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Regulation of the host range of human papovavirus JCV

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ABSTRACT **Human papovavirus JCV is associated with the human demyelinating disorder progressive multifocal leukoencephalopathy. In tissue culture, the virus is largely restricted to growth in primary human fetal glial cell. In this study, we demonstrate two levels of regulation of the viral host range. Expression of the early JCV mRNA, which encodes the essential viral protein, large tumor antigen (T antigen), depends on recognition of the early enhancer/promoter elements by tissue-specific factors found in both human and rodent glial cells. In the presence of JCV T antigen, viral DNA replication requires a species-specific factor, presumably a component of DNA polymerase, which is found in a wide range of primate cells. We further demonstrate that simian virus 40 T antigen has sufficient homology to efficiently substitute for the analogous JCV protein in initiating viral DNA replication.**

The human papovavirus JCV was first isolated from the brain of a patient with progressive multifocal leukoencephalopathy (PML) by Padgett *et al.* (1), who inoculated cultures of human fetal brain cells with extract of the diseased tissue. Subsequent isolations of JCV from the diseased brain tissue of other PML patients has led to the assumption that this papovavirus is the etiologic agent of PML (2, 3). In these patients, JC viral particles are detected in brain cells of glial origin—namely, oligodendrocytes, which represent cells that maintain the myelin sheath (4), and at a much lower level in astrocytes, which are sometimes transformed as a result of abortive viral infection (5). Intracranial inoculation of JCV induces medulloblastomas, glioblastomas, and unclassified tumors in hamsters (6, 7). JCV was also shown to cause brain tumors in owl monkeys (8, 9).

JCV also exhibits a highly restricted host range and tissue specificity in tissue culture. Studies on the propagation of JCV established that the virus is not expressed in primary human embryonic lung, kidney, intestine or liver, or testes, or in two diploid human fibroblast tissue culture cell lines (L-809, WI-38). Furthermore, JCV could not grow in established cell lines of simian, hamster, mouse, and mink origin (10). Primary human fetal glial (PHFG) cells represent the only cell line in which significant JCV expression is found (10, 11). In this study, we have further characterized the host range and tissue specificity of JCV. We have found that JCV will replicate in a number of primate cells, provided that either JCV large tumor antigen (T antigen) or simian virus 40 (SV40) T antigen is produced in that cell. In addition, we show that the restricted host range of JCV to brain tissue in cell culture is transcriptionally regulated, since significant activity of the JCV enhancer/promoter, as determined by chloramphenicol acetyltransferase (CAT) assays and RNA analyses, was found only in PHFG cells and hamster glial cells.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. PHFG cells were prepared from 15- to 20-week-old abortuses by procedures described (10). SVG and SV1 cells are established cell lines of human fetal brain and human embryonic kidney origin that have been transformed with an origin-defective SV40 mutant (12–14). SV40 T antigen is expressed constitutively in these cells. CV-1 is a continuous line of monkey kidney cells. COS cells were derived from CV-1 cells transformed with an origin defective SV40 mutant (15). The HeLa cell line was isolated from a carcinoma of the cervix (16). HJC cells are JCV-transformed hamster brain cells (6, 17). All cell types except PHFG cells were plated in 10-cm dishes for 24 hr prior to transfection. Cells were transfected by the calcium phosphate/DNA coprecipitate method (18). Test DNA plus carrier calf thymus DNA was inoculated at 30 μg in a final vol of 1 ml. The cells were incubated with the calcium phosphate/DNA coprecipitate for 4 hr and then washed thoroughly. PHFG cells were plated 2 weeks prior to transfection in Hepes-buffered minimal essential medium containing 10% fetal calf serum. When the cells reached 60% confluency, ≈4 days prior to transfection, the concentration of fetal calf serum was reduced to 3% to decrease fibroblast proliferation.

