U87MG GLIOBLASTOMA CELL LINE: AN IN VITRO CELL CULTURE SYSTEM TO PROPAGATE POLYOMAVIRUS JC

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By

PRAVEEN ANANTHULA

Thesis Committee:

Vivek R. Nerurkar, Chairperson
Allison Imrie
Andre Theriault
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Polyomavirus JC (JCV) is the causative agent of the demyelinating disease of the central nervous system, progressive multifocal leukoencephalopathy. The primary target of virus infection is oligodendroglial cells. JCV shows highly restricted host range, there are no established susceptible cell lines for JCV except primary human fetal glial (PHFG) cells that are difficult to procure and maintain. The restricted in vitro growth of JCV has hindered JCV pathogenesis studies to very few laboratories worldwide. Most published studies on JCV have employed SV40 derived cell lines or JCV/SV40 chimeras, which complicates biological and molecular characterization of JCV. We employed the U87MG glioblastoma cells for propagation of JCV. By using DNA replication, RT-PCR, real time PCR, in situ hybridization assays we were successfully demonstrated JCV propagation and progeny virus production in U87MG glioblastoma cells. These data are consistent with our studies of JCV replication, gene transcription and virion production in PHFG cells. Our data suggests that U87MG glioblastoma cell line will expedite the study on polyomavirus JC.
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LIST OF ABBREVIATIONS AND SYMBOLS

ATCC  American Type Culture Collection
ATP  Adinosine tri phosphate
BKV  BK virus
BSA  Bovine serum albumin
CNS  Central nervous system
CTL  Cytotoxic T-lymphocyte
DMSO  Dimethyl Sulfoxide
EMEM  Eagle's minimum essential medium
FBS  Fetal bovine serum
GFAP  Glial fibrillary acidic protein
GAPDH  Glyseraldehyde-3-phosphate dehydrogenase
GFP  Green fluorescent Protein
HAART  Highly active antiretroviral therapy
HBSS  Hank's balanced salt solution
HCMV  Human cytomegalo virus
IgG  Immunoglobulin G
JCV  JC virus
PCR  Polymerase chain reaction
PHFG  Primary human fetal glial
PML  Progressive multifocal leukoencephalopathy
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<tr>
<td>RE</td>
<td>Restriction endonucleases</td>
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<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<tr>
<td>RR</td>
<td>Regulatory region</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
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<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TAg</td>
<td>T antigen</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>VP1</td>
<td>Viral capsid protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>Mgcl$_2$</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>nM</td>
<td>Nanometer</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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CHAPTER 1. INTRODUCTION

JCV

The JC Virus (JCV) belongs to the family polyomaviridae. Its capsid is a 40 nm icosahedron containing a double-stranded, supercoiled DNA of about 5.13 kb [1]. JCV was first isolated in 1971 from a Hodgkin's lymphoma patient with the disease progressive multifocal leukoencephalopathy (PML) and the virus was named after the patient's initials 'JC' [2].

![Figure 1: JC virus. A computer graphic representation of the structure of JC virion. The shell consists of 72 pentamers of VP1, 60 hexagonally coordinated + 12 pentamerically co-ordinated (at the vertices)](image)

JCV viruria is found among 40% of the population in The United States of America [3]. Approximately 80% of the human population worldwide is JCV seropositive [4]. The primary infection occurs in childhood and doesn't show any symptoms in immunocompetent individuals [5]. Because of the asymptomatic nature of primary infection, the route of JCV transmission is not known. The oral
or respiratory routes are suspected to be the routes of transmission [6, 7]. Infection with JCV is associated with the establishment of lifelong latent or persistent infection, which may be activated upon suppression of the immune system [8].

**JCV Genome**

JCV shares significant genome homology with other polyomaviruses, simian virus 40 (SV40) and BK virus (BKV) [9].

JCV genome is functionally divided into an early region that encodes large and small TAg, a late region encoding viral capsid proteins, VP1, VP2, VP3 and agnoprotein along with a non-coding regulatory region (RR) [10].

The RR is located between early and late regions and contains the large T-antigen binding sites, origin of DNA replication and transcriptional control region (TCR). The RR also contains a bi-directional promoter/enhancer region that regulates early transcription from one strand and late transcription from the other strand [9].
Figure 2: Organization of the JCV genome shows early genes large and small TAg, late viral genes VP1, VP2, VP3 and agnoprotein and the non-coding regulatory region (Provided by Dr. Richard Frisque).

Role of JCV genes

The large TAg was shown to be a multifunctional phosphoprotein involved in JC viral DNA replication and TAg mediated activation of viral late genes whereas little is known about the small t-antigen, [11]. Recent studies found the expression of novel iso-forms of TAg and they are named $T'$ proteins which are produced due to alternative splicing of the early transcript. These $T'$ proteins appears to be involved in JCV DNA replication and interact with many cellular proteins which may play roles in different T-antigen functions [12]. The large T-antigen also interacts with p53, a tumor suppressor protein [13]. The viral late genes are VP1, VP2, VP3 are capsid proteins and these capsid proteins help in the attachment, adsorption, and penetration of JCV to the host cells. The function of the agnoprotein is not fully understood but some recent studies
indicate that agnoprotein seems to have roles in JCV gene transcription and replication [14].

JCV lytic cycle begins with the expression of early genes encoding the large TAg and small TAg. The large TAg initiates viral DNA replication and controls the transition from early to late gene transcription by activating late gene expression and by suppressing its early promoter. The above mechanism follows the onset of DNA replication in which the virus enters the late phase of infection, in the late phase capsid proteins are encoded, virions are matured and finally the host cell is lysed [15]

Figure 3: Lytic cycle of Polyomaviruses. Viral attachment, entry, transport to nucleus, viral DNA replication, transcription, translation and virion assembly (New England Journal of Medicine, 2002; 347:529).

Transcriptional control region

Transcriptional control region (TCR) is 279-bp in size and located in the non-coding region of the JCV genome, which contains the bi-directional promoter
and the origin of replication (ori). The TCR consists of multiple transcription factor binding sites in the promoter/enhancer region, which interact with the virus, and host cellular proteins that regulate the virus replication.

Different types of cellular transcription factors and their binding sites within the TCR of JCV have been identified. Among these, nuclear factor-1 (NF-1), [16], the glial transcription factors Tst-1 [17] and SP-1 [18], nuclear factor kappa B (NF-κB) [19], the single stranded DNA binding proteins YB-1 and Purα [20] and the activation factor 1 (AP-1) family members c-JUN and c-Fos [21] were shown to induce transcription of viral early and late genes.

