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A Cell-Free Replication System for Human Polyomavirus JC DNA

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The human polyomavirus JC virus (JCV) establishes persistent infections in most individuals and is the etiologic agent of progressive multifocal leukoencephalopathy. In this report, we describe the establishment of a soluble cell-free system that is capable of replicating exogenous plasmid DNA containing the JCV origin of replication. Replication in this system is completely dependent on the addition of JCV large T antigen (TAg). To prepare JCV TAg for replication analysis, a recombinant baculovirus containing the JCV TAg-coding sequence was generated. TAg expressed in insect cells was purified by metal chelate chromatography. JCV TAg supported initiation of JCV DNA replication in the presence of DNA polymerase α-primase, replication protein A, and topoisomerase I in a dose-dependent manner and was also capable of supporting DNA replication in crude human cell extracts. Point mutation of TAg-binding site I strongly diminished TAg binding and concomitantly reduced JCV DNA replication in vivo and in vitro by approximately 50%. Point mutation of TAg-binding site II or deletion of the early palindrome completely abolished replication of JCV origin-containing plasmid DNA in vivo and in vitro, marking these sequences as essential components of the JCV core origin. A comparison of several TAgs showed that simian virus 40 TAg, but not mouse polyomavirus (PyV) TAg, supported replication of a plasmid containing a JCV origin. These findings provide evidence that replication in the cell-free system faithfully mimics JCV DNA replication in vivo. Therefore, it may be a useful tool for future analysis of interactions between JCV and its host cell.

The primate papovavirus JC virus (JCV) establishes persistent infections in more than 70% of the human population and causes progressive multifocal leukoencephalopathy, a demyelinating brain disease that results from selective destruction of the myelin-forming oligodendrocytes, in humans. In normal individuals, its pathogenicity is presumably suppressed by the immune system since JCV causes progressive multifocal leukoencephalopathy only in strongly immunocompromised individuals (32, 33, 65). In recent years, the appearence of this disease has increased since a significant number of patients with AIDS have been affected (40, 65). Infection by JCV is restricted to a small number of cell types, including human glial cells (32–34, 65). In cell culture, JCV replicates inefficiently and exhibits an even narrower cellular host range, which make studies of the virus difficult (17, 30, 61). This restricted behavior occurs, in part, at the level of DNA replication (9, 11, 30, 31, 40). Whereas cell type specificity is determined mainly at the level of viral gene expression, DNA replication seems to be largely responsible for host specificity (9, 11, 16).

JCV shares sequence and structural homology with simian virus 40 (SV40), but in contrast to those of JCV, the DNA replication and life cycle of the closely related papovavirus SV40 have been well investigated (62, 65). SV40 DNA replication has served as an excellent model system for the study of virus-host interactions, since SV40 DNA replication, like JCV DNA replication, requires only one viral protein, the large T antigen (TAg), while the other replication proteins are supplied by the host (8, 23, 30, 58). The development of in vitro replication assays for SV40 DNA has allowed the identification and characterization of the cellular proteins that are required for replication in vitro (8, 23, 24, 27–29, 64, 67). In addition, SV40 DNA replication in vitro could be divided into several successive steps. First, TAg binds to multiple copies of a pentanucleotide sequence (GAGGC) in the origin of replication (ori) (15). In the presence of ATP, TAg assembles as a double hexamer at the ori core that consists of a 15-bp imperfect inverted repeat (early palindrome [EP]), the 27-bp dyad symmetry element (TAg-binding site II, containing four GAGGC sequence repeats), and the A/T-rich element (12, 15, 35, 45, 46). The ori core is flanked on the early transcribed side by TAg-binding site I, which contains two GAGGC sequences, and on the late transcribed side by transcription elements; these sequences adjacent to the ori core stimulate replication of SV40 DNA in vivo (13–15, 18). After binding of TAg, ori DNA becomes denatured within the EP, and in the presence of ATP and replication protein A (RP-A), the single-stranded DNA-binding protein, the helicase activity of TAg bidirectionally unwinds the DNA outward from ori with topoisomerase I or II, releasing torsional stress (3, 15, 51, 57 and references therein). Subsequently, the primase function of DNA polymerase α-primase synthesizes RNA primers at the origin of replication which are then elongated by DNA polymerase α (6, 36, 66). Through a polymerase switch mechanism, the sliding-clamp protein proliferating cell nuclear antigen (PCNA) and DNA polymerase δ are assembled on these initial RNA-DNA primers by replication factor C (RF-C) and form the leading-strand replication complex (26, 63, 67). Lagging-strand replication is thought to be mediated by DNA polymerase α-primase together with DNA polymerase δ and its accessory proteins (6, 44, 63, 64).

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Despite the close relationship, JCV DNA replication reveals significant differences from SV40 DNA replication; e.g., JCV has a narrower host range than SV40 (32, 33, 65). In addition, JCV DNA replication shows a greater dependence on ori flanking sequences than was determined with SV40 and these regulatory regions seem to play an important role during latency of JCV and its reactivation in the brain (32). To improve our understanding of the molecular basis which underlies JCV DNA replication and its restrictions, we established a replication system for JCV in vitro. To prepare purified JCV TAg for biochemical analysis, a recombinant baculovirus that expressed the JCV TAg-coding sequence was constructed and wild-type (wt) TAg was purified by metal chelate (Me^{2+}) chromatography. An initiation system that consisted solely of purified proteins and an ATP-regenerating system was used for primer synthesis at the viral origin of replication. In addition, recombinant TAg, origin-containing DNA, an ATP-regenerating system, and human cell extracts were sufficient to replicate viral DNA in vitro. By studying replication of JCV DNA in vivo and in vitro, we could show that flanking sequences of the minimal JCV origin of replication stimulated DNA replication while the EP and TAg-binding site II are essential for JCV DNA replication. Comparison of the replication activities of viral TAgs revealed that JCV TAg and SV40 TAg, but not PyV TAg, support DNA replication in vitro with a JCV origin.