Plasmids. pBJC is a plasmid containing the JCV genome *Mad-1* cloned at the *Eco*RI site in pBR322. pBJCT$^-$ is a T-antigen-negative plasmid obtained by cloning JCV at the *Bam*HI site in pBR322 in the early region and restricting the circular plasmid DNA with *Fnu*DII. This restriction enzyme only cleaves pBR322. As a result, 356 base pairs (bp) of pBR322 remain on the *Bam*HI ends of JCV. pBJCT$^-$ is transfected in a linear form and circularizes inside the cell to create a 356-bp pBR322 insertion in the JCV T-antigen amino terminus. pJC5'CAT (11) was obtained by cloning a JCV *Pvu* II/*Hin*dIII fragment containing the JCV enhancer/promoter elements at the *Bgl* II site of pCAT3M, which contains the CAT gene. pSV2CAT (19) contains the SV40 enhancer and promoter cloned upstream of the CAT gene. Plasmid pRSV-T, in which SV40 T antigen is expressed under the control of the Rous sarcoma virus long-terminal repeat was provided by Bruce Howard (National Institutes of Health).

Replication Assay. Linear pBJC or pBJCT$^-$ (2 μg) plus carrier calf thymus DNA (28 μg) were transfected into various cell lines. pBJC is linearized by cleavage with *Eco*RI and *Hha* I. *Hha* I exclusively cuts pBR322 at 31 sites, effectively eliminating plasmid DNA. *Eco*RI releases intact JCV. All cell lines except PHFG cells were harvested 24, 48, 72, and 96 hr posttransfection and the low molecular weight DNA was isolated (20). PHFG-transfected cells were harvested 7, 14, and 21 days posttransfection. This DNA was cleaved with one enzyme that linearizes JCV DNA as well as *Dpn* I. *Dpn* I is a multicutting enzyme that requires methylated DNA for its substrate and thus cleaves away the input plasmid DNA, but not DNA newly replicated in a eukaryotic

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Abbreviations: PHFG cells, primary human fetal glial cells; SV40, simian virus 40; CAT, chloramphenicol acetyltransferase; T antigen, large tumor antigen.

cell (21). The DNA was then electrophoresed through 1.5% agarose and transferred onto a nitrocellulose membrane (22). After hybridization with a full-length JCV nick-translated probe in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% $NaDodSO_4$/5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/denatured salmon sperm DNA (100 μg/ml), at 68°C overnight, the blot was washed in 3× SSC/0.1% $NaDodSO_4$ at 68°C and in 3× SSC at 68°C, and exposed to Kodak XAR5 film at −70°C.

CAT Assay. CAT assays were performed as described (19) except that all extracts were obtained 72 hr posttransfection.

S1 Nuclease Analysis. Whole cell RNA was prepared by the hot acid phenol procedure 72 hr posttransfection (23). DNA was removed by treatment with DNAse I (20 μg/ml) (Worthington Diagnostics) in the presence of RNasin (Promega Biotec, Madison, WI). RNA from PHFG cells (100 μg) and the other cell lines (50 μg) was probed for CAT mRNA with a single-stranded DNA probe uniformly labeled [^{32}P]dCTP (400 Ci/mmol; 1 Ci = 37 GBq) during strand synthesis from a single-standed M13 phage. RNA was analyzed by the S1 nuclease procedure as described (24).

RESULTS

Host Range of JCV Replication. The host range for JCV DNA replication was investigated by transfecting linear JCV DNA into the primate cell lines SVG, SV1, COS, HeLa, and CV-1 and the hamster line HJC. The level of JCV replication was assayed 72 hr posttransfection using the *Dpn* I assay as described. Substantial JCV DNA replication was detected in SVG, SV1, and COS cells (Fig. 1). All of these cells are of primate origin and express SV40 T antigen constitutively. JCV DNA did not replicate in HJC, CV-1, and HeLa cells. Although the HJC cell line expresses JCV T antigen, it is of rodent origin. CV-1 and HeLa are primate cell lines but do not express T antigen constitutively. When JCV DNA was cotransfected into CV-1 or HeLa cells with a plasmid producing SV40 T antigen (pRSV-T), replication did occur (Fig. 1). This result suggests, as might be expected, that T-antigen expression is a requirement for JCV replication in any cell, and its absence prevents viral DNA synthesis in HeLa and CV-1 cells. It is also clear that the highly related papovavirus T antigen from SV40 can support JCV replication in primate cells. In contrast, even JCV T antigen does not allow the homologous viral DNA to replicate in a rodent cell background (HJC).