![Figure 4: Schematic representation of the JCV Mad-1 regulatory region associating with some of the cellular factors which potentially interact with the viral DNA sequence (Journal of NeuroVirology, Issue: 2003; 9:236).](image)

Classification of JCV

Two types of JCV (archetype and rearranged type) have been identified based on the structure RR. The differences between these two types include the presence of tandem repeats of promoter-enhancer sequences in the rearranged
type, whereas archetype sequences are not arranged in repeats. The prototype strain of JCV, called Mad-1, has tandem repeats of 98 nucleotides, including duplication of the TATA sequences [9]. The archetypal form contains 23 and 66 base pairs (bp) [22].

Archetype is predominately detected in kidney and urine and the rearranged type is predominately detected in brain, tonsils and lymphocytes [5].

In addition to the variation in its RR, JCV also shows variations within its coding region as a result of virus evolution.

JCV genotypes have been defined in four different ways:

1. By restriction fragment length polymorphism analysis [23].
2. By sequencing a 610-bp fragment consisting of a highly variable both the early and late coding regions [24].
3. From the sequence of a short, 129-or 215-bp fragment in the VP1 gene upstream of the VT intergenic region [3].
4. By phylogenetic comparison of the entire viral coding region DNA [25].

Eight genotypes and several subtypes of JCV have been isolated so far. JCV Type 1 is mainly found in The United States in individuals belong to European ancestry [26]. JCV type 2 exists in Asians and Europeans. JCV Type 2 is divided into two sub types 2A and 2B. Type 2A is found in Japan, whereas Type 2B is found in Europe. Type 2B is represented by the strain GS isolated in Germany from PML brain (strain GS/B) and kidney (GS/K). JCV Type 3 is African in origin and has been found in Tanzanians and African Americans [27]. JCV Type 4 may be a recombinant in which a Type 3 replaces a short segment.
of Type 1 genome and has been found only in The United States among the European-Americans and African-Americans in Washington D.C. [28]. JCV Type 7 is predominately found in Asia and Type 8 is found in Polynesia [29]. JCV subtype 2B was found most frequently in the brain of PML patients, which may indicate that it has a higher potential to cause PML than other JCV Types [30].

**Tissue specificity**

Apart from the oligodendrocytes, where JCV preferentially replicates, JCV was detected in other cell types. JCV was found in circulating B-lymphocytes in PML patients [31] and to a lesser extent in peripheral blood leukocytes in healthy individuals [32]. JCV was found in human tonsillar stromal cells of healthy individuals, suggesting that tonsil might be a potential site for initial viral infection [7]. JCV DNA was also found in the kidney of PML patients [33] and in lung, kidney and spleen tissue of AIDS patients with PML [34]. Additionally JCV DNA was detected in gastrointestinal tract of healthy individuals [35].

**Propagation of JCV in primary cell culture and cell lines**

**Cultivation of JCV from human brain with PML**

The first successful propagation of JCV was done by inoculation of PHFG cells with the lysates obtained from PML patient brain tissue [36], after that, many human and animal cells have been tested to search for a better cell culture system for JCV research because PHFG cells are difficult to obtain and maintain.
JCV replication in human amniotic cells

In 1979 Miyamura et al. tested human amniotic cells for JCV replication. In their study they infected human amniotic cells with JCV that was propagated in human fetal brain cells [37]. Even though JCV replicated in human amniotic cells, virus yield was found to be very less. After eight passages in amniotic cells, there is no apparent adaptation of the JCV to human amniotic cells.

JCV adapted to human embryonic kidney cells

In continuation of the above studies, Miyamura and group adapted JCV to human embryonic kidney cells, which are more readily available. They found that JCV was adapted to human embryonic kidney cells and relatively higher yield of virus was found until eight passages. Albeit the human embryonic kidney cells has few disadvantages, the virus lost hemagglutinating activity after several passages [38].

Establishment of human fetal glial cell line

A useful strategy for prolonging the life span of human cell culture is transformation with SV40 DNA because the SV40 can alter both longevity and growth properties of human cells. Santoli et al. demonstrated that human brain cells in tissue culture can be transformed with SV40 to produce permanent lines. In their experiments Santoli et al. obtained cells from explants and monolayer sub cultures of adult human brain obtained from biopsies or autopsies of different patients who were suffering from diseases such as multiple sclerosis,
Creutzfeldt-Jakob and amyotrophic lateral sclerosis and transformed them with SV40 [39]. Similarly, Miranda et al. transformed human myoblasts, derived from mature muscle cells with SV40 to study molecular and biochemical alterations involved in human myopathies [40].

Even though the transformation was successful in all of the above attempts, few cells that were transformed with SV40 were exhibiting a crisis phase characterized by reduction in proliferation capacity, abnormal mitosis and cell detachment eventually leading to death. Moreover the life span of human brain cells that were transformed with SV40 lasted for only 13-39 passages [41].

To avoid above limitations, Major et al. used a DNA replication-defective mutant of SV40, (developed by Gluzman et al. Cite proper reference) to transform human embryonic kidney cells, which are the best host for BK virus and a potential host for JCV [41]. After successfully transforming human embryonic kidney cells and confirming that they are not exhibiting crisis phase, Major et al. tested whether human fetal glial cells can be transformed with DNA replication-defective mutant of SV40 [42].

In their experiments they transfected primary cultures of human fetal brain cells with origin-defective mutant of SV40 and the mutant DNA was able to transform cells to unlimited growth potential, and after sixth passage 100% of the cells were expressing T protein. This cell line was named SVG cell line [42]. SVG cells display the phenotypes of a continuous cell line because they grow to very high saturation density with an 18 hr generation time. The cell morphology was also not altered during the establishment of cell line and cells became
homogenous with typical astroglial characteristics whereas the original fetal brain cells contain astrocytes, oligodendrocytes, and fibroblast type of cells. The astroglial characteristics of SVG cell line was confirmed with GFAP expression, that is specific for astrocytic cultures [42].

JCV efficiently grows in SVG cell line. SVG cells although growing rapidly, did not decrease the length of time JCV required for multiplication in this cell culture, which was similar to PHFG cell culture and infected SVG cells produced JCV at same rate as PHFG cells [42].

Albeit SVG cell line supported the growth of JCV to a larger degree, there are few serious drawbacks with the cell line. SV40 T proteins in the cells interact with JCV regulatory sequences and produce viable virions. An additional problem with SVG cell line is the possibility of recombination occurring between integrated SV40 genome and input JCV DNA. The extensive homology between SV40 and JCV genomes favor this recombination event and leads to production of recombinant virus [43]. Because of the immunological cross reactivity of JCV and SV40 proteins, it might not be possible to identify such recombinants without close examination of the viral DNA using molecular techniques [43].