MATERIALS AND METHODS

Plasmids. The pUC18-based plasmid DNAs pJC433 (containing map positions 4981 to 279 of JCV strain Mad-1) and pJC180 and their derivatives were described earlier (53, 54; see also Fig. 4). Plasmid pJC389-wt was generated by inserting the full-length regulatory region of JCV strain Mad-1 (spanning nucleotides 5014 to 273) into the *Xho*I site of pBluescript II KS+ (Stratagene). Plasmid pJC389-I^- was derived from pJC389-wt by mutating TAg-binding site I at nucleotides 5086 to 5089 from CCTC to ATCT (pJC389-I^-, AA AAAG**ATCT**CACGCCC instead of AAAAAG**CCTC**CACGCCC), which creates a *Bgl*II site, and in pJC389-II^- within the sequence of TAg-binding site II at nucleotides 5127 and 5128, GG (Mad-1 sequence) was changed to TC (pJC389-II^-, GGAGGCGGA**TC**CG instead of GGAGGCGGA**GG**CG). Plasmid pJC389-I^-/II^- contained the mutations described here in both TAg-binding sites. Plasmid DNA used for DNA replication was purified by CsCl gradient centrifugation (48).

Expression and purification of JCV TAg. JCV TAg was expressed by using the newly created baculovirus expression vector pVL1392-JCVTAg, which contains the coding sequence of JCV TAg from pRSVT-Ag (47). Plaque-purified baculovirus JCV TAg was amplified and then used to infect High Five cells (Invitrogen, NV Leek, The Netherlands) at 10 PFU/cell. Insect cells were harvested 40 h postinfection, homogenized in lysis buffer (100 mM Tris/HCl [pH 7.5], 100 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 0.1% Nonidet P-40), and proteins were subjected to metal chelate (Me^{2+}) chromatography using Talon resins (Clontech, Heidelberg, Germany). After binding of the proteins, the resin was washed extensively with buffer 1 (20 mM Tris/HCl [pH 8], 100 mM NaCl, 3.5 mM 2-mercaptoethanol) including 0.5% lubrol (ICN, Eschwege, Germany), and 5 column volumes of buffer 1, which contained 5 mM imidazole. JCV TAg was eluted with 500 mM imidazole in buffer 1, and then fractions were dialyzed against 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0)–5 mM NaCl–1 mM dithiothreitol (DTT)–0.1 mM EDTA–30% glycerol.

JCV TAg was also purified by immunoaffinity chromatography using monoclonal antibody PAb419 (10) coupled to Sepharose 4B, essentially as described previously for SV40 TAg (5, 52).

Protein purification and protein manipulations. Expression and purification of DNA polymerase α-primase, topoisomerase I, and SV40 TAg were performed as previously described (5, 50, 55). Human recombinant RP-A (19) was purified as described by Nasheuer et al. (43) but without single-stranded DNA cellulose chromatography.

Protein concentration was determined as described by Bradford (4) by using a commercial reagent with bovine immunoglobulin G as the standard (Bio-Rad, Munich, Germany). Sodium dodecyl sulfate (SDS)-gel electrophoresis was carried out as previously described (25), with a 10-kDa ladder (Life Technologies, Eggenstein, Germany) or prestained molecular weight marker proteins (Sigma, Deisenhofen, Germany). After polyacrylamide gel electrophoresis, proteins were detected either by staining with Coomassie brilliant blue or by Western blot analysis as previously described (55). DNA polymerase α assays and DNA primase assays were performed as described by Nasheuer and Grosse (41, 42).

DNA-protein binding immunoassay. The DNA-protein binding assay was performed as previously described (21, 37). Each fragment (10^5 cpm, which was equivalent to approximately 5 ng of DNA) was incubated with 15 to 30 ng of purified TAg and 0.5 μg of poly(dI-dC) as a competitor in buffer A (20 mM HEPES [pH 7.8], 1 mM DTT, 5% glycerol, 2 mM EDTA) for 30 min on ice. Following this incubation, polyclonal anti-JCV TAg serum was added and the mixture was incubated for an additional 20 min. The immunocomplex was collected by adding 15 μl of a 50% protein A agarose slurry (Sigma). The pellet was washed three times with buffer A. The bound DNA-protein complex was analyzed by electrophoresis on a 4% polyacrylamide gel and autoradiography.

Alternatively, labelled DNA fragments were incubated with 10 or 75 ng of purified TAg and 0.5 μg of poly(dI-dC) in replication buffer (30 mM HEPES-KOH [pH 7.8]; 0.5 mM DTT; 7 mM magnesium acetate; 1 mM EGTA; 4 mM ATP; 0.3 mM CTP, GTP, and UTP; 0.1 mM dATP and dGTP; 0.05 mM dCTP and dTTP; 40 mM creatine phosphate; 80 μg of creatine kinase per ml) for 60 min at 37°C or on ice. Polyclonal anti-JCV TAg antibody and 15 μl of a 50% protein A agarose slurry was then added, and the mixture was incubated for 60 min at 37°C or on ice. The immunocomplex was washed five times with replication buffer, and the protein-bound DNA was analyzed as described above.