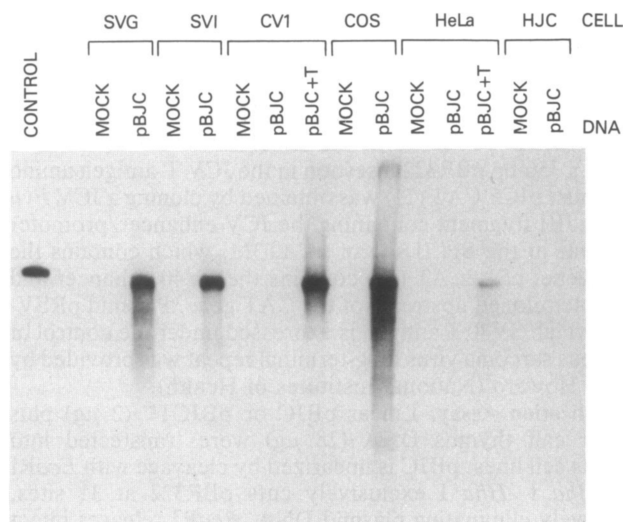

FIG. 1. Examination of JCV replication in human (SVG, SV1, HeLa), monkey (CV-1, COS), and hamster (HJC) cells. Two micrograms of linear JC DNA supplemented with 28 μg of carrier (calf thymus) DNA were introduced into cells as described (20). In some cases, JCV DNA was supplemented by cotransfection with 5 μg of a plasmid (pRSV-T) in HeLa and CV-1 cells (pBJC+T) that expresses SV40 T antigen. Low molecular weight DNA was isolated 72 hr posttransfection (22), and 20 μg were digested with *Dpn* I and *Bam*HI and electrophoresed in a 1.5% agarose gel. The DNA was blotted onto nitrocellulose membranes and hybridized with a full-length JCV nick-translated probe (24) in 6× SSC/0.1% $NaDodSO_4$/5× Denhardt's solution/denatured salmon sperm DNA (100 μg/ml) at 68°C overnight. The control lane contains unlabeled linear JCV DNA.

SV40 T Antigen Supports JCV Replication. To confirm that JCV DNA replication can be supported solely by SV40 T antigen, we constructed a mutant defective for the production of JCV T antigen. This was achieved by insertion of pBR322 at the *Bam*HI site in JCV, which interrupts the JCV T-antigen coding sequence 360 bp from its translation initiation codon. The resulting plasmid (pBJCT$^-$) was then restricted with *Fnu*DII, an enzyme that cleaves only in pBR322 DNA and leaves 356 bp of pBR322 DNA at the *Bam*HI site of JCV. This insertion causes a reading frameshift that does not permit JCV T antigen to be translated. To test that pBJCT$^-$ does not produce a functional T antigen, linear DNA cut with *Fnu*DII was transfected in PHFG cells. In parallel, wild-type JCV was transfected as a control. One, 2, and 3 weeks after transfection, low molecular weight DNA was isolated, and the level of JCV replication was determined by Southern blot analysis. DNA synthesis could not be detected with pBJCT$^-$ (Fig. 2), whereas wild-type JCV replicated efficiently in PHFG cells. This result establishes that no functional T antigen is encoded by pBJCT$^-$ as a result of the 356-bp pBR322 insertion in the amino terminus of JCV T antigen. We then examined the ability of pBJCT$^-$ to replicate in SVG cells that constitutively express SV40 T antigen, hence testing the ability of SV40 T antigen to support JCV replication. Linear pBJCT$^-$ and pBJC were transfected separately into SVG cells, and at several time points low molecular weight DNA was isolated and replication levels were determined. To differentiate pBJCT$^-$ and pBJC the low molecular weight DNA was cut with *Bgl* II. Since the former plasmid contains a 356-bp insertion, this cleavage generates 2.3-kilobase (kb) and 2.8-kb fragments with pBJC but 2.65-kb and 2.8-kb fragments with pBJCT$^-$.

