, including putative T Ag-binding site II, were removed. In this case, the T Ag binding of fragment C (1.41 kb) was diminished (Fig. 6, lane 2), pointing to the significance of the segment from nucleotide 3456 to 3379. In the construct JCV(GS)d115, the core ori sequence was truncated to nucleotide 3315 and contained no putative T Ag-binding domains. In this case, TAg binding of fragment C (1.35 kb) was reduced to non-specific levels (Fig. 6, lane 1). These findings were confirmed by densitometric quantification and demonstrate that JCV T Ag binding is limited to the ori between nucleotides 3315 and 3456. Binding increased depending on the presence of the putative consensus T Ag-binding domains I and II. This demonstrated JCV T Ag binding to both sites and restricted the contact to a 141 bp DNA segment.

The specificity of T Ag binding to ori sequences was further supported by binding analyses of wild-type JCV host-derived subtypes with differentially arranged upstream control elements (GS/B, GS/K; Loeber & Dörries, 1988). The control region contained duplicated

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**Fig. 4. DNA-binding activity of recombinant JCV T Ag.** (a) DNA binding was analysed with recombinant JCV T Ag immunoprecipitated with PAb 416 from 293 cell lysates with pJCV(GS/B)-GEM BglII fragments (300 ng; fragment C per lane). Lanes 4 and 5, uninfected cell lysates; lanes 2 and 3, cells infected with hybrid virus Ad5-JCV TAg; lanes 2 and 4, immunoprecipitated with PAb 416; lanes 3 and 5, with PAb 873. Bound DNA was extracted, radioactively labelled and separated on a 1% agarose gel. Lane 1, labelled pJCV(GS/B)-GEM BglII fragments A to D as marker bands (5 ng). Lengths of fragments in kb are indicated on the right. The smear between bands is probably due to nuclease activity in the control DNA batch. (b) Structure of the vector pJCV(GS/B)-GEM. TAg, coding sequences for the early proteins; VP, virus capsid proteins; CR, non-coding control region; TER, termination region of proteins. The BglII fragments of JCV plasmid pJCV(GS/B)-GEM 3 are labelled; dotted boxes, JCV DNA segments; open box, plasmid pGEM-3Z DNA sequences; shaded box, control region; arrows, direction of gene expression.

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**Fig. 5. Structure of the control region elements from wild-type JCV genomes and JCV deletion mutants.** Alignment of control elements in the non-coding region of JCV subtypes GS/B and GS/K as well as deletion mutants JCV(GS)d115 and d118 to d120. Mutants were constructed by exonuclease III digestion from the late side of the control region and religation. For binding experiments wild-type JCV subtypes were cleaved by the restriction enzyme BglII, whereas JCV deletion mutant DNA was cleaved by the enzyme combination BglII and ScaI. Nucleotide numbers in parentheses refer to the last JCV nucleotide (Loeber & Dörries, 1988) of each mutant as determined by nucleotide sequence analysis. Abbreviations: agno, putative late leader protein; pGEM-3Z, bacterial vector DNA sequences; arrows, direction of gene expression; I and II, T Ag-binding sites; TA, TATA box; TR1 and TR2, repeated enhancer/promoter elements (enh/pro) in JCV GS/B and single elements in JCV GS/K; hatched boxes, JCV GS/K-specific 58 and 46 bp sequence strings.
enhancer/promoter elements in the GS/B type and single enhancer/promoter elements flanked by a 58 and 46 bp sequence string in the GS/K type (Fig. 5). Binding
was performed by a reaction with Bg/II-restricted genomic DNA fragments. Fragment C, containing the JCV GS/B and GS/K control regions, varied in length (GS/B, 1.61 kb; GS/K, 1.56 kb). Both variant DNA fragments bound JCV TAg with comparable efficiency (Fig. 7a, lanes 3 and 4). Double bands of fragment C were observed in almost all experiments to a variable extent and might be caused by degradation in the radioactive labelling reaction (McKay, 1981). The specificity of the reaction was confirmed by binding of constant amounts of TAg to ascending amounts of DNA (Fig. 7a, lanes 10 to 7). Quantification by densitometry confirmed increasing TAg binding to fragment C corresponding to the amount of DNA used (Fig. 7b). No binding was observed after immunoprecipitation of extracts with a non-specific antibody (PAb 873; Fig. 7a, lanes 2 and 5) or with lysates from uninfected 293 cells (Fig. 7a, lanes 1 and 6). These findings confirm the specificity of recombinant TAg binding to naturally occurring JCV subtypes.

Discussion

The concentration of JCV TAg in transformed cell lines or after transient expression of cloned JCV full-length DNA in 293 cells is very low due to limited expression from the weak early JCV promoter. This makes functional studies impractical (O. Windl & K. Dörries, unpublished observations; Tavis & Frisque, 1991) and it was therefore necessary to construct a high-level expression system for JCV TAg. In contrast to transient expression of TAg by transfected bacterial vectors (Sock et al., 1993; Kerr et al., 1993) the adenovirus hybrid vector has the advantage of overexpressing JCV proteins in human kidney cells, because of its viral origins (Berkner, 1992). This cell type is permissive for JCV infection and it has been reported that splicing in SV40 and post-translational processing of the hybrid virus Ad5-SV40TAg is identical to that in SV40-infected cells (Salnich, 1981; McVey et al., 1989). As JCV is highly related to SV40, we expected correct processing, thus simplifying the construction of the hybrid vector.

