Identification of Critical Elements within the JC Virus DNA Replication Origin

KEVIN J. LYNCH AND RICHARD J. FRISQUE*

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

The T antigen of JC virus (JCV) does not interact productively with the simian virus 40 (SV40) origin of replication. In contrast, the SV40 T antigen does drive replication from the JCV origin as well as from its own. The basis for this restricted interaction was investigated by analyzing the structure of the JCV replication origin. The replication activities of JCV-SV40 hybrid origin plasmids were tested in cells constitutively producing either the JCV or SV40 T antigen. Results indicated that a region of the JCV origin critical for interaction with the JCV T antigen was positioned to the late side of the central palindrome of the putative core origin. A mutational analysis of this region indicated that the sequence of the A+T-rich tract was primarily responsible for determining the efficiency with which JCV can initiate replication from its origin. The tandemly repeated pentameric sequence AGGGA located proximal to the A+T-rich tract in the JCV enhancer element was found to stimulate JCV, but not SV40, T antigen-mediated replication. The effect on replication of other elements within the JCV enhancer was also dependent on the T antigen employed for initiation. A plasmid containing the replication origin of prototype BK virus was unable to replicate in cells containing JCV T antigen, again indicating the inflexibility of the JCV T antigen in interacting with heterologous origins.

Understanding the restricted lytic behavior of the human polyomavirus JC virus (JCV) has been a major focus of study in our laboratory. Although polyomaviruses generally have a restricted host range in vitro, JCV is extremely limited in this respect; it can be propagated well only in human fetal glial cells, and even in these cells its lytic cycle is prolonged (reviewed in reference 57). We have been attempting to identify the factors contributing to this restricted behavior, and our studies have centered upon the central step in the polyomavirus lytic cycle, that of DNA replication, which separates early gene expression from late protein synthesis, capsid assembly, and virion release.

The simian virus 40 (SV40) origin of replication consists of several cis-acting elements that flank a core origin spanning 64 nucleotides. The core contains the second of three T antigen-binding sites at its center, a palindromic sequence to the early side, and an A+T-rich sequence to the late side (4, 11, 18, 19, 54). Elements that augment but are not strictly required for DNA replication include the T antigen-binding region I to the early side of the core origin and the G+C-rich and enhancer repeat elements that lie to the late side of the core (14, 28, 32, 34). The viral T antigen initiates DNA replication in a stepwise fashion: T antigen recognizes and binds to pentameric sequences contained in binding region II in an ATP-dependent complex, the early palindromic sequence of the core origin is melted, a structural alteration occurs in the A+T-rich sequence, and elongation proceeds utilizing the ATPase-DNA helicase activity of T antigen in conjunction with various host cell factors (5, 10, 20, 42, 44, 52, 55, 56, 58). The exact function of the auxiliary elements of the origin in replication is not yet known.

We have utilized the molecular similarity between JCV and SV40 to address questions relating to the differences in biological characteristics of the two viruses. JCV and SV40 have 69% sequence homology at the nucleotide level; sequences within the noncoding region have diverged to the greatest extent and include those specifying the auxiliary and core domains of the viral origin of replication (Fig. 1) (22). The T antigens of the two viruses have 72% homology at the amino acid level.

Recently the SV40 T antigen was shown to initiate DNA replication from both the SV40 and JCV origins, whereas the JCV protein was able to drive replication only from its own origin (K. J. Lynch and R. J. Frisque, Virology, in press). The specific DNA binding activity of the JCV T antigen was found to be less than that of the SV40 protein (binding to site II of either DNA was especially weak), which presumably contributes to the inefficient DNA replicating activity of JCV overall. However, the JCV T antigen did recognize the JCV and SV40 binding regions with similar efficiencies, indicating that the failure of the JCV T antigen to drive replication from an SV40 origin was due to a defect in a replication step after the initial binding of the protein to the DNA. In the present study we have begun to identify sequences within the JCV origin of replication that are required for a productive interaction with the JCV T antigen.

MATERIALS AND METHODS

Cells. COS-1 cells (CV-1 African green monkey kidney cells transformed by origin-defective SV40 [25]) and cPOS-2 cells (primary human fetal glial [PHFG] cells transformed by origin-defective SV40 [Lynch and Frisque, in press]) were grown in Dulbecco modified Eagle (DME) medium supplemented with 2 to 10% fetal bovine serum. POJ-19 cells (PHFG cells transformed by origin-defective JCV [37]) were grown in DME supplemented with 10% fetal bovine serum.

DNAs. Plasmid pKP55 was used to construct all viral origin-bearing plasmids with the exceptions of pJLo and pJLod4, which were constructed with the plasmid vector pKP45 (33). pJLo consists of the 200-nucleotide-pair (np) origin-containing HindIII-SphI fragment of SV40, and pJLod4 contains the corresponding fragment of the origin-defective SV40 mutant 8-4 (26). pBKo contains the 555-np HindIII origin fragment of prototype BK virus (BKV) (24). The plasmids pKP55, pBKo, pJLo, and pJLod4 were gifts
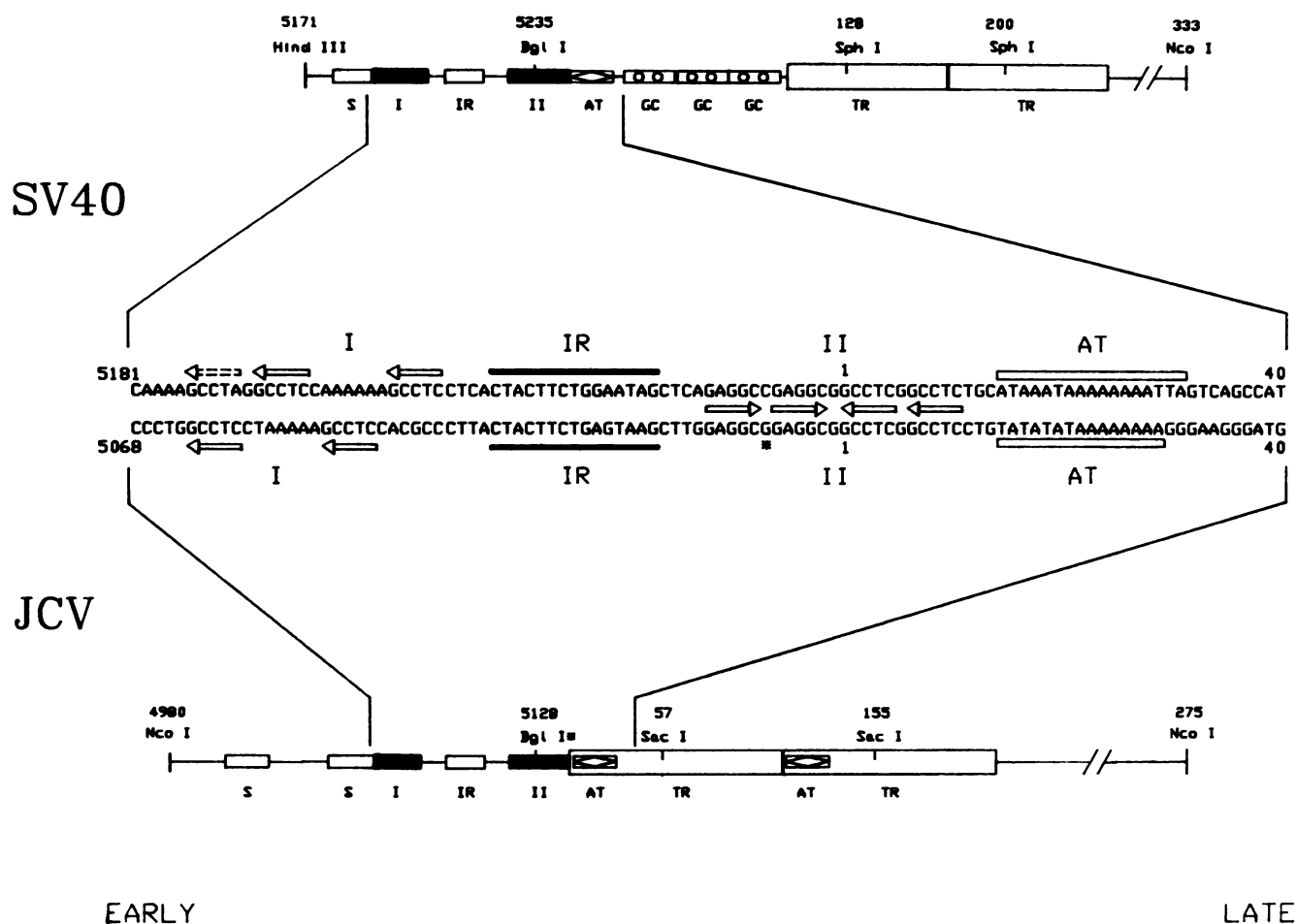
* Corresponding author.