The above limitations severely question the use of SVG cell line in molecular and pathological characterization of JCV.

**Derivation and characterization of POJ cell line**

The need for a convenient and effective cell system for the growth of JCV in vitro impelled Frisque et al. to endeavor to derive a permissive cell line, by
transforming PHFG cells with ori defective JCV [43]. Even though, above strategy is similar to Major's approach, aim of the Frisque's experiment was to derive a permissive cell line to propagate wild type JCV [43].

In their experiments, they Frisque et al. constructed deletion and insertion mutants of JCV using site-specific mutagenesis technique. Several of these mutants were sequenced and their biological functions were investigated. The mutants allowed them to identify sequences required for the replication of JCV genome [43]. After detecting the sequences required for the JCV replication and establishing ori defective mutants, they used above mutants to transform PHFG cells [43].

The POJ cell system was tested for JCV replication and they found that above cell line supported the lytic growth of JCV and JCV T protein was constitutively expressed in POJ cell line [43].

POJ cells represent the first permissive cell system for JCV that constitutively express wild type T protein [43].

Frisque's efforts to establish a cell system in which JCV produces wild type T protein has got enormous amount of impact on JCV research because this cell system does not complicate the molecular and biological characterization of JCV.

Even though the POJ cells are unique in JCV research, the cell system had some experimental limitations. To establish POJ cells, three requirements should be accomplished, ori-defective mutants had to be constructed, PHFG cells had to be transformed with the mutant DNAs and the cells had to retain their susceptibility for JCV. Although the first step is considered to be straightforward,
problems associated with second and third steps are considered to be the major limitations in establishing POJ cell line [16].

**COS cell line in JCV research**

CV-1 (African Green monkey kidney cell line), an established line of simian cells permissive for lytic growth of SV40, were transformed by an origin defective mutant of SV40, which codes for SV40 [44]. In this process various COS cell lines such as COS 1, 3 and 7 were established [44]. There is no *in vitro* model for replication of archetype JCV. In 1998 Hara et al. demonstrated that various archetype JCV DNA clones can initiate efficient JCV replication in COS-7 cells [45]. Moreover they demonstrated that archetype JCV in urine can be isolated by using COS-7 cells [45].

Even though COS-7 cell line supported the growth of archetype JCV, the same limitations which are applicable to SVG cell line, are also applicable to COS-7 cell line and questions the use of SV40 transformed CV-1 cell line in molecular and pathological characterization of JCV [16]

**Human fetal astrocytes in JCV multiplication**

Previous studies have reflected that JCV growth is efficient in cultures obtained from human fetal brain; nevertheless these cultures contained mixed population of astrocytes and progenitor oligodendrocytes.
In 1989, Major et al. separated mixed populations of glial cells from human fetal brain in culture and produced a pure population of astrocytes and characterized the cell line with GFAP expression, GFAP is a protein specific for astrocytes [46]. They then tested above cultures for JCV replication and found that JCV was efficiently multiplying in astrocytic cell cultures obtained from human fetal brain. These astrocytes can also be passaged for several times without losing astrocytic character [46]. Even though JCV multiplied efficiently in astrocyte cultures, a previous report from Padgett suggested that serially passaged JCV in astrocytes, produced non-viable genomic deletions that leads to production of defective viruses [47].

Propagation of JCV in human neuroblastoma cell line

Neuroblastoma cell line IMR-32 was inoculated with JCV strains Mad-1 and Tokyo-1 respectively; the virus was passaged in IMR-32 cells. With repeated passages, it was demonstrated that virus multiplication increased with number of passages. Their experiments confirmed that IMR-32 was a permissive cell line for JCV [48].

In an other attempt by same group, JCV, which was propagated in IMR-32 cell line, was inoculated into different kinds of cell lines including IMR-32 and U87MG and they found efficient JCV replication only in IMR-32 cells, but they did not explain any reason why JCV propagated in IMR-32 can't replicate in other cell types, on the other hand, they did not test the virus replication in conventional cell systems like PHFG and SVG cells [12].
Figure 5: A graphical representation of JCV dissemination in human body (Journal of NeuroVirology 2003; 9: 236).

Progressive multifocal leukoencephalopathy (PML)

JCV is the etiological agent of the disease PML. PML is a chronic, progressive and fatal demyelinating disease, resulting from the lytic infection of oligodendoglial cells in immunosuppressed individuals. PML is an opportunistic disease and it is mostly found in patients suffering from immunosuppressive diseases like Hodgkin's lymphoma and lympho-proliferative diseases. In addition
it is also found in individuals who are undergoing antineoplastic therapy [49] and 2-5% of the acquired immunodeficiency syndrome (AIDS) patients develop PML [49]. Neuropathological features of PML include multifocal demyelination, enlarged oligodendrocytes with nuclear inclusions, astrocytes with lobulated hyperchromatic nuclei and mitotic bodies [50].

Symptoms of PML are visual deficit, ataxia, and lack of muscle coordination, confusion and dementia [51].

Since the symptoms of PML are similar to toxoplasmosis, meningitis, or AIDS dementia complex, PML is difficult to diagnose. Presently PML disease is not treatable but studies have shown that there are improved neurological conditions in AIDS associated PML patients undergoing highly active antiretroviral therapy (HAART) [52]. Few studies indicated that, a nucleotide analogue cidofovir could be administered in combination with HAART for better neurological conditions in PML patients [53, 54].

Figure 6: Brain section of a PML patient. Patchy demyelination, particularly widespread in the right hemisphere with discoloration in some areas of white matter with residual hemorrhage.
Immune response

JCV is a slow replicating virus that causes persistent infection, so the presence of CTL memory response is important against JCV. Primary infection with JCV occurs in childhood; close to 90% of healthy adults have anti-JCV immunoglobulin G (IgG) [8]. JCV remains latent in the kidney, when the host becomes immunocompromised; JCV reactivates, severely depresses the cellular immune response and causes PML. Intrathecal synthesis of anti-JCV IgG was demonstrated in 76% of the PML patients but this humoral immune response neither prevents the development of PML nor modifies the time course of the disease [8].

Rationale for using U87MG cells in JCV research

The objective of present study was to identify an alternative cell culture system to PHFG cells in JCV pathogenesis and molecular studies.

JCV is a slow replicating virus and it efficiently replicates only in PHFG cells, which are difficult to procure and maintain. To overcome this limitation, researchers employ SVG cell line and/or SV40/JCV chimeras that confound the molecular and biological characterization of JCV. Lack of a suitable cell culture system is hampering pathogenesis related JCV studies. Because PHFG cells are difficult to obtain and maintain, U87MG glioblastoma cell line, which can be easily procured and maintained for several passages was selected for studying JCV replication and progeny virus production. Based on
the finding that JCV can be propagated *in vitro* in PHFG cells and in vivo JCV is detected in the glial cells, we attempted to transfect and infect U87MG glioblastoma cell line with molecular clone of Mad1A JCV and JCV, respectively.