Initiation of replication on JCV DNA. Initiation reactions were performed as previously described (5, 49, 56), with slight modifications. Briefly, the JCV initiation assay (40 μl) was assembled on ice and contained 0.25 μg of pJC433 DNA (JCV origin-containing DNA), the amounts of JCV TAg indicated in Results, and 0.30 mM HEPES-KOH (pH 7.8)–7 mM magnesium acetate–1 mM EGTA–1 mM DTT–0.2 mM UTP–0.2 mM GTP–0.01 mM CTP–4 mM ATP–40 mM creatine phosphate–1.5 μg of creatine kinase–0.3 μg of topoisomerase I–0.2 mg of heat-treated bovine serum albumin per ml–0.4 primase U of human DNA polymerase α-primase–20 μCi of [α-^{32}P]CTP (3,000 Ci/mmol; NEN-DuPont).

SV40 initiation reactions (40 μl) were carried out as described above but contained 0.25 μg of pUC-HS DNA (SV40 origin DNA) and 0.6 μg of SV40 TAg. The reaction products were analyzed as previously described (5).

Preparation of S100 extracts and viral DNA replication in vitro. S100 extracts were prepared from logarithmically growing human HeLa or 293S cells as previously described (5, 49, 69). Replication of viral DNA in vitro was performed as previously described (5, 49), with slight modifications. Briefly, the JCV assay (60 μl) consisted of crude insect cell extracts that contained the amounts of recombinant or purified JCV TAg indicated in Results, 0.25 μg of JCV origin DNA (pJC433 DNA or the plasmid DNA indicated in Results), 300 μg of S100 from human cells in replication buffer, and 5 μCi each of [α-^{32}P]dCTP and [α-^{32}P]dTTP (3,000 Ci/mmol; NEN Life Science Products, Cologne, Germany). The replication products were prepared and analyzed as described by Brückner et al. (5).

In vivo DNA replication assay. DNA replication was analyzed in U138 glioblastoma cells as previously described (53, 54). Briefly, cells were plated at a density of 5×10^5/6-cm-diameter dish in Dulbecco's modified Eagle medium with 10% fetal calf serum and transfected on the following day with 2 μg of the test plasmid by the calcium phosphate technique. JCV TAg was supplied by cotransfection of 1 μg of the Rous sarcoma virus expression vector pRSV-JCT. To detect even minor replication products with the core origin mutants pJC389-II and -I^-/II^- (see Fig. 7A), U138 cells were harvested at 84, instead of 60, h posttransfection (53) and low-molecular-weight DNA was extracted, incubated with *Dpn*I and analyzed by Southern blotting (20, 54).

RESULTS

Purification of recombinant JCV TAg. The cDNA of JCV TAg was expressed with baculovirus vectors. Large amounts of JCV TAg were produced in insect cells (Fig. 1A, compare lanes 1 and 2). This wt JCV TAg efficiently bound to Me^{2+} chromatography columns and was highly purified by Me^{2+} chromatography (Fig. 1A, lane 4). The yield of the affinity purification was about 1.2 mg from 10^9 insect cells. In control experiments with wt baculovirus-infected insect cells, only minor amounts of proteins were eluted under these conditions (Fig. 1A, lane 3).

The monoclonal antibody PAb419 against SV40 TAg (10) also recognizes JCV TAg in Western blots and immunoprecipitations (data not shown). However, immunopurification of JCV TAg requires stringent washing conditions which reduce the yield of purified JCV TAg to about 0.09 mg/10^9 insect cells. Despite extensive washing of the resin, immunoaffinity-purified JCV TAg was less pure than TAg purified by Me^{2+} chromatography (Fig. 1A and B, lane 5).

Initiation of JCV DNA replication by purified proteins. The expression and purification of JCV TAg enabled us to test whether it can initiate DNA replication in vitro. The initiation of DNA replication in vitro required functional TAg, since in its absence, initiation of viral DNA replication was not de-

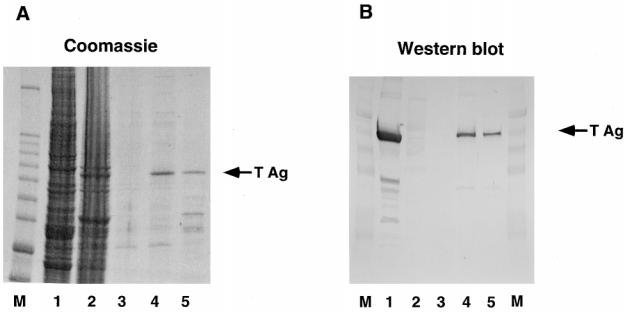

FIG. 1. Purification of recombinant JCV TAg. JCV TAg was expressed in High Five cells with recombinant baculoviruses. (A) Proteins (50 μg) from insect cells infected with wt baculovirus were separated by SDS-gel electrophoresis and stained with Coomassie brillant blue (lane 1). In lane 2, extracts from cells infected with baculoviruses expressing wt JCV TAg were analyzed. These extracts were then applied to Me^{2+} columns and eluted with 500 mM imidazole (0.6 μg of proteins from control extracts after Me^{2+} chromatography, lane 3; 1.5 μg of affinity-purified wt TAg, lane 4). In lane 5, immunoaffinity-purified JCV TAg (3 μg) was analyzed by SDS-gel electrophoresis. Lane M shows the 10-kDa molecular mass ladder. (B) These fractions were also analyzed by Western blotting with a polyclonal antiserum elicited against SV40 TAg at a dilution of 1:5,000. Lanes: 1, crude extracts containing recombinant JCV TAg; 2, control extracts without JCV TAg; 3, control proteins after Me^{2+} chromatography; 4 and 5, JCV TAg purified by Me^{2+} or immunoaffinity chromatography, respectively; M, prestained molecular mass marker.