FIG. 1. Structure of the SV40 and JCV origins of replication. Elements that are indicated in the drawings of the entire origin regions include the following: small open boxes, dyad symmetries (S); filled boxes, T antigen-binding sites (I) and (II); boxes containing diamonds, TATA boxes (AT); boxes containing circles, G+C-rich repeats (GC); large open boxes, enhancer elements (TR). Numbers above the elements denote nucleotide positions of the restriction enzyme sites indicated; the BglI site marked with an asterisk denotes the restriction site created in the JCV mutant M1(BglI). The center of the figure is a comparison of the nucleotide sequences that include the core origin of SV40 and the putative core origin of JCV. The arrows indicate positions of T-antigen recognition sequences; the dashed arrow in the SV40 origin denotes a weak recognition sequence (15, 45). The inverted repeat regions (IR) are marked by solid bars, and the A+T-rich tracts (AT) are marked by open bars. The G at nucleotide position 5124 of the JCV sequence (asterisk) is a C in the mutant M1(BglI).

from Joachim Li and Thomas Kelly. pSVo was produced by joining the 108-np NcoI-BglI fragment of the hybrid polyomavirus pM-1(SV40) (9) to a 341-np fragment of SV40 spanning np 5236 through 333 produced by digestion with BglI and partial digestion with NcoI. pdI892o was produced in a similar manner to pSVo but replaces the 341-np BglI-NcoI fragment of SV40 with the 322-np BglI-NcoI fragment of the viable SV40 mutant dl892, containing a deletion of nucleotides 34 to 52 (48). Plasmid pM1o contains the 425-np origin-containing NcoI fragment of the prototype strain of JCV, Mad1 (22). By digesting pM1o with SacI, one copy of the 98-np JCV enhancer repeat was deleted to yield pM1(d98)o. pM1(BglI)o contains the 425-np NcoI fragment of JCV mutant M1(BglI), in which a G-to-C transversion was introduced into the Mad1 genome at nucleotide 5124 by oligonucleotide-directed site-specific mutagenesis (Lynch and Frisque, in press). This created a unique BglI site at the same position as that found within the replication origin of SV40. Three hybrid replication origin plasmids were constructed by exchanging JCV and SV40 sequences at the BglI site within T antigen-binding region II. The hybrid origin

construct pMSVo consists of the 148-np NcoI-BglI fragment spanning nucleotides 4980 to 5128 of M1(BglI) and the 341-np BglI-NcoI fragment of SV40. The hybrid origin construct pMSo is similar to pMSVo but replaces the 341-np BglI-NcoI fragment of SV40 with the 322-np BglI-NcoI fragment of dl982. The hybrid origin pSMo contains the 108-np NcoI-BglI fragment of M-1(SV40) and the 279-np BglI-NcoI fragment of M1(BglI). pM8o contains the 434-np NcoI origin fragment of the JCV variant Mad8Br (39), and pM8(d83)o was created by digesting pM8o with SacI and recircularizing the larger fragment by ligation, thereby deleting one of the 83-np enhancer repeats. The numbering system used throughout this paper for JCV and SV40 follows those established by Frisque et al. (22) and Buchman et al. (6), respectively.

Replication origin mutations; exonuclease III mutagenesis. A series of progressively larger deletions were introduced into pM1(d98)o, beginning with the removal of sequences from the SacI site at nucleotide 57/155. This plasmid (10 μg) was first digested with SacI, extracted with phenol and chloroform-isoamyl alcohol (24:1), and precipitated with

ethanol. The DNA was suspended in 100 μl of 1× S1 nuclease buffer (40.5 mM potassium acetate [pH 4.6], 34 mM NaCl, 1.4 mM ZnSO$_4$, 6.75% glycerol) and digested with 6 U of S1 nuclease (Pharmacia LKB Biotechnology, Inc.) for 30 min at room temperature. The reaction was terminated by the addition of 20 μl of S1 nuclease stop buffer (4 M ammonium acetate, 100 mM EDTA [pH 6.7]) and incubation at 70°C for 10 min. The DNA was extracted with phenol and chloroform-isoamyl alcohol, precipitated with ethanol, and suspended in 60 μl of buffer (66 mM Tris hydrochloride [pH 8.0], 0.66 mM MgCl$_2$). A portion of the DNA (20 μl) was digested with 100 U of exonuclease III (New England BioLabs). At 15-s intervals 2.5-μl samples were transferred into 7.5 μl of S1 nuclease mix (2.4 U of S1 nuclease, 1× S1 buffer) on ice to stop exonuclease III activity. S1 digestion then proceeded by incubation at room temperature for 30 min and was stopped by the addition of S1 nuclease stop buffer and incubation at 70°C for 10 min, followed by phenol and chloroform-isoamyl alcohol extraction. The DNAs were precipitated with ethanol, suspended in 1× restriction enzyme buffer, and digested with *Pvu*II, which cleaves pKP55 once and leaves blunt ends. This was done to create uniform sequences of the vector proximal to the inserted origins. The DNAs were incubated with Klenow fragment of DNA polymerase I and deoxynucleoside triphosphates to ensure that the remaining ends treated with exonuclease III were blunt. After phenol and chloroform-isoamyl alcohol extraction, precipitation with ethanol and suspension in 1× ligase buffer and 200 U of T4 DNA ligase (New England BioLabs), the DNAs were ligated and used to transform *Escherichia coli*. DNAs from resulting clones were screened by restriction enzyme analysis, and their sequences were determined by the double-stranded dideoxy-chain termination method (46).

Replication origin mutations, cassette mutagenesis. Mutagenesis of the JCV replication origin was also performed by substituting synthetic complementary oligonucleotides for regions of the JCV origin. Briefly, the JCV origin plasmid pM1(BglI)o was cleaved either with *Bgl*I and *Sac*I or with *Bgl*I and *Cla*I. The large fragment containing pKP55 and a portion of the JCV origin was isolated. The oligonucleotides were synthesized on a Milligen/Biosearch model 7500 DNA synthesizer; complementary sequences were heated to 90°C for 3 min and allowed to anneal by cooling the mixture gradually from 70°C to room temperature. The double-stranded oligonucleotides containing a *Bgl*I end and a *Sac*I or *Cla*I end were ligated to the plasmid sequences in a 10:1 molar excess of oligonucleotides. After ligation and transformation, the structures of the recombinant DNAs were verified by restriction enzyme and sequence analyses (46). All plasmids were propagated in *E. coli* DH-1 and purified by standard procedures (38).