**Hypothesis**

Based on our preliminary data and published reports we hypothesize that U87MG glioblastoma cells are capable of supporting the replication of polyomavirus JC and infectious virions can be produced in these cells

**Specific Aim 1**

To transfect U87MG glioblastoma cells with JCV type 1A infectious clone and test for JCV DNA replication, gene transcription and progeny virus production.

**Specific Aim 2**

To infect U87MG glioblastoma cells with prototype JCV Mad 1A and test for JCV DNA replication, gene transcription and progeny virus production.
CHAPTER 2. METHODS

Culturing of U87MG glioblastoma cells

U87MG human glioblastoma cells, were derived from a primary brain tumor (astrocytoma) of a 44-year-old Caucasian woman, were procured from American Type Culture Collection (ATCC, Manassas, VA). These adherent cells contain epithelial morphology. The cells were propagated in Eagle's Minimum Essential Media (EMEM) (ATCC), supplemented with 10% fetal bovine serum (ATCC) and 1% penicillin/streptomycin (Sigma, St. Louis, MO) mixture and maintained at 37°C and 5% CO₂.

Sub-culturing of U87MG glioblastoma cells

For sub-culturing, the medium was removed and the cells were washed twice with Hank's balanced salt solution (HBSS) (ATCC) and trypsinsed with 0.25% trypsin, 0.03% EDTA solution (ATCC) at 37°C until the cells were detached from the bottom of the flask. Usually it takes 5 min to detach the cells from the bottom of the flask, after which fresh culture medium with FBS was added to neutralize the trypsin and the media was aspirated and dispensed into new culture flasks.
Storage of U87MG glioblastoma cells

For future experiments cells were stored in liquid nitrogen by freezing them in freezing media containing 85% Eagle’s Minimum Essential Media 10% FBS and 5% DMSO (Sigma, St. Louis, MO). The cell pellet of one 75-cm²-cell culture flask was resuspended in 5 mL of above freezing medium. One mL of the cell suspension was transferred into one o-ring tube and placed at -80°C in a freezing container over night. The next day tubes were transferred into liquid nitrogen.

Isolation, extraction and purification of JCV Mad 1 Type 1A molecular clone

Digestion of DNA

Restriction endonucleases (RE) are used to excise JCV DNA clones from the plasmid and also to digest the vector. Restriction endonucleases are bacterial enzymes that cleave double-stranded DNA at specific sites.

In the first step of digestion, DNA was digested by adding the enzyme EcoRI (1.5 U/µg of DNA) in NE buffer 4 (New England Biolabs, Beverly, MA) and BSA (New England Biolabs) and incubated at 37°C for three hr, after confirming that the JCV DNA is excised from the plasmid, in the later step enzyme Hhal (1.5 U/µg of DNA) (New England Biolabs) was added to the above reaction mixture and incubated at 37°C overnight to digest the vector.
**Agarose gel electrophoresis**

The principle for the operation of gel electrophoresis lies in the charge associated with DNA, as it is composed of a negatively charged phosphate group. The gel-electrophoresis apparatus uses a positive and a negative charged pole generated by electrical currents. The DNA is loaded in on the negatively charged pole and pulled through the gel toward the positive pole.

After the digestion, DNA fragments were separated on a 1% agarose gel. To prepare samples for electrophoresis, 1 µL of 6x gel loading dye was added for every 10 µL of DNA solution, mixed well and electrophoresed at 50-100 volts until dye markers have migrated an appropriate distance. After the electrophoresis DNA was visualized illuminating with UV light and the data was analyzed by Bio-Rad's phosphorimager (Bio-Rad, Hercules, CA).

**Extraction of DNA from the gel**

DNA was extracted from agarose gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA) using manufacturer's protocol; the principle of the purification of DNA from agarose gel lies in spin-column technology. During the centrifugation, DNA binds to a silica-membrane in the presence of high salt while contaminants pass through the column. Later the pure DNA is eluted with Tris-buffer.

**Measurement of DNA concentration**
A fraction of the above DNA was diluted in 10 mM Tris-Cl, pH 8.5, and its concentration was determined using a spectrophotometer (Beckman Instruments, Fullerton, CA) by measuring the absorbance at 260 nm. The ratio of readings at 260 nm and 280 nm was used to estimate the purity of the DNA sample.

**Ligation**

T4 DNA ligase catalyzes DNA ligation between double-stranded DNA ends with 3' hydroxyl and 5' phosphate ends. The DNA ends can be either blunt or cohesive. Ligation requires ATP that is contained in the supplied ligation buffer.

The linear JC viral DNA was ligated using T4 DNA ligase (40 U/µg DNA) (New England Biolabs) and the 10X T4 buffer (New England Biolabs) and the reaction was incubated at 16°C overnight.

**Transfection experiment**

U87MG glioblastoma cells were transfected with JCV DNA and pcDNA3.1/GFP (Invitrogen, Carlsbad, CA) separately using Lipofectamine™2000, a lipid-based transfection agent, which was proved to give higher transfection efficiencies. A day before transfection 5 X 10^5 U87MG glioblastoma cells were seeded per 35-mm cell culture plate and grown to 80-90% confluency. On the day of transfection 5 µL of Lipofectamine™2000
(Invitrogen, Carlsbad, CA) and 500 ng of DNA (per plate) was mixed with 250 μL each of opti-MEM (Invitrogen) were kept at room temperature for 5 min, followed by mixing together and incubated at room temperature for 20 mins to allow the formation of DNA-lipid complexes. Cells were washed twice with 2 mL of opti-MEM. The above DNA-lipid complexes in 500 μL aliquots were added into the culture plates, incubated at 37°C and 5% CO₂ for 6 hr. After 6 hr transfection medium was replaced with 3 mL of EMEM supplemented with 30% FBS and 1% penicillin/streptomycin mixture and incubation was continued as above, later cells were harvested for DNA and RNA extraction on days 0, 3, 5, 8 and 14 post-transfection (p.t.).

**Infection experiment**

5 X 10⁵ U87MG cells were seeded per 35-mm plate a day before infection, and cells were grown to 80-90% confluency. Cells were washed twice with pre-warmed opti-MEM and infected with 500 HA/400μL/per plate prototype Mad 1A JCV in opti-MEM for 4 hr at 37°C and 5% CO₂. After the incubation, cells were washed 3 times in 1 mL HBSS. Cells were harvested for DNA and RNA extraction on days 0, 3, 5, 8 and 14 post-Infection (p.i.)