tected (Fig. 2, lane 1). In addition, proteins from control extracts of mock-infected insect cells that were eluted from the Me^{2+} column did not support initiation of JCV DNA replication in vitro and the radioactive material which was detected by autoradiography (Fig. 2, lane 3) was only slightly above the background that was determined in the absence of TAg (Fig. 2, lane 1). In the complete assay, SV40 TAg supported the initiation of DNA replication at the SV40 origin of DNA replication and this served as a positive control for the activity of the purified proteins (Fig. 2, lane 2; the amount of initiation products synthesized in the presence of SV40 TAg was arbitrarily defined as 10 U). The JCV TAg purified by Me^{2+} chromatography was highly active in the initiation of JCV DNA replication (Fig. 2, lanes 4 to 6). A 0.6-μg sample of purified JCV TAg was sufficient to synthesize oligoribonucleotide primers at the JCV origin of DNA replication (Fig. 2, lane 4; 9.2 arbitrary U). Addition of 1.2 or 1.8 μg of JCV TAg only slightly increased the amount of synthesized primers (Fig. 2, lanes 5 and 6; 13 arbitrary U each). The amount of initiation products synthesized by 0.6 μg of JCV TAg at the JCV origin sequence was similar to that synthesized by 0.6 μg of SV40 TAg at the SV40 origin DNA (Fig. 2, compare lane 4 [9.2 arbitrary U] with lane 2 [10 arbitrary U]).

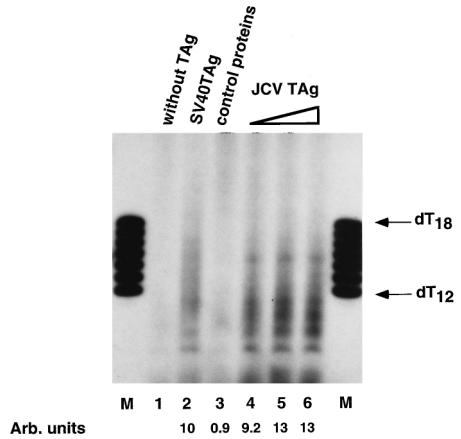

FIG. 2. Initiation of JCV DNA replication in vitro. In the presence of purified DNA polymerase α-primase, RP-A, topoisomerase I, and an ATP-regenerating system, recombinant JCV TAg is able to support initiation of JCV DNA replication in vitro of a plasmid containing JCV origin DNA. Lanes: 1, radioactive material detectable in the absence of JCV TAg; 2, initiation products of a complete reaction but with 0.6 μg of SV40 TAg and pUC-HS (plasmid DNA containing the SV40 origin of replication; positive control); 3, complete initiation reaction including the JCV origin of DNA replication and proteins that were purified by Me^{2+} chromatography from control cells (Fig. 1, lane 3); 4 to 6, products synthesized in complete reactions and increasing amounts of purified JCV TAg (0.6, 1.2, and 1.8 μg). Quantification was performed by using the PhosphorImager system. To calculate the amount of synthesized products, the radioactive material in lane 1 (nonspecific background) was subtracted from each lane and the amount of products determined in lane 2 (SV40 initiation) was arbitrarily displayed as 10. The arbitrary units are shown below the lanes. Lane M contained 5′-end-labelled oligo(dT_{12-18}) markers, as indicated at the right.

DNA replication by recombinant JCV TAg with S100 extracts. After having shown TAg-dependent initiation of DNA replication, we asked whether recombinant JCV TAg is also active in supporting the elongation step of DNA replication. In the presence of HeLa cell extracts, plasmid pJC433 DNA, with a full-length JCV origin of replication was incubated with increasing amounts of insect cell extracts that contained recombinant JCV TAg. Crude extract (25 μg of total protein) that contained about 1.5 μg of JCV TAg (determined by quantitative Western blotting [data not shown]) supported DNA replication, and *Dpn*I-resistant replication products were easily detected (Fig. 3A, lane 4). Increasing the amount of JCV TAg raised the amount of *Dpn*I-resistant replication products (Fig. 3A, compare lanes 4, 6, 8, and 10). The incorporation of radioactively labelled deoxynucleoside monophosphates (dNMPs) also increased in a concentration-dependent manner (Fig. 3B, columns 2 to 5). HeLa extracts did not replicate JCV DNA in the presence of insect cell extracts that contained 50 μg of protein, and the incorporation of dNMPs was just above the background (Fig. 3A, lanes 1 and 2, and B, column 1).

Sequence requirements for JCV DNA replication in vitro. The efficient initiation and propagation of DNA replication in vitro enabled us to study the sequence requirements of JCV DNA replication in the cell-free system. As already shown above, the full-length origin was efficiently replicated in vitro by using HeLa extracts supplied with unpurified TAg in insect cell extracts (Fig. 3). Incorporation of dNMPs with the constructs that had a deletion in the early region up to nucleotide 5072 (construct pJC443Δ91) was also high (Fig. 4B), while deletion of TAg-binding site I significantly reduced DNA synthesis from this plasmid (construct pJC443Δ112) to about 50% of that determined with full-length ori (Fig. 4B). The construct pJC443Δ136, which lacked the EP of the JCV origin, was not replicated in vitro (Fig. 4B). In addition, we tested whether the late region of JCV influences DNA replication in vitro. The construct that lacked the 98-bp repeat region (pJC180) was replicated as efficiently as or slightly more efficiently than the full-length construct (Fig. 4C, compare pJC433 with pJC180). Deletion of TAg-binding site I also caused a reduction of DNA replication by a factor of 2 (Fig. 4C, pJC180Δ112), and removal of a part of the early palindrome abolished DNA replication in vitro (Fig. 4C, pJC180Δ131).