DNA transfection. Transfections of human and monkey cells with viral origin-containing plasmid DNAs were performed by using a modification of the DEAE-dextran technique (50). Cells were grown on 60-mm plates to approximately 80% confluency, washed once with serum-free DME medium, and incubated at 37°C for 1.5 to 2 h with 1 ml of a solution (DME, 50 mM Tris hydrochloride [pH 7.5]) containing DEAE-dextran (250 μg/μl; molecular weight, 500,000; Pharmacia) and the plasmid DNA (100 ng/ml). After the incubation period, the cells were washed with serum-free DME and refed with DME supplemented with 10% fetal bovine serum.

DNA replication assay. The ability of the various origin plasmids to replicate in JCV and SV40 T antigen-expressing cell lines was measured by using the *Dpn*I replication assay.
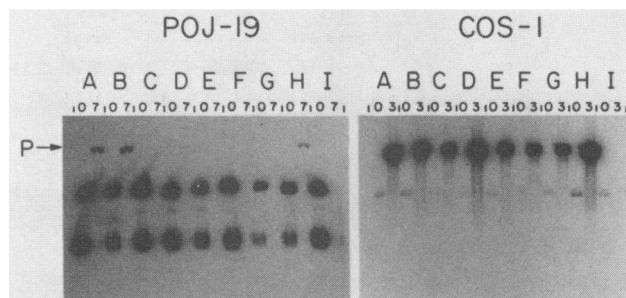

FIG. 2. DNA replication activity of prototype, mutant, and hybrid origins in POJ-19 and COS-1 cells. Subconfluent plates of cells were transfected with 100 ng of plasmid DNA. At 0 and 7 days posttransfection (0 and 3 days posttransfection for experiments with COS-1 cells), low-molecular-weight DNA was isolated from the cells by the method of Hirt (29). This DNA was cleaved with *Eco*RI and *Dpn*I, electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose filter, and probed with linearized, ^{32}P-labeled pKP55. Letters denote plasmids pM1o (A), pM1(BglI)o (B), pSVo (C), pJLo (D), pdI892o (E), pMSVo (F), pMSo (G), pSMo (H), and pJLod4 (I). Numbers over the lanes indicate the day posttransfection that the DNAs were extracted from the cells. P indicates the position of the progeny plasmid DNAs. DNA markers (not shown) were included on each gel to allow comparisons of the sizes and quantities of the progeny DNAs. The autoradiograms were exposed for 2 h (POJ-19 experiment) or for 45 min (COS-1 experiment).

Low-molecular-weight DNA was extracted by the Hirt procedure (29) at various times after transfection (days 0 and 3 posttransfection for COS-1 and cPOS-2 cells, days 0 and 7 for POJ-19 cells). The extract (25%) from a 60-mm plate was digested with *Dpn*I and *Eco*RI. The restriction enzyme *Dpn*I was used to distinguish DNA replicated in bacterial versus mammalian cells based upon its ability to cleave only the fully methylated DNA produced in bacteria. The second restriction enzyme, *Eco*RI, linearized the plasmid DNA, thereby facilitating quantitation. The digestion products were separated by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose filters by the method of Southern (51). The immobilized DNA was hybridized with linearized [α-^{32}P]dCTP-labeled (oligo labeling kit; Pharmacia) pKP55 DNA. After exposure of the filters to Kodak XAR-5 film, the radioactive bands were excised and quantitated by counting in an LS5801 liquid scintillation counter (Beckman Instruments).

RESULTS

Replication of hybrid viral origins. Previous studies have indicated that cells containing SV40 T antigen will support the replication of DNA with the SV40 or JCV origin (21, 33, 35), whereas cells producing JCV T antigen will only support efficient replication of DNA containing the JCV origin (Lynch and Frisque, in press). In an attempt to delineate the elements of the JCV replication origin that are critical to an effective interaction with its T antigen, the replicating activities of hybrid origins containing SV40 and JCV sequences were tested in cells expressing the JCV or SV40 T protein. This activity was measured with the *Dpn*I assay at times when DNA replication reached its peak (Lynch and Frisque, in press): 3 days posttransfection with COS-1 cells (containing SV40 T antigen) and 7 days posttransfection with POJ-19 cells (containing JCV T antigen). Autoradiographs of representative experiments are shown in Fig. 2, and quantitative

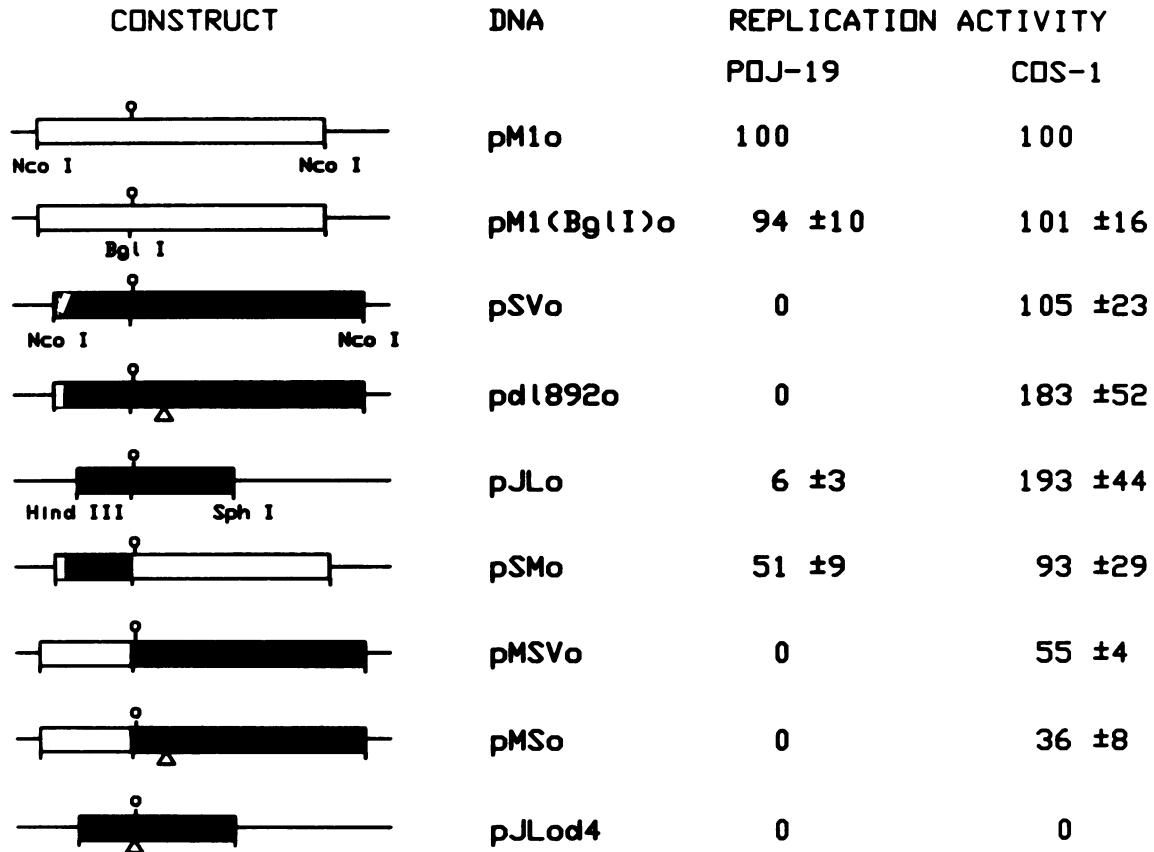| CONSTRUCT	DNA	REPLICATION ACTIVITY	
		POJ-19	COS-1
	pM1o	100	100
	pM1(BglI)o	94 ±10	101 ±16
	pSVo	0	105 ±23
	pdl892o	0	183 ±52
	pJLo	6 ±3	193 ±44
	pSMo	51 ±9	93 ±29
	pMSVo	0	55 ±4
	pMSo	0	36 ±8
	pJLod4	0	0