**DNA extraction**

Viral DNA was extracted at different time points p.t. and p.i. using a modified protocol for the Qia Prep® spin mini prep Kit (Qiagen, Valencia, CA)
which is designed for purification of up to 15 μg of high-copy plasmid DNA from
*E. coli*, was used for extraction of viral DNA from JCV- transfected and infected
glioblastoma cells [55]. The protocol was slightly modified for extraction of low
molecular weight DNA from transfected and infected mammalian cells. Briefly,
after washing the plates once with HBSS, 250 μL buffer P1 and P2 were added
directly to the plates and incubated for 5 min at room temperature. The cell
lysate was then gently transferred to a microcentrifuge tube, using a rubber
policeman, and digested with 20 μL of proteinase K (Sigma) (final concentration
800 μg/mL) at 55°C for 1 to 2 hr. Cellular DNA and cell debris were precipitated
by adding 350 μL of buffer N3, and the tube was mixed gently but thoroughly,
followed by incubation on ice for 5 min and centrifugation at 13,000 RPM for 10
min. The supernatant was loaded onto the spin column and centrifuged at
13,000 RPM for 1 min. The spin column was washed once each with buffer PB
and PE. An additional 1 min centrifugation was performed to remove residual
wash buffer. To elute viral DNA, 80 μL elution buffer (10 mM Tris-HCl, pH 8.5)
was added to the center of the column, the column was incubated at 37°C for 5
min and centrifuged at 13,000 RPM for 1 min. The final elution step was repeated
with additional 80 μL of elution buffer.

*Dpnl* replication assay

Replication of JCV DNA was analysed by the *Dpnl* replication assay. Forty μL of DNA was digested with 20 U/μL *EcoRI* and *Dpnl* (New England
Biolabs, Beverly, MA) in 10× DpnI buffer (1 M NaCl, 100 mM Tris pH 7.5, 100 mM MgCl₂, 100 mM β-mercaptoethanol, 3 mg BSA).

The digested DNA was dried in a DNA speed vac (Savant Instruments, Farmingdale, NY) resuspended in 10 µL of 0.75× loading dye (Promega, Madison, WI) and separated on a 1% agarose gel containing 0.3 µg/mL ethidium bromide (Sigma, St. Louis, MO) in TBE buffer for approximately 18 hr at 35 Volts.

**Southern hybridization**

The Southern blotting technique involves transfer of DNA fragments separated in electrophoretic gels to membrane filters for detection of specific base sequences by complementary probes. Southern blots are used to identify specific DNA sequences. After the separation of DNA fragments on 1% agarose gel, the gel was incubated in denaturation buffer on a shaker for two times (30 min each). The gel was then washed with transfer buffer for 15 minutes. The transfer was done using downward capillary, alkaline transfer system (Schleicher & Schuell, Keene, NH). DNA was transferred to the membrane within three hours. After the transfer, membrane was neutralized in 1X neutralizing buffer and it was UV cross-linked using a Stratalinker (Stratagene, La Jolla, CA) and stored at 4°C.

**³²P Labeling**

Random oligonucleotide primers can be used for the synthesis of uniformly labeled DNA probes. The oligonucleotides serve as primers for initiation of DNA synthesis by DNA polymerases on single-stranded templates.
The use of random sequence hexanucleotides makes it possible to prime DNA synthesis at numerous sites along a denatured template DNA. The primer-template complex is then a substrate for the Klenow fragment of *E.coli* DNA polymerase I. By using $[^{32}\text{P}]$ dCTPs (Roche Applied Science, Indianapolis, IN) as precursors, the newly synthesized DNA probe can be radiolabeled to a very high specific activity.

For the synthesis of $^{32}\text{P}$-labeled full-length JCV as a probe for hybridization the Rediprime™ II Random prime-labeling system (Amersham Biosciences, Piscataway, NJ) was used. One hundred ng of the template DNA was diluted in a total volume of 45 μL in TE buffer (Qiagen, Valencia, CA) and the protocol was followed as recommended by the manufacturer.

**Hybridization**

The radioactive DNA probe was hybridized to the immobilized DNA on the nylon membrane as followed. To block nonspecific DNA binding sites on its surface, the membrane was rolled into a hybridization tube, 15 mL of pre-hybridization solution, containing Denhardt's reagent (Sigma) was added and the membrane was kept in rotating in rotated hybridization incubator at 68°C for at least 45 minutes. After prehybridization, the labeled DNA probe was added to another 15 mL of pre-hybridization solution and poured into the hybridization tube containing the membrane. An overnight incubation at 68°C with rotation allowed the probe to bind to target sequences in the immobilized DNA. The membrane was placed in a plastic tray the next day for the following two washing steps with...
wash solution I (2X SSC+0.1% SDS) at room temperature for 10 min, each. The membrane was then transferred to the hybridization tube and wash solution II (2X SSC+0.1% SDS) was added and incubated with rotation at 68°C for 15 min. Wash solution II was replaced by wash solution III (1X SSC+0.1% SDS) and incubated for another 10 min at 68°C after that wash solution III is replaced by wash solution IV (0.1X SSC+0.1% SDS). After this final washing step, the membrane was dried on paper towels and wrapped in plastic wrap for exposure.

Exposure

Storage phosphor screen technology is more sensitive, quantitative and more accurate than X-ray film technology. When a radioactive emission strikes the screen, phosphor oxidation occurs and a stable, high-energy state is formed. Following exposure, the screen is placed in a high-resolution laser scanner. The charged areas of the latent image on the screen return to the ground level when screened, releasing energy in the form of emitted photons of visible light, which are collected and counted by a photo multiplier tube. A computer builds a digitalized image of the sample that can be analyzed using appropriate software.

The hybridized membrane was exposed to a Kodak storage phosphor screen in a standard X-ray cassette. After exposure, radioactivity in the hybridized bands was detected using the Molecular Imager by storage phosphor screen technology and the screen was erased with fluorescent light for 15 min prior to re-exposure.

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RNA extraction:

The cells, that were transfected and infected with JCV genotype 1A, or mock-transfected/infected were washed twice with HBSS and were lysed directly on the plates with lysis buffer (RNeasy Mini Total RNA Isolation Kit, Qiagen®). The lysate was transferred to an eppendorff tube for total RNA extraction using the RNeasy kit according to the manufacturer's protocol. Genomic DNA contamination was removed by digesting the RNA twice on column with 30 Kunitz units of RNase free Dnase (Qiagen®) for 30 min each. The yield of each RNA sample was determined by spectrophotometer.