To study the effect of the JCV core origin flanking regions in more detail, we compared DNA replication in the presence of increasing concentrations of purified JCV TAg. A 0.25-μg sample of TAg supported incorporation of dNMPs, and DNA synthesis in vitro increased in a dose-dependent manner (Fig. 5). Replication of a DNA that lacked TAg-binding site I (pJC433Δ112) showed lower levels of DNA synthesis than the full-length construct. In the presence of 0.25 μg of TAg, the

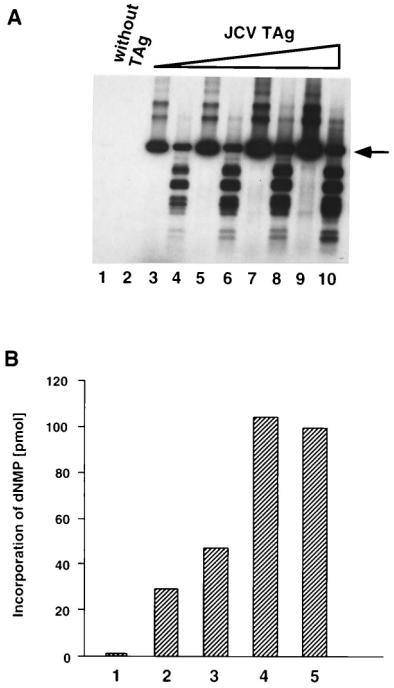

FIG. 3. DNA replication by recombinant JCV TAg. Increasing amounts (25, 50, 75, and 100 μg) of insect cell extracts that contained recombinant JCV TAg were added to S100 HeLa extracts in the presence of plasmid DNA with a complete JCV origin of replication (pJC433). (A) DNA synthesis products were analyzed for complete DNA replication by digestion with 10 U each of *Eco*RI and *Dpn*I (even-numbered lanes). In parallel, the products were linearized with 10 U of *Eco*RI (odd-numbered lanes). Lanes: 1 and 2, 50 μg of extracts from insect cells infected with wt baculovirus; 3 to 10, JCV DNA replication with extracts from insect cells that contained recombinant JCV TAg; 3 and 4, 25 μg of insect cell extracts; 5 and 6, 50 μg of extracts; 7 and 8, 75 μg of extracts; and 9 and 10, 100 μg of extracts from insect cells that contained recombinant JCV TAg. The arrow at the right indicates the linearized DNA synthesis products. (B) Incorporation of dNMPs into JCV DNA from panel A was measured by scintillation counting Columns: 1, 50 μg of cell extracts without recombinant TAg; 2 to 5, 25, 50, 75, and 100 μg, respectively, of cell extracts containing recombinant JCV TAg.

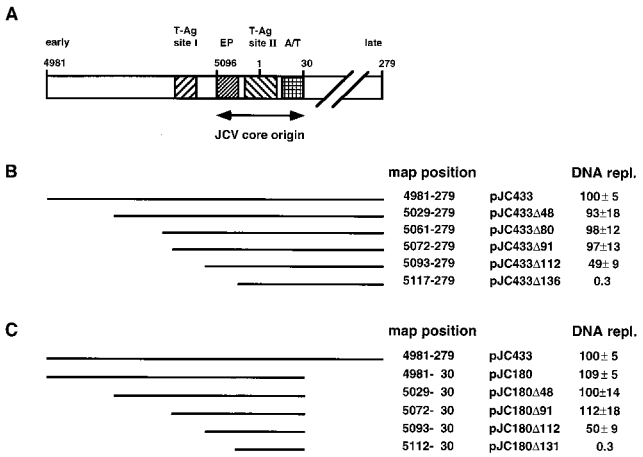

FIG. 4. DNA sequences controlling JCV DNA replication in vitro. Deletion mutant forms of the JCV origin of replication were analyzed using the cell-free JCV DNA replication system (see Materials and Methods) by addition of optimal amounts of unpurified JCV TAg. (A) Plasmid pJC433 contains map positions 4981 to 279 of JCV strain Mad-1, which enclose TAg-binding site I (nucleotides 5073 to 5090), the EP (5096 to 5114), TAg-binding site II (5119 to 11), the A/T-rich region (15 to 29), and the 98-bp repeats. The regions between positions 5096 and 5130 and positions 1 and 29 comprise the JCV core origin of DNA replication (17, 30, 53). (B) The deletion constructs pJC433Δ48 (5029 to 279), pJC433Δ80 (5061 to 279), pJC433Δ91 (5072 to 279), pJC433Δ112 (5093 to 279), and pJC433Δ136 (5117 to 279) were used for JCV DNA replication in vitro. The incorporation of radioactive dNMPs was determined with each DNA, and then incorporation was compared to the replication activity of plasmid pJC433, which was set arbitrarily at 100. (C) The pJC180 plasmids contain nucleotides 4981 to 30 (map positions) of the JCV genome, which encompass the region from TAg-binding site I (nucleotides 5073 to 5090) and the JCV core origin but not the 98-bp repeats. The pJC180 deletion constructs contained the following map positions: pJC180Δ48, 5029 to 30; pJC180Δ91, 5072 to 30; pJC180Δ112, 5093 to 30; pJC180Δ131, 5112 to 30. The experiments were carried out as described above. The DNA replication assays were performed in a minimum of four independent experiments and with two plasmid DNA preparations (see Materials and Methods).

dNMP incorporation with pJC433Δ112 reproducibly reached about 34% of that measured with pJC433 DNA, while in the presence of high TAg concentrations (1.5 μg), replication with pJC433Δ112 DNA rose to about 52% of that determined with full-length ori (Fig. 5).