FIG. 3. Structure and replication activity of JCV-SV40 hybrid origin plasmids. Structures of the origin plasmids are indicated to the left. JCV DNA is shown as an open box, SV40 DNA is represented by a filled box, and the adjoining sequences of the plasmid are shown as a thin line. The open circle above each construct indicates the position of the center of the central dyad symmetry (T antigen-binding site II). The small open region at the early (left) ends of the pSVo, pdl892o, and pSMo constructs represents a 5-nucleotide span of JCV DNA used as an *Hinf*I-*Nco*I linker (9). The triangles beneath pdl892o, pMSo, and pJLod4 indicate deletions in the sequence as described in Materials and Methods. The data tabulated to the right represent results from replication assays with these plasmids in POJ-19 and COS-1 cells. Radioactive bands representing *Dpn*I-resistant, full-length plasmid DNA were excised from nitrocellulose filters and counted in a liquid scintillation counter. All values were expressed relative to the replication activity of pM1o (arbitrarily given a value of 100). Throughout these studies, the replicating activity of the origin constructs was found to be significantly higher in the two cell lines expressing SV40 T antigen compared with that measured in POJ-19 cells (20- to 40-fold and 60- to 90-fold higher in cPOS-2 [Fig. 4] and COS-1 cells, respectively). Bands were taken from samples harvested 7 days posttransfection in POJ-19 cells and 3 days posttransfection in COS-1 cells. The values shown are an averages of three independent experiments ± the standard errors of the means.

data plus diagrams of the origin constructs are shown in Fig. 3. As expected, plasmids containing either prototype origin replicated in COS-1 cells; the JCV origin (pM1o) replicated about as well as its SV40 counterpart (pSVo). Plasmids containing a smaller SV40 origin fragment (pJLo) or the origin of the viable SV40 mutant dl892 (48) (pdl892o) replicated approximately twice as well as pSVo, whereas the origin-defective mutant pJLod4 failed to replicate. Substitution of the guanine at position 5124 of the JCV sequence with a cytosine in the mutant pM1(BglI)o did not affect the replicating activity of the origin. pM1(BglI)o was used as a source of JCV sequences in the construction of JCV-SV40 hybrid origins. The plasmid pSMo, containing early origin sequences of SV40 coupled to late elements of JCV, replicated as efficiently as the wild-type SV40 origin in COS-1 cells. The constructs containing early elements of the JCV origin and late sequences of either SV40 (pMSVo) or dl892 (pMSo) both replicated in COS-1 cells, albeit to a lower level than either intact origin.

The ability of JCV T antigen to effect replication of these origin-containing plasmids was tested in POJ-19 cells. Plas-

mids containing JCV origins, pM1o and pM1(BglI)o, were able to replicate, whereas replication of the SV40 origin plasmids either was undetectable (pSVo and dl892o) or occurred at a very low levels (pJLo) in these cells (Fig. 2 and 3). The hybrid pSMo replicated to levels approximately half that of the prototype JCV origin, but neither pMSo nor pMSVo exhibited detectable replicating activity. These data indicated that sequences positioned to the late side of the putative T antigen-binding site II of the JCV origin were required for effective interaction with the JCV T antigen and/or that elements positioned to the late side of binding region II of the SV40 replication origin inhibited JCV T antigen-mediated DNA replication.

Deletion analysis of the JCV replication origin. To identify sequences to the late side of the JCV origin that influence DNA replicating activity, a series of mutants was created by progressively deleting sequences from the *Sac*I site (nucleotide 57) toward the putative core origin by using exonuclease III digestion. The replication activities of these deletion mutants were measured in COS-1, POJ-19, and cPOS-2 cells. cPOS-2 cells are transformed human fetal glial cells that
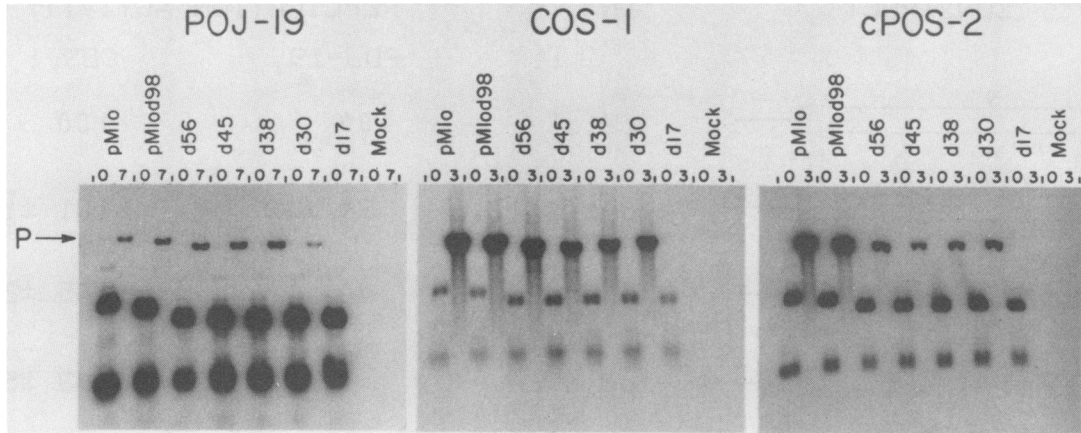
FIG. 4. DNA replication activity of JCV origin deletion mutants in POJ-19, COS-1, and cPOS-2 cells. The procedures and the identification of markers are as described in the legend to Fig. 2 and in Materials and Methods. Low-molecular-weight DNAs were harvested at days 0 and 3 posttransfection for the cPOS-2 cells. The exposure times for the autoradiograms were 5 h for the POJ-19 experiment, 6 h for the COS-1 experiment, and 12 h for the cPOS-2 experiment.

express the SV40 T protein. These cells allowed us to investigate whether elements present in the JCV replication origin functioned in a cell type-specific manner. Representative autoradiographs from experiments with each cell line are shown in Fig. 4, and a diagram of the deletion scheme and the quantitative data are shown in Fig. 5. The deletion mutant pM1(d98)o contains only one of two copies of the 98-np enhancer element found in the prototype JCV