RT-PCR

Bio-Rad iScript One-Step RT-PCR Kit (Bio-Rad, Hercules, CA) was employed for synthesizing cDNA. The cycling conditions are 25°C for 5 min; 42°C for 40 min; and 80°C for 5 min. For two-step RT-PCR, cDNA was synthesized in a 20 μL reaction mixture containing 1 μg of RNA 4 μL of 5x iscript reaction Mix, 1 μL of iscript reverse transcriptase and molecular grade water.

For the second step of RT-PCR, 1 μL of each of the above cDNA were included in a 20 μL of reaction mixture containing 1X PCR buffer, 2.5 mM MgCl₂, 0.8 mM each dNTP, 2.5 U Taq polymerase (Applied Biosystems), 25 pmol each of the forward and reverse primers (Table 1), both internal controls (cDNA from mock-transfected cells) and no template controls (only RNA samples) were also amplified. The RT reactions were carried out on a Gene Amp PCR system 9700
(Applied Biosystems). The GAPDH primer set was also used to amplify housekeeping gene GAPDH as an internal positive control for normalization.

The amplicons were run on a 2% agarose gel and the ethidium bromide fluorescence was visualized after scanning using the Bio-Rad Molecular Phosphor imager.

**In-situ hybridization:**

U87MG cells were grown \((0.5 \times 10^6)\) on sterile 13 mm cover slip in a petri dish, and cultured overnight at 37°C, 5% CO2. On the following day the cells were transfected with JCV DNA (500ng/35 mm dish) using lipofectamine 2000 and incubated for 6 hr in Opti-MEM.

The cells were washed 3 times with 1X PBS before fixing onto the cover slip with 1 mL of fixative (methanol/acetone: 1:1). The fixative was removed by vacuum-suction, washed 3 times with 1X PBS and air-dried. The cover slips were washed with DEPC-treated water and incubated in a humid chamber at 55°C for 30 min. Sixty μL of ready-to-use nick translated DNA probe reagent (0.5 μg/mL) was added onto the fixed-cells on cover slip, and placed on top of the mounting slide (cells were upside down). The slide was heated on a heating block at 95°C for 5 min before transferring to 37°C for 24 hr. The cover slip was washed in pre-warmed 50% formamide/1X SSC at 50°C for 3 times, 20 min each time followed with 2 times washed with pre-warmed 1.0X SSC at 50°C, 15 min each wash. The cover slip was then rinsed with 1.0X TBS for 3 times, 5 min each time.
To develop color, 80 µL of streptavidin/AP was added onto the slide, and covered with cover slip and incubated for 10 min at 37°C. The cover slip was then rinsed with TBS 3 times, 5 min each time. Two hundred µL of NBT/BCIP substrate was added and stored at room temperature for the color development. The reaction was stopped by adding distilled water. The cells were mounted by adding 20 µL of aqueous mounting medium onto the cover slip.

Preparation of lysate

The glioblastoma cell line, U87MG was transfected with JCV molecular clone, and lysed on day 14 p.t. using Autotune series high intensity ultrasonic processor. Naïve PHFG cells and U87MG glioblastoma cells were infected with 0.4 mL of the above lysate and DNA was extracted on days 0, 3, 5, 8 and 14 and viral replication was confirmed using the method described under DNA replication section. RNA was also extracted from the above cells at above time points using the method described under the RNA extraction section and viral gene transcription was confirmed using the method described under RT-PCR section.

Quantitative real time PCR

Quantitative real time PCR assay was conducted to measure JCV TAg copy number in different p.t. and p.i. DNA samples using Bio-Rad iCycler iQ™ Multicolor Real time PCR Detection system.
DNA was diluted to 1:20 (5 µL of DNA was added to 95 µL of molecular grade water) and 5 µL of above diluted DNA was used as a template and the master mix was prepared by adding Bio-Rad 2X IQ™ SYBER® Green super mix and 12.5 pmol each of forward and reverse primers specific for JCV TAg gene [JCT-1 (Forward): 5' AGA GTG TTG GGA TCC TGT GTT TT 3'; JCT-2 (Reverse): 5' GAG AAG TGG GAT GAA GAC CTG TTT 3']. Thermal cycling was initiated first with a denaturating step of 10 min at 95°C, followed by 35 cycles of 95°C for 10 sec and 60°C for 15 sec.

Standard curve was constructed using serial dilutions of JCV (Type 1A) plasmid DNA. The range of detection of viral copy number was determined by using 10-fold serial dilutions of JCV plasmid in the range of 100 pg to 10 fg that represent 1.8 x 10^6 to 1.8 x 10^2 copy numbers. JCV DNA copy numbers was calculated by plotting JCV copy equivalents vs. Ct value of the sample.
CHAPTER 3. RESULTS

Measurement of transfection efficiency of U87MG cells

U87MG glioblastoma cells were transfected with pcDNA3.1/GFP plasmid (green fluorescent protein) to determine whether the cell line can be transfected using Lipofectamine-2000, after 24 hr p.t., 40% of the cells were fluoresced green (Figure 1).

In situ hybridization

In order to demonstrate the localization of replicated JCV DNA in U87MG glioblastoma cells, we conducted in situ DNA hybridization using biotin labeled JCV DNA probes. Localization of JCV in the U87MG cells was detected as early as day 3 p.t. The signal intensity was increased at day 5 and no signal was observed in mock-transfected cells with and without probe (Figure 3).

Replication of JCV in transfected and infected U87MG glioblastoma cells based on DpnI assay

In this experiment we used JCV molecular clone Type 1A to transfect U87MG glioblastoma cells and viral DNA was extracted at different time points after transfection and DpnI replication assay was conducted, in which the extracted DNA was digested with EcoRI and DpnI. Upon digestion with EcoRI, replicated JCV DNA is linearized and is visible as unit-length molecule of 5.1 kb whereas DpnI selectively digests transfected DNA that has been methylated
during prokaryotic replication, where as DNA replicated in eukaryotic cells is unmethylated and resistant to DpnI digestion. The above digested DNA was probed with $^{32}$P-labeled JCV DNA and JCV replication was found as early as day 3, the replication efficiency was increased as time increases and a strong JCV signal was found on day 8 post transfection. (Figure 2A). Mock-transfected cells did not show any signal.

In order to demonstrate that U87MG glioblastoma cells are susceptible to JCV, we infected the above cells with prototype JCV Mad 1A virus. Viral DNA was extracted at different time points after infection and replicated DNA (5.1 kb) was detected using the method described above. The result indicated that JCV replication started as early as day 3 and increased with time up to day 14 (Figure 4A).