JCV TAg binding site II is required for DNA replication in vivo and in vitro. In addition to the sequences flanking the core origin of replication and the early palindrome, we wished to analyze the influence of the TAg-binding sites upon JCV DNA replication in vivo and in vitro. To this end, we introduced point mutations within each binding site of JCV TAg (I^-, mutation in site I [AAAAAG**ATCT**CACGCCC instead of AAAAAG**CCTC**CACGCCC]; II^-, site II mutation [GG AGGCGGA**TC**CG instead of GGAGGCGGA**GG**CG]; I^-/II^-, both sites mutated). To analyze the effect of each mutation on the binding of TAg to DNA under various conditions, we performed immunoprecipitation assays with purified JCV TAg and several full-length origin fragments which either contained exclusively wt sequences or carried mutations in the TAg-binding sites (Fig. 6). As an internal control, a shorter fragment was included which consisted of TAg-binding sites I and II.

First, JCV TAg was incubated with DNA in buffer A on ice (Fig. 6A). Interaction of TAg with a fragment that still contained binding site I but had site II mutated was only slightly reduced in comparison with the DNA with exclusively wt TAg recognition sites (Fig. 6A, compare lane 4 with lane 1). In contrast, mutation of binding site I strongly diminished binding of JCV TAg to DNA (Fig. 6A, lane 3), even though TAg-

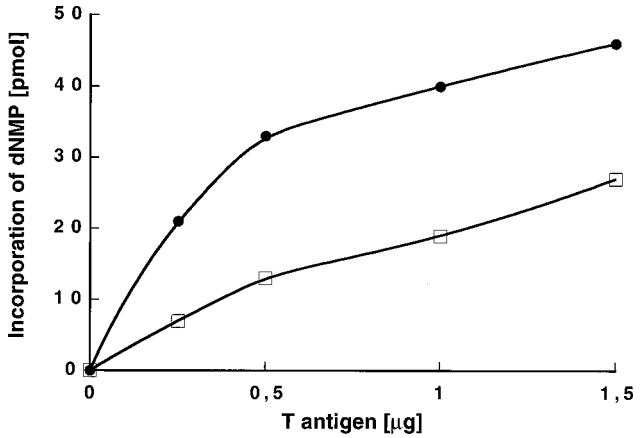

FIG. 5. JCV TAg-binding site I stimulates DNA replication in vitro. Plasmid pJC433 (●), which contained the full-length JCV origin, and its derivative pJC433Δ112 (□), without TAg-binding site I, were used for DNA replication in S100 extracts from HeLa cells in the presence of various concentrations of purified JCV TAg.

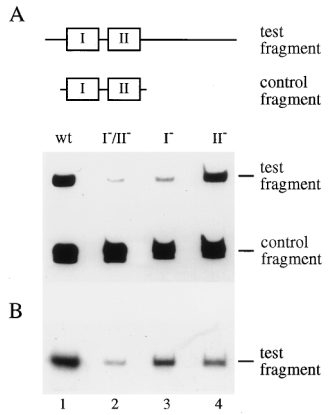

FIG. 6. Binding of JCV TAg to the origin of replication. This assay was performed as previously described (37). (A) Fragments within the 389-bp region spanning the complete regulatory region of JCV strain Mad-1 (nucleotides 5014 to 273). Lanes: 1, wt 389-bp regulatory region; 3, derivative containing inactivated TAg-binding site I (I^-); 4, derivative containing inactivated site II (II^-); 2, derivative with both sites inactivated (I^-/II^-) as described in Materials and Methods. A radiolabelled 75-bp fragment containing JCV TAg-binding sites I and II (nucleotides 5071 to 14 of Mad-1) served as a control fragment (lanes 1 to 4). Each fragment was incubated with 15 to 30 ng of purified TAg and competitor DNA in buffer A for 30 min on ice. (B) Labelled DNA fragments incubated with 75 ng of purified TAg and competitor DNA in replication buffer for 60 min at 37°C. After washing of the immunobeads, the bound DNA-protein complex was analyzed by gel electrophoresis and autoradiography.

binding site II was still present. The combination of both mutations (I^-/II^-) almost abolished the binding of TAg to DNA (Fig. 6A, lane 2). Under these experimental conditions, binding site II contributed only weakly to the overall binding of TAg to JCV ori. In a second approach, the binding activity of TAg was tested under replication conditions and the incubation was performed in replication buffer at 37°C (Fig. 6B). When high protein concentrations were used, the interaction of TAg with DNA was significantly reduced by mutation of binding site II (Fig. 6B, lanes 1 and 4) and TAg bound to site II as tightly as to site I (Fig. 6B, compare lane 4 with lane 3). In addition, protein concentrations and temperature were varied. A low protein concentration or incubation on ice diminished the interaction of TAg with binding site II, even in replication buffer, and the binding patterns were comparable to that presented in Fig. 6A (data not shown).

The abilities of these DNAs to support JCV DNA replication in vivo and in vitro were also investigated and compared (Fig. 7). Mutation of TAg-binding site I significantly reduced the replication capacity of the DNA to about 32% ± 6% in vivo (Fig. 7A, lane 4, and B, column 4) and to 67% ± 10% in vitro (Fig. 7C, column 4). Mutation of TAg recognition site II in the core origin eliminated the replication of JCV DNA in vivo (Fig. 7A, lane 5, and B, column 5) and virtually did so in vitro (Fig. 7B, column 5; 2.2% ± 0.5%, which was slightly above the background). DNA with both TAg-binding sites mutated was not replicated in vivo (Fig. 7A, lane 6, and B, column 6) and hardly showed any replication in vitro (Fig. 7C, column 6; 1.3% ± 0.4%). Plasmid pJC433Δ112 (deleted site I), which showed 27% ± 6% replication activity in vivo (Fig. 7A, lane 2, and B, column 2) and 50% ± 3% activity in vitro (Fig. 7C, column 2; also, Fig. 4B), was replicated in parallel and used for comparison.