construct pM1o. In POJ-19 cells, this deletion had the effect of increasing the replication activity of the JCV origin. Progressive deletion of nucleotides resulted in additional increases in replication activity of the mutant origins in POJ-19 cells until sequences spanning the short tandem repeat 5'-AGGGAAGGGA-3' (constructs d38 and d30, Fig. 5) were deleted, and activity dropped approximately sixfold. Activity became undetectable when sequences between nu-
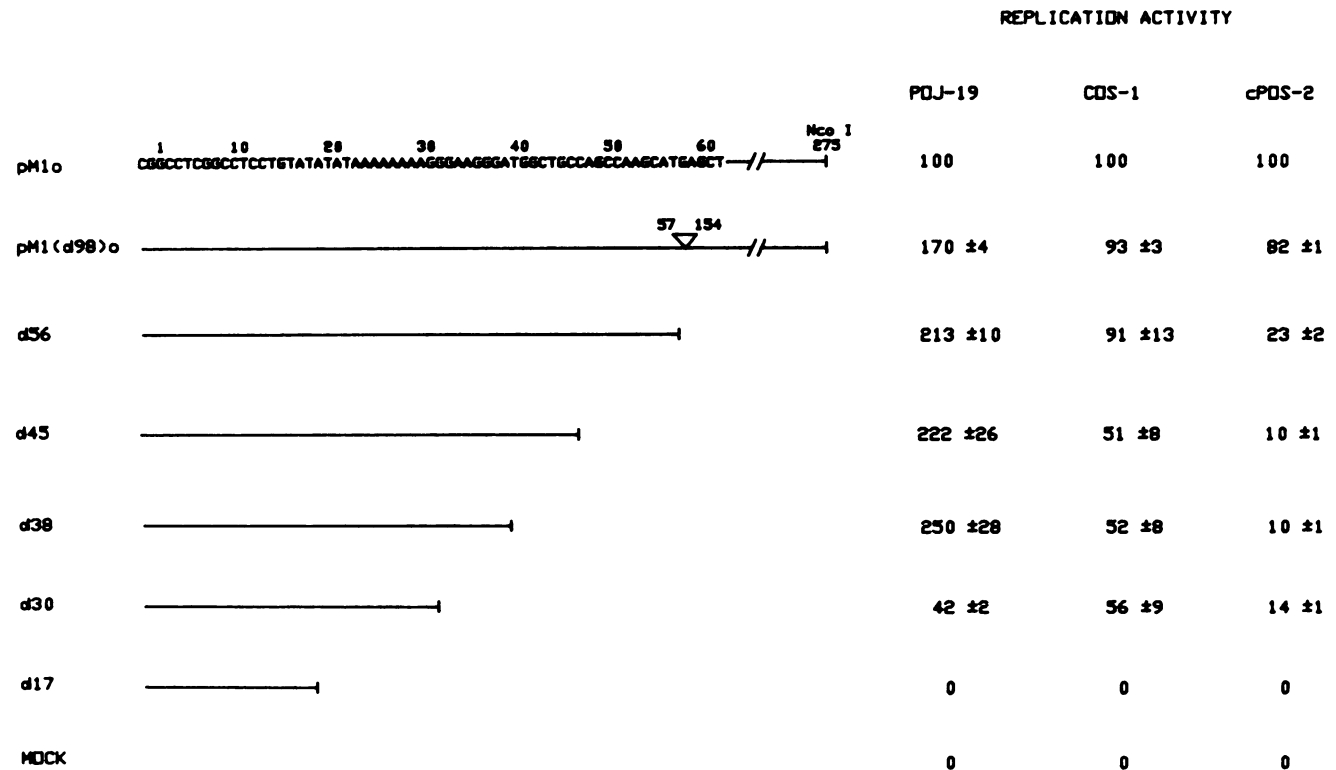

FIG. 5. Structure and replication activity of JCV origin deletion mutants. The left side of the figure indicates the sequences deleted in each mutant; the triangle on pM1(d98)o indicates the deletion of one of the 98-np tandem repeats (the nucleotides deleted are noted above the triangle). Replication activities were determined as described in the legend to Fig. 3 and are the averages of three independent experiments ± standard errors of the means.
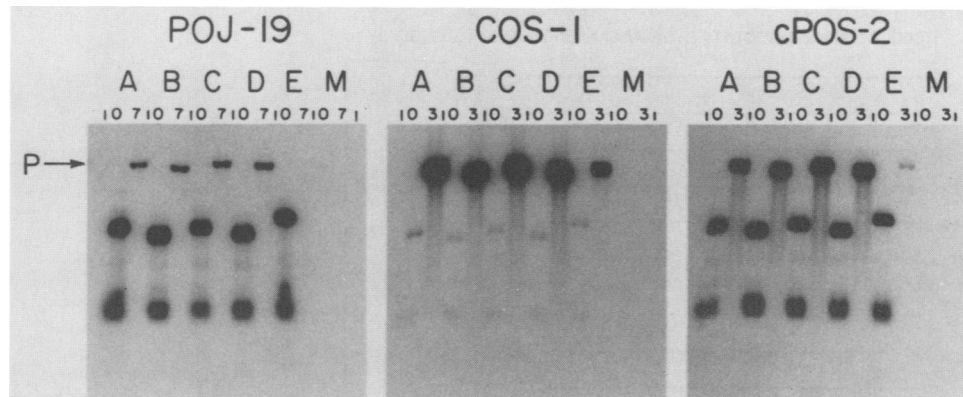
FIG. 6. DNA replication activity of plasmids containing JCV variant Mad8Br and BKV origins in POJ-19, COS-1, and cPOS-2 cells. The replication assay and the identification of markers are described in the legend to Fig. 2 and in Materials and Methods. The letters denote plasmids pM1o (A), pM1(d98)o (B), pM8o (C), pM8(d83)o (D), and pBKo (E) and mock-infected cells (M). The autoradiograms were exposed for 13 h (POJ-19 experiment) or 5 h (COS-1 and cPOS-2 experiments).

cleotides 17 and 30 were removed. The replication activity of the mutant JCV origins in COS-1 and cPOS-2 cells was strikingly different from that measured in POJ-19 cells. In these SV40 T antigen-containing cells, pM1(d98)o and pM1o replicated with similar efficiency. In COS-1 cells replication activity remained at wild-type levels until the deletion reached nucleotide 45, when activity was approximately half the pM1o level. Activity in these cells was not reduced further by the removal of the AGGGA tandem repeat; however, as in POJ-19 cells, replication became undetectable with the deletion of sequences between nucleotides 17 and 30. Replication patterns observed in cPOS-2 cells were similar to those seen in COS-1 cells, except that the initial reduction in activity occurred with the deletion of sequences between nucleotides 155 and 275 [between pM1(d98)o and d56]. Thus, the effect that different regions of the JCV origin had on replication activity appeared to depend primarily on which T antigen was used to initiate replication.

Replication of JCV variant and BKV origins. A large difference in replication activity occurred with the deletion of a small region of the JCV origin lying immediately proximal to the A+T-rich sequence. This region is disrupted in JCV type II variants (39); in the Mad8Br variant, a 23-np sequence, 5'-TAGGGAGGAGCTGGCTAAAACTG-3', is inserted between nucleotides 36 and 37 of the prototype Mad1 origin. A closely related 17-np sequence, 5'-CAGG GAGGAGCTGCTTA-3', is found in the BKV (wild type) replication origin from nucleotides 37 to 53. This latter

sequence, termed the rep element, is required for replication of BKV origin plasmids in COS-1 cells (16, 17). It was of interest to determine whether these related sequences influenced viral DNA replication in the present study. The replication activities of the BKV origin-containing plasmid pBKo and of two plasmids containing the replication origin of Mad8Br were tested in POJ-19, cPOS-2, and COS-1 cells. Representative experiments are shown in Fig. 6, and quantitative data are presented in Table 1. The Mad8Br origin plasmid replicated with an efficiency that was similar to that of pM1o in all three cell lines tested, and plasmid pM8(d83)o, which has a deletion of one of the Mad8Br enhancer elements, behaved in a manner similar to the analogous prototype deletion plasmid pM1(d98)o. The BKV origin plasmid pBKo replicated poorly when compared with pM1o in cPOS-2 and COS-1 cells, and replication was undetectable in POJ-19 cells. These experiments indicated that the intervening sequence found in the JCV type II variants did not significantly affect DNA replication with either SV40 or JCV T antigen in vivo. The failure of the BKV origin plasmid to replicate in POJ-19 cells demonstrated the inability of JCV T antigen to initiate DNA replication from a second heterologous origin.