**JCV transcription in transfected and infected glioblastoma cells**

To demonstrate viral transcription, we amplified JC viral early and late genes using RT-PCR. Viral transcripts were seen as early as day 1 for TAg and day 3 for agnoprotein and VP1 in transfected cells (Figure 2B). In infection experiments viral transcripts were seen as early as day 3 for TAg and day 5 for agnoprotein and VP1 (Figure 4B). All JC viral transcripts increased steadily over 14 days. TAg, the early replication gene appeared first followed by the late genes VP1 and agno.
Production of Progeny Virus

The glioblastoma cell line, U87MG was transfected with JCV molecular clone and lysed on day 14 p.t and naïve primary human fetal glial cells and U87MG glioblastoma cells were infected with the above lysate and DNA was extracted on days 0, 3, 5, 8 and 14 and viral replication was confirmed using the method described under the DNA replication section. RNA was also extracted from the above cells at above time points using the method described under the RNA extraction and viral gene transcription was confirmed using the method described under RT-PCR and the DNA replication assay results indicated that JCV replication started as early as day 3 and increased with time up to day 14 and RT-PCR results indicated that all JC viral transcripts increased steadily over 14 days.

Real time PCR based analysis of JCV copy number in PHFG and U87MG cells post transfection/infection:

To further confirm and compare the profile JCV replication in these two cell types, JCV copy number was determined at different time points by real time PCR, both, p.t. and p.i. with 500 HA of the virus and with lysate obtained from JCV transfected U87MG cells at day 14 p.t (figure 7).

Though the viral copy numbers exhibit a continuous increase from day 3 to day 14, our data indicates burst of virus production at around day 8 p.i. It was also noted that in both the infection experiments, using 500 HA of virus and the
lysate, the pattern of increase in viral copy numbers at different time points in U87MG cell line was very similar to PHFG cells.

These results are in line with our previous results of JCV transcription in both the cell types. This further strengthens the observation that U87MG cells can support JCV life cycle very similar to PHFG cells.
CHAPTER 4. DISCUSSION

The first successful propagation of JCV was conducted by inoculation of primary human fetal glial (PHFG) cells with the lysates obtained from PML patient brain tissue [36]. Since then several human and animal cells have been tested to search for a better cell culture system for JCV replication because PHFG cells are difficult to obtain and maintain. Human amniotic cells have been shown to be susceptible to JCV, but the yield of virus was found to be very less [37]. JCV, which was adapted to human embryonic kidney cells, lost hemaglutination activity after several passages [38]. JCV replication has been demonstrated in transformed cell line of human fetal glial cells established by introducing the origin defective mutant of SV40 (SVG cell line) [42] and a SV40 transformed African green monkey kidney cell line COS-7 [45]. POJ cell line (PHFG cells transformed with origin defective JCV) [43], IMR-32, a neuroblastoma cell line [48] and human fetal astrocytes in culture [46] were also used to study JCV. Human cytomegalovirus (HCMV) was also demonstrated to induce the replication of JCV DNA in human fibroblasts which are not permissive for the replication of JCV alone [56] and HCMV induced productive replication was shown in U373MG glioblastoma cell line [57].

JCV is difficult to propagate compared to other polyomaviruses, SV40 and BK. The restricted growth of JCV has limited in vitro pathogenesis related studies of PML. For past 30 years various attempts have been made to propagate JCV in cell lines and susceptible primary cells [2, 41-46, 56]. Although
many human cell cultures have been tested, JCV efficiently grows only in PHFG cells [58]. Even though JCV is permissive to different human cell lines, it does not efficiently replicate in these cells.

Several JCV pathogenesis related studies have employed JCV/SV40 chimeras and/or transformed primary human fetal glial cells with ori defective SV40 or JCV mutants (cite references). Moreover archetype JCV replication has been demonstrated in COS 7 cell lines [44]. The principal disadvantage of using these cell lines is, SV40 or JCV ori defective mutants drive JCV replication.

The data presented here describe our attempt to identify glioblastoma cell line (U87MG) as a better alternative to PHFG cells for replication of JCV. The principal reason to employ U87MG glioblastoma cell line to study JCV pathogenesis is that the PHFG cells are difficult to obtain and maintain. On the other hand glioblastoma cell line can be procured easily and maintained over several number of passages.

In the present study we tested our hypothesis, whether non-transformed glial cell line (U87MG) can be transfected and infected with JCV infectious clone and JCV respectively, in order to propagate and produce infectious JCV.

We used both receptor-independent (transfection) and -dependent (infection) approaches to demonstrate efficient replication of JCV in U87MG cells. Dpn I replication assay and Southern hybridization using JCV full-length clone we demonstrated that JCV efficiently replicated in U87MG glioblastoma
cells (Figure 2). Since most JCV pathogenesis related experiments can be completed by day 14, we chose day 14 as our last time point. In independent experiments we have demonstrated JCV replication past day 21 in U87MG cells (data not shown). Lack of JCV replication in mock-transfected U87MG cells using Southern hybridization and JCV full-length clone demonstrated that U87MG cells are not transformed with SV40 or JCV genome.

Previous in vitro and in vivo studies using tumor specimens have suggested that JCV replication does not always equate to JCV transcription. To validate JCV gene transcription we amplified JCV mRNA transcripts of early and late genes. As expected JCV TAg, an early replication regulatory gene product appeared first followed by the late genes VP1 and agno. Additionally we demonstrated localization of JCV genome using in situ hybridization.

Infection of U87MG glioblastoma cells with prototype JCV Mad 1A resulted in replication of JCV as demonstrated by replication and RT-PCR based assays, respectively. Even though U87MG cells demonstrated similar replication profile in both transfection and infection experiments, there was a difference in transcription profile. In transfection experiments the early gene appeared on day 1 p.t. and the late genes appeared on day 3 p.t., whereas in infection experiments the early gene appeared on day 3 p.i. and late genes appeared on day 5 p.i. The difference in JCV transcription profile may be attributed to receptor-independent as opposed to -dependent experimental conditions.

JCV replication in U87MG cells is not sufficient for demonstration of the efficiency of propagation of JCV and progeny virus production. To demonstrate
progeny virus production we inoculated naïve U87MG and PHFG cells with day 14 lysate from JCV transfected U87MG cells. Replication and transcription of JCV was observed using Dpn I replication and RT-PCR, assays, respectively. These data clearly demonstrate that JCV lifecycle gets completed successfully and the progeny virus is produced in U87MG glioblastoma cell line.