SV40 TAg antigen supports in vitro replication of plasmid DNA with a JCV origin. DNA replication of JCV, PyV, and SV40 follows similar mechanisms (8, 15, 65). Since it is known that SV40 TAg efficiently supports replication of JCV DNA in vivo (53), we wanted to know whether DNA replication in vitro occurs with different viral TAgs using a JCV origin of DNA replication. PyV TAg did not support DNA replication with a JCV origin. In the presence of PyV TAg, plasmid DNA containing the JCV origin was replicated neither in human S100 extracts nor in mouse replication extracts, although in parallel experiments, PyV TAg was highly active in replication of a plasmid that contains a PyV origin of replication (Fig. 8 and data not shown). In contrast to these results, SV40 and JCV

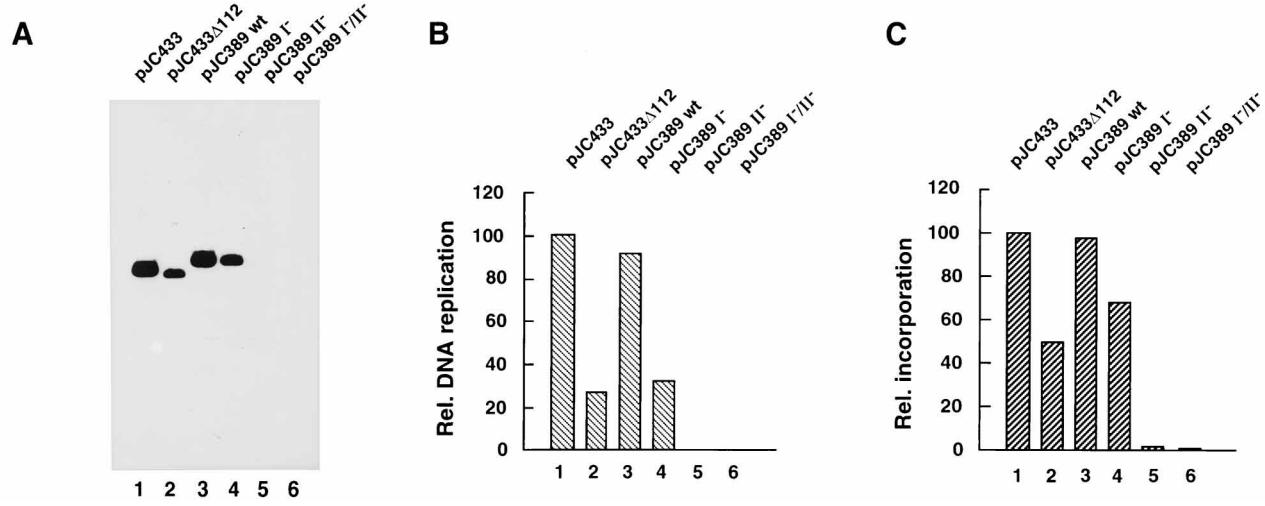

FIG. 7. JCV TAg-binding site II is essential for JCV DNA replication. DNA replication was analyzed in U138 glioblastoma cells or in HeLa cell extracts. (A) U138 glioblastoma cells were plated and transfected on the following day with 2 μg of the test plasmid and 1 μg of Rous sarcoma virus expression vector pRSV-JCT. At 84 h posttransfection, they were harvested and low-molecular-weight DNA was extracted (20). Plasmid DNAs were digested with *Dpn*I and analyzed by Southern blotting followed by autoradiography. Plasmid pJC389-wt contained nucleotides 5014 to 273 of JCV strain Mad-1 (lane 3), pJC389-I^- had a mutated form of TAg-binding site I (lane 4), pJC389-II^- contained a mutation within the sequence of TAg-binding site II (lane 5), and pJC389-I^-/II^- is a recombination with both of the mutations (lane 6). Plasmid DNAs pJC433 (full-length ori) and p433Δ112 (I^-) served as controls (lanes 1 and 2). (B) Quantification of panel A was performed with the PhosphorImager system. (C) In the presence of high concentrations of JCV TAg, acid-precipitable incorporation of dNMPs by HeLa extracts was determined with these DNA constructs and quantified by liquid scintillation counting.

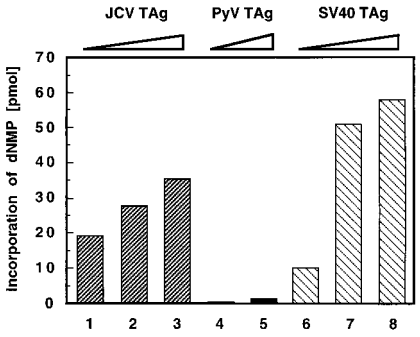

FIG. 8. SV40 TAg supports replication of plasmid DNA containing a JCV origin. Increasing amounts of polyomavirus TAg were added to S100 extracts from human 293S cells. Acid-precipitable replication products were collected on glass fiber filters, and incorporation of radioactive dNMPs was measured by scintillation counting. Background radioactivity was subtracted, and the average incorporation of three in vitro replication experiments is presented in columns 1 to 8. Columns 1, 2, and 3 show the dNMP incorporation with 0.5, 1, and 1.5 μg of JCV TAg, respectively; columns 4 and 5 show that with PyV TAg (0.5 and 1 μg); and columns 6, 7, and 8 present the incorporation in the presence of 0.5, 1, and 1.5 μg of SV40 TAg, respectively.