Replication of origin mutants produced by cassette mutagenesis. The core origins of JCV and SV40 are very similar; however, there are five nucleotide differences within or near the A+T-rich region of the cores. SV40 sequences that differ from those of JCV include a deletion of the cytosine at nucleotide 12, a T-to-C transition at nucleotide 15, a T-to-A transversion at nucleotide 19, and two G-to-T transversions at nucleotides 30 and 31 (JCV numbering [22]). To test the possibility that these differences influence the ability of JCV T antigen to effectively interact with the origin, core origins containing combinations of the JCV and SV40 sequences were produced by using complementary synthetic oligonucleotides annealed and inserted into pM1(BglI)o (see Materials and Methods) and (Fig. 7). This approach was also used to further investigate the influence on DNA replication of sequences lying immediately to the late side of the A+T-rich region. The first set of mutants were composed of early sequences of the JCV origin spanning nucleotides 4980 to 5128 and late sequences from nucleotides 155 to 275, with various synthetic sequences inserted between these segments. The first letter of the name (B) signified the *BglI* end

TABLE 1. DNA replication activities of Mad8Br and BKV origin plasmids in POJ-19, COS-1, and cPOS-2 cells

DNA	Replication activity[a] ± SEM		
	POJ-19	COS-1	cPOS-2
pM1o	100	100	100
pM1(d98)o	129 ± 4	91 ± 5	86 ± 4
pM8o	105 ± 14	87 ± 9	90 ± 10
pM8(d83)o	149 ± 25	90 ± 8	73 ± 18
pBKo	0	19 ± 2	6 ± 2

[a] Replication activities were determined as described in the legend to Fig. 3. The value for pM1o was set arbitrarily as 100. Values are averages of three independent experiments in POJ-19 and COS-1 cells and two experiments in cPOS-2 cells. Descriptions of the plasmids are given in the text. There was no replication activity in mock-infected cells.
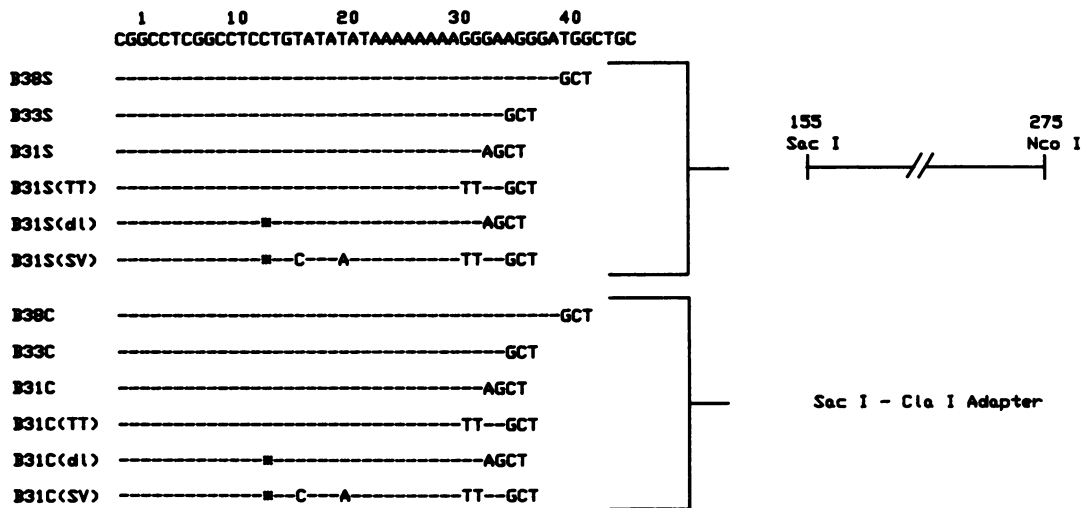
FIG. 7. Structure of replication origins created with synthetic oligonucleotides. The sequence at the top of the figure is that of Mad1 prototype JCV with the nucleotide numbers indicated (22). Nucleotides of the mutant sequence that are homologous to the prototype sequence are denoted with dashes, and deleted nucleotides are indicated by asterisks. The SacI-NcoI fragment joined to the S series of mutants (the upper set of mutants), represents prototype JCV DNA, nucleotides 155 to 275. The C-series mutants (the lower set of mutants) lack the JCV SacI-NcoI fragment; the JCV core origin sequences are directly linked to pKP55 via a synthetic SacI-ClaI linker.

of the synthetic DNA fragment; the number after the letter denoted the last nucleotide included in the fragment that was homologous to the prototype JCV sequence. The last letter in the name indicated the restriction enzyme site present at the other end of the synthetic fragment (either S [SacI] or C [ClaI]). Letters within parentheses after the name indicated the changes introduced into the prototype JCV sequence. The replication activity of these mutants was compared with that of the parent plasmid pM1(BglI)o in COS-1, POJ-19, and cPOS-2 cells [previous experiments indicated that the activity of pM1(Bgl)o was equivalent to that of the wild-type JCV origin plasmid (Fig. 3)]. To compare the activity of these mutants with that of the complete SV40 late origin DNA, pMSVo was included in the assays. The results of these experiments are presented in Fig. 8 and Table 2. Consistent with earlier experiments, pMSVo replicated in COS-1 and cPOS-2 cells but not in POJ-19 cells. B38S replicated as well as pM1(BglI)o in COS-1 cells, somewhat lower in cPOS-2 cells, but 2.5-fold better than the parental plasmid in POJ-19 cells. This was consistent with results seen with the deletion

mutants (Fig. 5). The activities of B33S and B31S were similar to those of B38S in all three cell types; a slight decrease in activity was observed consistently with B31S in POJ-19 cells. The activities of mutants B31S(dl), B31S(TT), and B31S(SV), which convert 1, 2, and 5 nucleotide positions, respectively, in the JCV core origin to an SV40-like core, were measured in these same cells. Within the plasmid B31S(TT), two guanines at positions 30 and 31 in JCV were changed to two thymines, which are present at the corresponding position in SV40. This mutation slightly increased replication activity when compared with that of B31S in COS-1 cells, doubled activity in cPOS-2 cells, and decreased activity in POJ-19 cells. B31S(dl) deleted a cytosine at np 12 of the JCV sequence, which increased the homology between JCV and the corresponding region of the SV40 origin. This mutation decreased replication activity to various degrees in all three lines when compared with that of the prototype sequence of B31S. The mutant B31S(SV) contains four substitutions in addition to the deletion at np 12, creating an A+T-rich region identical to that of SV40. This
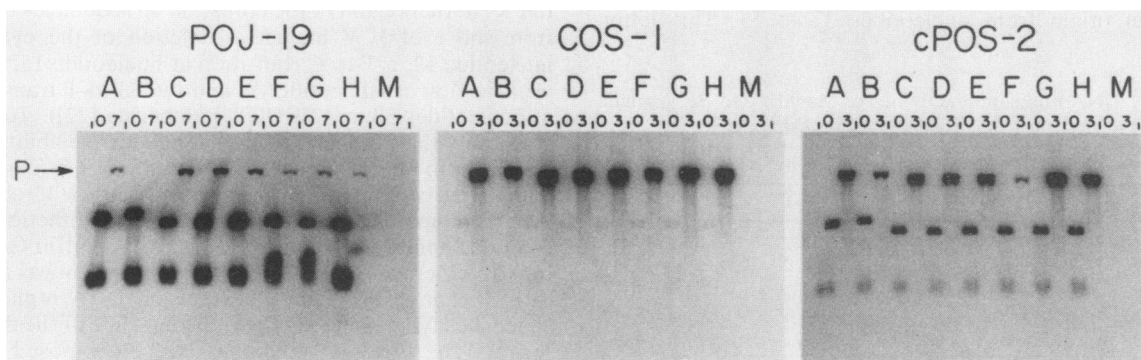

FIG. 8. DNA replication activity of the S series of mutants in POJ-19, COS-1, and cPOS-2 cells. The replication assay and the identification of markers are as described in the legend to Fig. 2 and in Materials and Methods. The letters denote plasmids pM1(BglI)o (A), pMSVo (B), B38S (C), B33S (D), B31S (E), B31S(dl) (F), B31S(TT) (G), and B31S(SV) (H) and mock-infected cells (M). Autoradiograms were exposed for 4 h (POJ-19 experiment), 1 h (COS-1 experiment), or 1.5 h (cPOS-2 experiment).