Both pBJC and pBJCT$^-$ were shown to replicate with roughly equal efficiencies by 72 hr posttransfection in SVG cells (Fig. 3). The ability of a T-antigen-defective JCV DNA molecule to replicate in SVG cells demonstrates conclusively that SV40 T antigen can substitute for JCV T antigen in supporting JCV DNA replication. This finding is contradic-

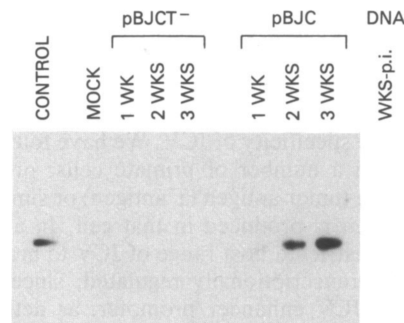

FIG. 2. Requirement of JCV T antigen for JCV DNA replication in PHFG cells. pBJCT$^-$, a plasmid with 356 bp of pBR322 inserted at the *Bam*HI site in the coding region for the amino terminus of JC T antigen and wild-type JCV DNA linearized at the *Bam*HI site were introduced separately in PHFG cells. One, 2, and 3 weeks posttransfection, low molecular weight DNA was extracted and digested with *Pst* I, which renders both JCV DNAs linear.

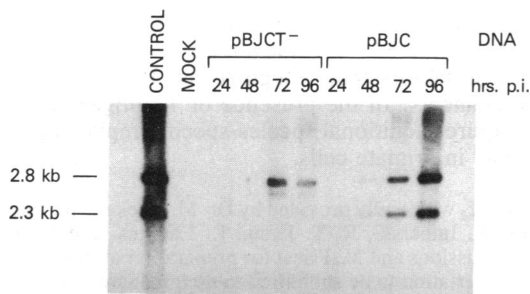

FIG. 3. SV40 T antigen supports JCV DNA replication. The JCV T-antigen-defective plasmid pBJCT$^-$ and wild-type JC linearized at its *Bam*HI site were introduced into SVG cells that constitutively express SV40 T antigen. Southern transfer of the low molecular weight DNA harvested 24, 48, 72, and 96 hr posttransfection and digested with *Bgl* II was hybridized with a nick-translated JCV DNA probe. Wild-type JCV generates two fragments of 2.8 kb and 2.3 kb when cut with *Bgl* II, while pBJCT$^-$ produces a 2.8- and a 2.65-kb fragment as a result of pBR322 insertion.

tory to a recent observation, which suggested that JCV T antigen is specifically required for viral DNA synthesis (12).

Transcriptional Activity of the JCV Enhancer/Promoter in Primate and Rodent Cells. We next attempted to assay the transcriptional activity of the JCV enhancer/promoter in various cell lines and determine its relationship to JCV DNA replication. The pJC5′CAT plasmid, in which the JCV enhancer/promoter was cloned upstream of the CAT gene, provided a sensitive initial evaluation. Production of CAT enzyme is dependent on transcription governed by the JCV regulatory sequences. Plasmid pJC5′CAT was transfected into PHFG, SVG, SV1, COS, HeLa, and HJC cells. A negative control plasmid, pCAT3M, which lacks the enhancer/promoter but is otherwise identical to pJC5′CAT, and a positive control, pSV2CAT, which contains the SV40 enhancer and promoter cloned upstream of the CAT gene, were transfected separately into the same cell lines as pJC5′CAT. Cell extracts obtained 72 hr after transfection were subjected to a 30-min reaction in the presence of acetyl CoA and [^{14}C]chloramphenicol to determine CAT enzyme levels. As expected, the SV40 enhancer was transcriptionally active in all the primate and rodent cells tested (Table 1). CAT activity ranged from 16.8% in HJC cells to 47.1% in SV1 cells.