Expression of recombinant JCV TAg from the Ad5-JCVTAg hybrid virus was considerably higher than that from the homologous promoter in the JCV-transformed cell line HJC or in JCV-transfected 293 cells. Comparison with recombinant SV40 TAg expression in the same system revealed that the protein yield obtained with the Ad5-JCVTAg vector is comparable to that reported earlier for SV40. Estimated amounts ranged up to 4% of total protein in an herpes simplex virus–Ad5 hybrid vector (Lamarche et al., 1990; Berkner, 1992) thus providing a protein level that facilitates enrichment and purification.

Immunoreactivity and the identical size of the recombinant protein to that in productively infected cells and in transformed cell lines (Haggerty et al., 1989; Major & Traub, 1986; Lynch & Frisque, 1991) corroborates the other evidence indicating that endogenous JCV-specific transcription and splicing signals worked well and that protein expression is not affected by surrounding adenovirus sequences (Berkner, 1992). Specific binding of TAg molecules to JCV DNA fragments encompassing the core ori within the genomic control region indicated functional activity. As this property is dependent on the phosphorylation state of TAg (Prives, 1990) we can assume that post-translational modifications of the protein in 293 cells are similar to those in vivo. In SV40, TAg-binding activity is localized to an amino acid stretch identical to that in JCV TAg, and it is believed to be a prerequisite for the intrinsic helicase activity of TAg (Simmons et al., 1990b). Our analyses demonstrate that the recombinant TAg is naturally spliced, intact in length, has functional activity and is expressed in sufficient amounts to allow studies on JCV DNA replication.

TAg-dependent DNA replication is influenced in vivo by cell-specific expression of the protein and probably by interaction with cellular factors in a host- and cell-specific manner (DePamphilis, 1993). In the JCV system, sequences on the late side of JCV ori elements were identified that have a significant effect on the activity of DNA replication (Lynch & Frisque, 1990; Sock et al., 1991, 1993). However, the first essential task of TAg in the polyomavirus life cycle is the initiation of DNA replication by binding to DNA ori sequences (Borowiec et al., 1991; DePamphilis, 1993). To understand the cascade of regulatory events leading to JCV DNA replication we first determined the borders of the DNA binding region, using the reaction of TAg to ori sequences of truncated control region elements. Since the presence of cellular components in the binding reactions might change TAg–ori interactions, binding was performed with immunopurified target-bound TAg, thus avoiding interference by cellular components (Hinzpeter et al., 1986). The data demonstrate that a residual 24 bp on the early side of the control region do not mediate specific TAg binding. This localizes the borders of ori sequences to the early side of the control region at nucleotide position 3315, similar to that described by Lynch & Frisque (1990, 1991) and Sock et al. (1991, 1993). The following 64 nucleotides, encompassing TAg-binding site I with two consensus pentanucleotides, mediate specific TAg binding. Binding efficiency to site I was clearly reduced in comparison to that observed in a mutant containing the complete putative ori sequences with central dyad symmetry, consensus TAg-binding site II and the AT rich domain.
In SV40, similar consensus sites and flanking sequences of the inverted region have been described as essential segments (Borowiec et al., 1991; DePamphilis, 1993) and our results suggest that JCV T Ag binds strongly to the same elements. Earlier reports failed to detect JCV T Ag binding to putative JCV T Ag-binding site II, although experiments confirmed strong T Ag binding to site II in the SV40 system under the same experimental conditions (Tavis & Frisque, 1991). Apart from the presence of cellular proteins in those experiments, lysates of a JCV-transformed cell line were used that contained several-fold lower amounts of JCV T Ag than SV40 T Ag in the respective cell line. Provided that affinity of binding site I was higher than that of binding site II, as described in the SV40 system (Stetter et al., 1988; Deb & Deb, 1989), binding to site I would dominate under suboptimal conditions. The use of DdeI to generate fragments containing sites I and II suggests another explanation. DdeI cleaves between the consensus sequences, placing the binding sites at the extreme ends of the DNA fragments. In our hands, binding experiments with oligonucleotides containing JCV T Ag-binding site II were not convincing and it appears likely that the deletion of early flanking sequences might interfere with JCV T Ag binding.

DNA-binding experiments to increasing amounts of DNA fragments containing the complete non-coding region of wild-type JCV subtypes in the cell protein-free system confirmed the specificity of recombinant T Ag binding to the variant control regions. This finding, however, does not contradict the possibility that T Ag binding might be influenced by interference of cellular factors (Lynch & Frisque, 1990, 1991; Sock et al., 1991, 1993; DePamphilis, 1993).

In summary, the data demonstrate that overexpression of the JCV early gene segment in a eukaryotic cell line produced a recombinant protein of JCV T Ag-specific length and antigenicity that specifically bound to the JCV ori within the genomic control region. This will allow future studies addressing questions about the interaction of T Ag with DNA sequences in the course of DNA replication and the influence of different cellular factors from permissive and semi-permissive cells.

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