TABLE 2. DNA replication activities of the S series of cassette mutant origin plasmids in POJ-19, COS-1, and cPOS-2 cells

DNA	Replication activity[a] ± SEM		
	POJ-19	COS-1	cPOS-2
pM1(BglI)o	100	100	100
pMSVo	0	63 ± 6	23 ± 2
B38S	269 ± 25	128 ± 7	84 ± 7
B33S	300 ± 25	110 ± 8	70 ± 10
B31S	189 ± 11	112 ± 7	69 ± 15
B31S(TT)	104 ± 3	139 ± 4	154 ± 16
B31S(dl)	77 ± 2	67 ± 7	9 ± 3
B31S(SV)	47 ± 2	130 ± 10	97 ± 16

[a] Replication activities were determined as described in the legend to Fig. 3, except that pM1(BglI)o rather than pMlo served as the reference plasmid and its replication activity was arbitrarily given a value of 100. Values are averages of three independent experiments in POJ-19 and COS-1 cells and two experiments in cPOS-2 cells.

TABLE 3. DNA replication activities of the C series of cassette mutant origin plasmids in POJ-19 and COS-1 cells

DNA	Replication activity[a] ± SEM	
	POJ-19	COS-1
pM1(BglI)o	100	100
pMSVo	0	68 ± 6
B38C	204 ± 20	91 ± 12
B33C	182 ± 25	77 ± 16
B31C	110 ± 10	68 ± 12
B31C(TT)	127 ± 64	91 ± 11
B31C(dl)	26 ± 5	6 ± 1
B31C(SV)	12 ± 5	53 ± 16

[a] Replication activities were determined as described in the legend to Fig. 3, except that pM1(BglI)o rather than pMlo served as the reference plasmid and its replication activity was arbitrarily given a value of 100. Values are averages of three independent experiments.

mutant behaved similarly to B31S(TT) in COS-1 and cPOS-2 cells, with replication activity slightly higher than that of B31S. Although the replication level of B31S(SV) was significantly lower than that of B31S in POJ-19 cells, B31S(SV) was able to replicate to detectable levels.

The possibility that JCV sequences lying between nucleotides 155 and 275 had enhanced the replication activity of these constructs was apparent when the results with the cassette and deletion mutants were compared (e.g., d30 [Fig. 5] and B31S [Table 2] activities in POJ-19 cells). Such an effect would interfere with our investigation as to the possible effects exerted by the core sequences. To eliminate this complication, one additional set of origin plasmids was created from the cassette mutants by excising the SacI-ClaI fragment of the plasmids (nucleotides 155 to 275 of JCV were removed in addition to some vector sequences) and recircularizing the construct via a synthetic 15-np SacI-ClaI adapter sequence (Fig. 7). These mutants were tested for replication activity in POJ-19 and COS-1 cells (Fig. 9, Table 3). The truncated origins B38C, B33C, and B31C all exhibited a lower activity in these cell lines when compared with that of the S-series mutants [the activity of pM1(BglI)o was used as a reference]. This decrease ranged between 25 and 40%. The effects of the SV40-like mutations were more pronounced with these constructs. The activity of B31C(TT) was slightly higher in both cell lines when compared with that of B31C,
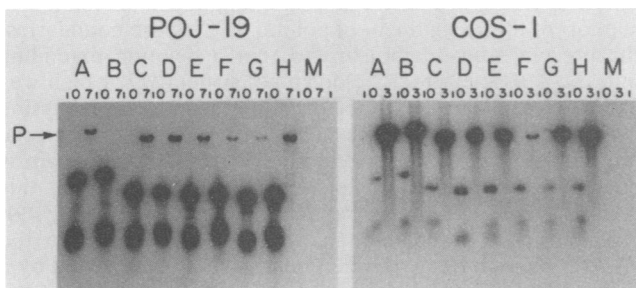

FIG. 9. DNA replication activity of the C series of mutants in POJ-19 and COS-1 cells. The replication assay and the identification of markers are as described in the legend to Fig. 2 and in Materials and Methods. The letters denote plasmids pM1(BglI)o (A), pMSVo (B), B38C (C), B33C (D), B31C (E), B31C(dl) (F), B31C(SV) (G), and B31C(TT) (H) and mock-infected cells (M). The autoradiograms were exposed for 16 h (POJ-19 experiment) or 6 h (COS-1 experiment).

and the deletion of nucleotide 12 in the JCV origin dramatically decreased replication in both cell lines. In COS-1 cells the replicating behavior of B31C(SV), which contained an SV40-like A+T-rich region, approached that of the hybrid construct pMSVo, which contained the intact late side of the SV40 origin; in POJ-19 cells replication of B31C(SV) was low but still detectable, unlike that of pMSVo. These results indicated that sequences primarily within the A+T-rich region of SV40 contributed to the inability of JCV T antigen to efficiently initiate DNA replication from the SV40 origin of replication; auxiliary sequences of the SV40 origin may have further inhibited this interaction.

DISCUSSION

JCV exhibits an unusually restricted lytic behavior in vitro; the virus can be propagated in only a limited number of human cells, and the lytic cycle is prolonged. The inefficient DNA replicating activity of the virus is thought to contribute, in part, to this limited lytic activity (9, 21). In a recent study we have shown that the JCV T antigen fails to drive replication from an SV40 origin (Lynch and Frisque, in press). The goal of the present study was to identify the sequence differences that allow the JCV T antigen to discriminate between the JCV and SV40 origins. Initially, hybrid JCV-SV40 replication origins were constructed to determine whether sequence differences to the early or late side of the center of the core origins were responsible for the different T antigen interactions. Subsequent experiments relied upon a site-specific mutagenesis approach to more precisely localize those sequences.

Hybrid replication origins were constructed by utilizing a unique BglI site within the core origins of SV40 and M1(BglI), a viable mutant of JCV. Sequences to the early side of the central dyad symmetry, which contain T antigen-binding site I and the inverted repeat of the core origin, interacted with similar efficiency with either T antigen; the presence of heterologous early origin sequences reduced replication activity only about twofold when either viral T antigen was used (Fig. 3; compare activities of pSVo or pSMo with those of either pMlo, pMSVo, or pMSo in COS-1 cells and compare pMlo and pSMo activities in POJ-19 cells). It was somewhat surprising to find that the activities of the early regions of the two viral origins were similar, since sequence differences affecting important replication signals within this region are apparent (Fig. 1). One such signal, T antigen-binding site I, stimulates replication activity of the SV40 core origin five- to sevenfold (14, 27, 30,

34, 40, 53). Within JCV binding site I, only two of the three pentanucleotide recognition sequences found in the SV40 site are present. A second replication signal, the inverted repeat to the early side of T antigen-binding site II, represents an important component of the core origin (5, 10, 11, 13, 42); it is here that melting of the DNA duplex initially occurs after T-antigen binding. This repeat also includes the binding site for a monkey cell factor suspected of being involved in SV40 DNA replication (55). Although the JCV and SV40 binding sites differ at only a single nucleotide position (position 5098 in JCV), a mutation at this position in the SV40 site reduces DNA replication fivefold (11).

Replication experiments with the hybrid JCV-SV40 origins did indicate that sequences to the late side of T antigen-binding site II had a strong influence on the ability of the JCV T antigen to effect replication. The only origin plasmid that contained SV40 late sequences and exhibited detectable replication activity in POJ-19 cells was pJLo, a construct in which all but 22 nucleotides of the SV40 enhancer have been deleted (Fig. 3) (33). These results suggested that SV40 enhancer sequences may be partly responsible for the inability of the JCV T antigen to interact productively with SV40 origin plasmids. The finding that the pJLo plasmid also replicated better than the full-length origin plasmid pSVo in COS-1 cells supported previous studies indicating that SV40 enhancer sequences inhibit SV40 DNA replication in the presence of the G+C-rich promoter elements (7, 31, 32, 34). The SV40 G+C-rich repeats behave as auxiliary replication elements; their presence stimulates replication of an SV40 origin plasmid severalfold (4, 23, 32, 34). In agreement with this finding, the replication efficiency of pJLo (SV40 nucleotides 1 through 128; includes G+C-rich repeats) appeared to be higher than that of B31C(SV) (SV40 nucleotides 1 through 31; no G+C-rich repeats) in COS-1 cells (Table 3, Fig. 9). In the presence of JCV T antigen, however, the G+C-rich elements failed to stimulate DNA replication; B31C(SV) and pJLo appeared to replicate with similar efficiencies in POJ-19 cells.