TAg supported DNA replication with the JCV origin in human cell extracts (Fig. 8). The comparison of the replication activity of SV40 and JCV TAg showed that in the presence of 0.5 μg of JCV TAg, incorporation of dNMPs was two to three times as high as that with SV40 TAg (Fig. 8, compare columns 1 and 6), while at higher protein concentrations, replication with JCV TAg was significantly lower than that with SV40 TAg (Fig. 8, compare columns 2, 3, 7, and 8).

DISCUSSION

In this report, we describe for the first time the replication of JCV DNA in vitro and characterize this cell-free system. Understanding of the related SV40 DNA replication was greatly advanced by establishing an in vitro replication system. To study the functions of JCV TAg biochemically, the preparation of purified JCV TAg in large quantities is essential. Recently, its expression in 293 cells and insect cells was described (2, 68). Independently, we expressed JCV TAg in insect cells with recombinant baculoviruses and established a one-step purification scheme using commercially available resins. Me^{2+} chromatography simplifies purification of JCV TAg, since in contrast to SV40 TAg, only a few well-characterized monoclonal antibodies against JCV TAg are available (1, 2).

Recombinant JCV TAg is highly active in the initiation reaction with DNA polymerase α-primase, RP-A, topoisomerase I, and the JCV origin of replication. Its activity at the JCV origin of replication is comparable to that of SV40 TAg at the SV40 origin of replication (Fig. 2). JCV TAg also efficiently supported replication of plasmid DNA containing an origin of JCV in crude human cell extracts. By comparing the replication activities of different polyomavirus TAgs with JCV DNA, it could be shown that at low protein concentrations, JCV TAg was reproducibly more active than SV40 TAg. Interestingly, increasing the concentration of both TAgs resulted in greater dNMP incorporation with SV40 TAg than that with JCV TAg. These findings suggest that there is a rate-limiting step in the replication of a plasmid containing a JCV origin with SV40 TAg which can be overcome by the addition of larger amounts of protein and that this seems to be less emphasized in the case of JCV TAg. The finding that SV40 supports replication of JCV DNA more efficiently is in agreement with earlier findings on JCV and SV40 DNA replication in vivo, since in these studies JCV TAg was less active than SV40 TAg, even with the JCV origin of replication (53). However, the differences in the replication activities of JCV TAg and SV40 TAg were not as striking in vitro as in vivo. This discrepancy could be due to factors which are absent in the initiation assay and in the replication extracts and which modulate JCV or SV40 DNA replication in glial cells. Alternatively, the disparity may be a result of differences in the various TAg protein levels in the in vivo experiments. Another possibility is that posttranslational modification of JCV TAg expressed in glial cells could differ from that of baculovirus-expressed TAg. By using the well-studied SV40 TAg as a model, it was shown that phosphorylation modulates SV40 TAg replication activity both positively and negatively (7, 38, 39) and that the phosphorylation state of the protein expressed in insect cells is optimal for its replication function (22). Recently, it was shown that phosphorylation of JCV TAg exhibits a high degree of similarity to that of SV40 TAg (59, 60) but it still needs to be proven that phosphorylation modulates both TAgs comparably. A reproducible source and purification scheme for JCV TAg, which is active in DNA replication, makes such investigations possible.

DNA replication and its initiation at the origin are complex reactions and require the cooperation of several proteins and protein complexes (8, 13, 14, 23, 58). Initiation of DNA replication and DNA synthesis are modulated by DNA sequences at the origin or flanking it and by factors binding to these sites. In addition, DNA elements neighboring the origin seem to be crucial for the replication of JCV DNA, as well as for that of other papovaviruses (13, 14, 32). We report here that flanking sequences on the late side of JCV core ori which stimulate JCV DNA replication in vivo only slightly influenced replication of JCV DNA in vitro (Fig. 4). However, this lack of modulation in vitro by elements adjacent to the A/T-rich element of core ori was also observed with SV40 DNA replication in vitro (29). Recent findings in this system suggested that under specific conditions, the stimulatory function of sequences at the late side was detected but that factors which bind to these elements might be absent in replication extracts or be unstable under replication conditions (13, 18).

Deletions or mutations of sequences at the early side of JCV ori reduced replication activity in vivo and in vitro in comparison to wt DNA. A mutation of TAg-binding site I that strongly diminished the interaction of TAg with this DNA reduced DNA replication (Fig. 6 and data not shown). At high TAg concentrations, the DNA replication reaction did not decrease to the same extent as observed in vivo. However, at low TAg concentrations DNA replication was reduced about threefold, which is in the range that was observed in experiments in vivo. The role of binding site I during viral DNA replication is still unclear. The interaction of TAg with its binding site I could support recruitment of TAg to binding site II. Alternatively, binding of TAg to site I might either be involved in facilitating destabilization of double-stranded DNA at the EP or support a later step, when TAg acts as a DNA helicase at the replication fork.

Mutations of JCV core ori abolished DNA replication in vivo and in vitro. Interestingly, the interaction of JCV TAg with TAg-binding site II was greatly influenced by the assay conditions. Incubation of the binding assay on ice, a low protein concentration, or omission of nucleotides resulted in preferred interaction of JCV TAg with binding site I, whereas raising the temperature to 37°C, increasing the protein concentration, and addition of nucleotides caused an increased affinity of JCV TAg to binding site II such that both binding sites were nearly equally well recognized. Recently, it was also

shown that baculovirus-expressed JCV TAg efficiently binds to site II under replication conditions (2). These results suggest that JCV TAg probably interacts as a higher-order structure with binding site II, similarly to SV40 TAg, which recognizes binding site II as a double hexamer in an ATP-dependent manner (2, 35).

In summary, the newly established in vitro DNA replication system closely resembles DNA replication in vivo and therefore will allow detailed functional studies of JCV DNA replication and its complex regulation. This cell-free system might facilitate studies of viral host specificity and virus-host interactions.

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