Table 1. CAT activity with JCV enhancer/promoter in primate and rodent cell lines

Plasmid	Transfected cell line					
	PHFG	SVG	SV1	HeLa	COS	HJC
pJC5′CAT	20.5	4.3	1.8	2.6	6.0	71.9
pCAT3M	1.7	1.2	1.2	1.6	1.4	1.9
pSV2CAT	15.0	20.9	47.1	24.9	35.8	16.8

Transcriptional activity of the JCV enhancer in human (PHFG, SVG, SV1, HeLa), monkey (COS), and hamster (HJC) cells. Twenty micrograms of pJC5′CAT DNA was introduced into cells by the calcium phosphate technique (20). Cell extracts were prepared 72 hr posttransfection and CAT activity was determined. pSV2CAT, a plasmid that contains the SV40 enhancer and promoter cloned upstream of the CAT gene, was used as a positive control. pCAT3M, an enhancer/promoter-negative CAT construct, was used as a negative control.

Plasmid pCAT3M, without an enhancer/promoter, did not express significant CAT activity in any cell line. pJC5′CAT expressed very little CAT activity in SVG and COS cells (3.1% and 4.6% above background level of pCAT3M, respectively) and virtually no activity in SV1 cells and HeLa cells (0.6% and 1.0% above pCAT3M, respectively). However, substantial activity was observed in HJC and PHFG cells (71.9% for HJC cells and 20.5% for PHFG cells). To confirm that the higher levels of CAT activity in HJC and PHFG cells corresponded to higher levels of transcriptional activity induced by the JCV enhancer/promoter, steady-state CAT mRNA levels were determined by quantitative S1 nuclease analysis. A 458-nucleotide probe used for the S1 nuclease analysis was synthesized from a single-stranded M13 recombinant plasmid; the fragment protected by CAT RNA is 256 nucleotides (Fig. 4*B*). Consistent with the CAT assay results presented above, CAT mRNA could be detected after transfection with pJC5′CAT only in HJC and PHFG cells (Fig. 4*A*). Together, these results demonstrate that the JCV enhancer/promoter is transcriptionally active only in cells of glial origin, but in glial cells of both primate and rodent species.

DISCUSSION

The highly restricted host range and tissue specificity of JCV have been investigated to determine the mechanisms that