Once it was established that the JCV T antigen could discriminate between JCV and SV40 sequences to the late side of the two replication origins, a mutational approach was adopted to identify more precisely the critical nucleotide differences. The initial experiments involved a deletion analysis of the JCV promoter-enhancer region. It has been reported that deleting the SV40 enhancer sequences reduces DNA replication in the absence of the G+C-rich repeats (14, 32, 34). When the corresponding JCV sequences were deleted, however, this inhibitory effect was not observed; the JCV origin plasmids pM1(d98)o and pM8(d83)o replicated more efficiently than did the undeleted parental constructs pM1o and pM8o (Table 1, Fig. 6). This positive effect was seen in POJ-19 cells but not COS-1 or cPOS-2 cells. Furthermore, this effect was observed either in the absence (prototype Mad1 origin) or the presence (variant Mad8Br origin) of a G+C-rich element.

As larger deletions were introduced into the promoter-enhancer region of the JCV constructs, their replication activity relative to the intact origin remained elevated in POJ-19 cells but decreased in COS-1 and cPOS-2 cells. The region of the JCV enhancer between nucleotides 33 and 58 has been found to interact with an NF-1-like factor (1). The transcriptional activator NF-1 has been shown to stimulate adenovirus (41, 43) and SV40 (8) DNA replication. It is interesting to note that when sequences overlapping the consensus NF-1 recognition site were deleted from the JCV origin plasmid (nucleotides 45 to 56), replication activity

dropped twofold in both COS-1 and cPOS-2 cells but increased slightly in POJ-19 cells.

DNA replication driven by the JCV T antigen decreased sixfold when sequences between nucleotides 38 and 30 (constructs d38 and d30, Fig. 5) were deleted from the JCV origin region. In contrast, replications of these two plasmids were indistinguishable when driven by the SV40 T antigen expressed in two different cell types. This deletion removed the short tandem repeat 5'-AGGGAAGGGA-3', a sequence identified by Kahlili and co-workers (personal communication) to be a binding site for a brain-specific transcriptional factor. This same region of the JCV genome has been shown to adopt a unique non-B DNA structure (2). In type II variants of JCV (e.g., Mad8Br) the tandem repeat is disrupted by the addition of a 23-np sequence. This insertion does recreate two copies of the AGGGA pentanucleotide (39), though, which may account for the similar replicating activities of the prototype and Mad8Br origin plasmids (Table 1). A sequence closely related to the JCV AGGGA repeat, called the rep element, is also found within the BKV origin region; however its presence is not sufficient to promote JCV T antigen-mediated replication of the BKV origin. This finding suggests that four nucleotide differences identified between the late halves of the JCV and BKV core origins (BKV differences from JCV are a C-to-T transition at nucleotide 12, G-to-A transitions at nucleotides 14 and 30, and an insertion of a T between nucleotides 19 and 20 [47]; JCV numbering [22]) are primarily responsible for this nonproductive interaction.

To investigate further the influence of the AGGGA sequence on JCV DNA replication, mutants were created that contained 0, 1, or 2 copies of this pentanucleotide. The construct containing one copy of this sequence replicated approximately as well as that containing two copies. When the pentanucleotide sequence was omitted from the origin plasmids, replication decreased to the basal levels measured for the intact JCV origin construct, pM1(BglI)o. This effect was more pronounced with the C series of mutants (Table 3) than with the S series of mutants (Table 2). Significantly, the S mutants include nucleotides 155 to 275 of JCV, which contain an AGGGA sequence and a potential NF-1-binding site. It is possible that these sequences may compensate for the loss of the short tandem repeat.

Recent work indicates that the JCV core is included within a 68-np fragment that corresponds to the 64-np SV40 core. A plasmid containing this fragment (JCV nucleotides 5094 to 31) replicates in POJ-19 cells at 17% of the efficiency of pM1o (J. E. Tavis and R. J. Frisque, unpublished results). In support of this assignment of potential JCV core boundaries, the present study demonstrated that a deletion extending into the A+T-rich tract yielded a construct (d17) which was unable to replicate in any of the three T antigen-expressing cell lines (Fig. 5). In the presence of the SV40 T protein, the integrity of the A+T-rich region of the SV40 core origin is essential for replication; deletions or substitutions in this region reduce or abolish replication activity (12, 40, 49). Recent reports indicate that the DNA is bent within the SV40 A+T-rich tract, and that bending may be induced by a cellular DNA-binding protein (3, 12, 36). This region of the core origin has also been implicated in the activation of the helicase activity of the T antigen, after the protein has bound to the core and caused localized melting of the early inverted repeat (42). The spacer and A+T-rich sequences of SV40 differ with those of JCV at five nucleotide positions (12). Conversion of these sequences to an SV40-like A+T-rich tract had a negative effect on JCV T antigen-mediated

replication (Tables 2 and 3). Converting nucleotides 30 and 31 from GG (JCV) to TT (SV40) to create B31C(TT) had little effect on replication activity in POJ-19 or COS-1 cells. In contrast, replication activity of B31C(dl), in which the cytosine at position 12 of the JCV origin was deleted to increase homology with the SV40 A+T-rich tract, was reduced significantly in both POJ-19 and COS-1 cells relative to that of B31C. By introducing all five mutations into the JCV A+T-rich region to create the SV40 construct B31C(SV), replication activity relative to that of B31C(dl) was reduced further in POJ-19 cells but increased in COS-1 cells. Deletion of the cytosine alone may have adversely affected replication by altering the spacing of the origin elements, but the addition of the four substitutions may have compensated for this spacing difference to allow a more favorable interaction with the SV40 T antigen but not the JCV T antigen.

The JCV T antigen is less tolerant of differences in origin structure than is the SV40 protein. Previous observations (Lynch and Frisque, in press) indicate that the JCV T antigen does bind to the JCV and SV40 origin sequences with similar efficiencies; therefore a step in replication after this initial binding must determine whether such an interaction will be productive. Several differences between the JCV and SV40 core and auxiliary origin sequences found to the late side of the central dyad symmetry contribute to the inability of JCV T antigen to replicate the SV40 origin; differences within and immediately adjacent to the A+T-rich region appear to be the major determinant.

It is apparent that the A+T-rich tract of JCV had to be present on a JCV origin plasmid to detect a basal level of replication in human and monkey cells expressing the JCV or SV40 T antigen. The presence of the AGGGA pentanucleotide adjacent to the A+T-rich sequence promoted JCV T antigen-mediated replication of the JCV origin severalfold. In the presence of additional enhancer sequences, the replication of JCV origin constructs declined until the basal level of replication was again observed with a construct containing the entire JCV promoter-enhancer region. JCV sequences encompassing the A+T-rich and AGGGA elements may effect the unusual structure reported for the JCV promoter-enhancer region (2). Such a structure may represent a more favorable substrate for the helicase activity of the JCV T antigen. Perhaps the conformation of the corresponding region in SV40 does not allow a favorable interaction with the JCV T antigen to stimulate this activity. Although this property of the JCV T protein may explain its inability to interact productively with the SV40 origin, it may also suggest that the relatively inefficient DNA replicating activity of JCV is a consequence of constraints imposed by the JCV T protein-DNA interaction.

ACKNOWLEDGMENTS

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