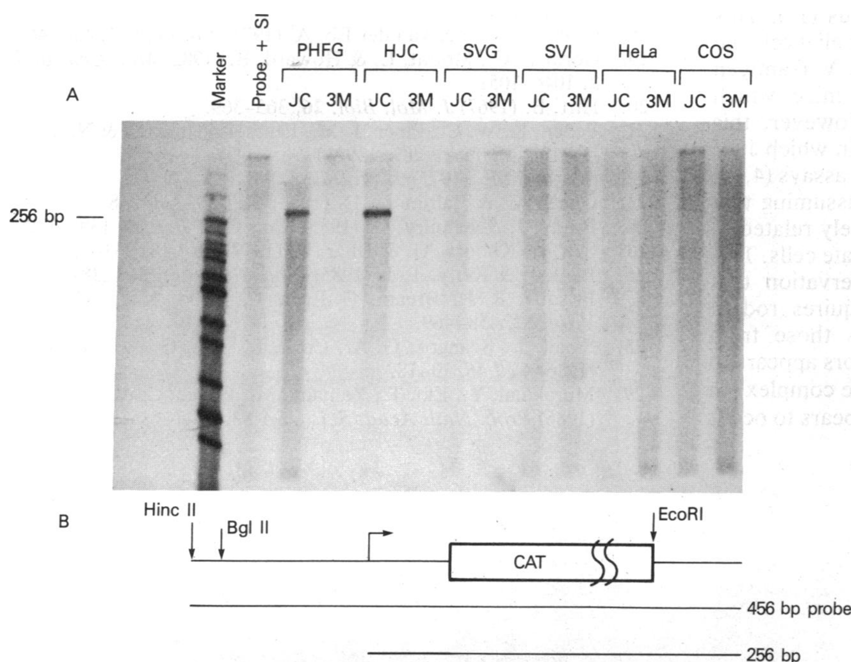

FIG. 4. Tissue-specific expression of early RNA in JCV-transfected cells. (*A*) S1 nuclease analysis of CAT mRNA produced when pJC5′CAT is introduced in human (PHFG, SVG, SV1, HeLa), monkey (COS), and hamster (HJC) cells. Twenty micrograms of pJC5′CAT (JC) and 20 μg of pCAT3M (3M) were transfected in the various lines and 72 hr posttransfection total mRNA was isolated by the hot acid phenol procedure (25). One hundred micrograms of mRNA from PHFG cells and 50 μg of mRNA from all the other cell lines were hybridized to a uniformly ^{32}P-labeled CAT mRNA probe obtained with pM13CAT2 (see *Materials and Methods*). RNA·DNA hybrids were treated with S1 nuclease and electrophoresed through a 6 M urea denaturing polyacrylamide gel. CAT mRNA could be detected only in PHFG and HJC cells as shown by protection of a 256-base CAT coding fragment. (*B*) Diagram of a portion of pM13CAT2 plasmid used to generate a 458-base single-stranded probe for S1 nuclease analysis of CAT mRNA. The 256-bp fragment homologous to CAT mRNA is shown.

govern various levels of expression of the viral genome and their potential application to tissue-specific gene expression. We have established that JCV can replicate in all of the primate cells we have tested in cell culture, provided that JCV T antigen or SV40 T antigen is endogenously produced in that cell or is coexpressed from JCV DNA or a separate transfected plasmid.

Replication of a plasmid containing an intact JCV replication origin, but a defective T-antigen coding region, confirms that the endogenous SV40 T antigen is sufficient to support JCV DNA synthesis in COS, SV1, and SVG cells. As expected, this defective plasmid could not replicate in PHFG cells in the absence of an endogenous T antigen. Together, these results indicate that not only JCV T antigen, but also SV40 T antigen, is capable of supporting JCV DNA replication in primate cells.

Earlier studies in this laboratory and others had demonstrated a functional complementation between the T antigens of SV40 and human papovavirus BKV (25). More recently, *in vitro* replication assays performed with a HeLa cell extract supplemented with SV40 T antigen showed that a plasmid containing the JCV origin of replication could replicate with 20% (26) the efficiency of SV40 DNA in this system. These findings most likely derive from the fact that JCV T antigen and SV40 T antigen are 72% homologous; the greatest homology exists at the amino terminus of the molecule, which is critical for the specificity of the replication function (27).

The ability of JCV DNA to replicate in a naive cell (i.e., one that does not express T antigen from an integrated or a cotransfected gene) requires prior expression of JCV T antigen from the introduced JCV DNA template. This, in turn, requires cellular recognition of the early JCV regulatory signals. Using CAT assays, we showed that JCV was transcriptionally active only in PHFG and HJC cells both of glial derivation. S1 nuclease analysis of CAT mRNA from the various cell lines tested produced similar results. Based on this set of experiments, we conclude that all of the primate cells used in this study have the necessary factors to support JCV DNA replication and that the restriction of JCV propagation to glial cell is due to a transcriptional control for the expression of JCV T-antigen RNA. It seems clear that this transcriptional control element is enhancer and/or promoter sequences and that regulation is mediated through tissue-specific factor(s) that bind to critical JCV sequences and allow for expression of early genes of the virus (11). This factor(s) also appears to be present in the rodent glial cell line HJC, which produces substantial amounts of JCV T-antigen level in the oligodendroglial cells of transgenic mice, which efficiently express JCV early mRNA (28). However, this factor(s) appears to be absent from SVG cells in which JCV was not transcribed, at levels detectable by our assays (4, 5).

The lack of JCV replication in HJC cells, assuming that they contain a functional JCV T antigen, is likely related to the absence of replication factors from nonprimate cells. This concept is in agreement with a recent observation that polyoma virus DNA replication *in vitro* requires rodent cell-specific factors, whereas SV40 requires those from primate cells (29). At least one set of these factors appears to be a species-specific DNA polymerase–primase complex. In summary, the host range restriction of JCV appears to occur on at least two levels in the lytic cycle. Transcription of early JCV RNA, essential for T-antigen production, requires glial cell-specific factors that recognize the JCV transcriptional control elements. In the presence of T antigen, DNA synthesis requires additional species-specific replication factors found only in primate cells.

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