Based on the real time PCR data irrespective of the cells used (U87MG or PHFG) the virus production was very similar when the same HA units or volume of lysate was used to infect these cells (Figure 7D). Burst of virus production was seen on day 5. The amount of virus produced was directly proportional to the inoculum used. The lysate used for infection was approximately 10 HA (data not shown), which produced more virus by day 14 when compared to 500 HA of virus (80 fold vs. 10 fold). At 500 HA almost 100% of the cells are infected by day 1 whereas at 10 HA few cells are infected, which results in several more rounds of infection by day 5. In short by using 500 HA virus no cells remain uninfected resulting in significantly less production of new virions. This result was similar for both the cell types, U87MG and PHFG.

Based on our studies we propose that U87MG glioblastoma cell line can be used to conduct JCV pathogenesis related studies. Moreover U87MG cell line is a convenient and alternative cell culture system for PHFG cells, which are difficult to procure and maintain. U87MG glioblastoma cell line is easily available and supports JCV replication, gene transcription and progeny virus production as demonstrated in this study.
Our studies are optimistic and we believe that the U87MG glioblastoma cell line will expedite the study of polyomavirus JC, and the availability of this cell line will assist in understanding the pathobiology of PML.
Table 1. Primer Sequences and Cycling Conditions for Amplification of JCV and GAPDH genes

<table>
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<tr>
<th>Gene</th>
<th>Type</th>
<th>Position</th>
<th>Primer Sequence</th>
<th>Temperature and time</th>
<th>Cycles</th>
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<td>3006-3028</td>
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<td></td>
<td>R</td>
<td>3401-3380</td>
<td>5'-GCT ATT CAA GGG GCC AAT AGA C-3'</td>
<td>95°C for 30 sec; 57°C for 30 sec</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>72°C for 15 sec</td>
<td>1</td>
</tr>
<tr>
<td>*F</td>
<td></td>
<td>4268-4320</td>
<td>5' AGA GTG TTG GGA TCC TGT GTT TT 3'</td>
<td>95°C for 5 min</td>
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* Primers used for quantitative real-time PCR
Figure 1. Determination of transfection efficiency of U87MG glioblastoma cell line. U87MG cells were transfected with pcDNA3.1/GFP plasmid and cells were observed 24 hr p.t. using a fluorescence microscope (40x magnification). Approximately 40% of the cells fluoresced green.
Figure 2. Replication of JCV molecular clone Type 1A in U87MG glioblastoma cell line. (A). DNA was extracted on days 3, 5 and 8, and DpnI replication assay was conducted, digested DNA fragments were separated on a 1% agarose gel, transferred to a nylon membrane and hybridized with a full-length $^{32}$P-labeled linear JCV DNA. After washing, membrane was exposed to the phosphor screen for 45 min, followed by scanning using a Bio-Rad molecular imager. (B). RNA was extracted from JCV transfected U87MG glioblastoma cells on days 0, 1, 3, 5, 8 and 14. One $\mu$g of RNA was used for cDNA synthesis using Bio-Rad iScript RT-PCR Kit. RT-PCR was conducted using primers specific for JCV T-antigen, agno gene, VP1 gene and housekeeping gene GAPDH in a Gene Amp Thermal Cycler 9700. The amplicons were separated on a 2% agarose gel and the ethidium bromide fluorescence was visualized after scanning using the Bio-Rad Molecular Phosphorimager.
Figure 3. Detection of JCV by In situ hybridization. The glioblastoma cell line, U87MG, was transfected with the full-length JCV molecular clone Type 1A and cells were fixed on days 3 and 5 with 1:1 methanol and acetone mixture and hybridize with JCV Bioprobe.
**Southern hybridization**

A Days 3 5 8 14 + 5.1-kb

**RT-PCR**

B Days 0 3 5 8 14 +
- T-antigen (350-bp)
- Agno (185-bp)
- VP1 (190-bp)
- GAPDH (400-bp)

**Figure 4.** Replication of JCV Type 1A in U87MG glioblastoma cell line. (A). DNA was extracted on days 3, 5, 8 and 14 p.i. and digested with EcoRI, digested DNA fragments were separated on a 1% agarose gel, transferred to a nylon membrane and hybridized with a full-length $^{32}$P-labeled linear JCV DNA. After washing, the membrane was exposed to the phosphor screen for 45 minutes, followed by scanning with a Bio-Rad molecular imager. (B). RNA was extracted from JCV infected U87MG glioblastoma cells on days 0, 3, 5, 8 and 14. One μg of RNA was used for cDNA synthesis using BioRad iScript RT-PCR Kit. RT-PCR was conducted using primers specific for JCV T-antigen, agno gene, VP1 gene and housekeeping gene GAPDH in a Gene Amp Thermal Cycler 9700. The amplicons were separated on a 2% agarose gel and the ethidium bromide fluorescence was visualized after scanning using the BioRad Molecular Phosphorimager.
Figure 5. Production of Progeny Virus: The glioblastoma cell line, U87MG was transfected with JCV molecular clone, and lysed on day 14 p.t. Naïve glioblastoma cells were infected with the above lysate (A). DNA was extracted on days 0, 3, 5, 8 and 14 and viral replication was confirmed using the method described in figure 2A and 3A. (B). RNA was extracted from JCV infected U87MG glioblastoma cells on days 0, 3, 5, 8 and 14. One μg of RNA was used for cDNA synthesis and cDNA was synthesized using BioRad iScript RT-PCR Kit. RT-PCR was conducted using primers specific for JCV T-antigen, agnogene, VP1 gene and housekeeping gene GAPDH in a Gene Amp Thermal Cycler 9700. The amplicons were run on a 2% agarose gel and the ethidium bromide fluorescence was visualized after scanning using the BioRad Molecular Phosphorimaget.
Figure 6. Production of Progeny Virus: The glioblastoma cell line, U87MG was transfected with JCV molecular clone, and lysed on day 14 p.t. Naïve PHFG cells were infected with the above lysate. (A). DNA was extracted on days 0, 3, 5, 8 and 14 and viral replication was confirmed using the method described in figure 2A and 3A. (B). RNA was extracted from JCV infected PHFG cells on days 0, 3, 5, 8 and 14. One µg of RNA was used for cDNA synthesis and cDNA was synthesized using BioRad iScript RT-PCR Kit. RT-PCR was conducted using primers specific for JCV T-antigen, agnogene, VP1 gene and housekeeping gene GAPDH in a Gene Amp Thermal Cycler 9700. The amplicons were run on a 2% agarose gel and the ethidium bromide fluorescence was visualized after scanning using the BioRad Molecular Phosphorimager.
Figure 7A. PCR Amplification Cycle Graph. Quantitative real-time PCR assay amplification plot was obtained from SYBR Green-based assay, using previously diluted linear JCV (Mad1) DNA. JCV DNA concentration was measured using the Beckman Coulter spectrophotometer and was converted to JCV copy numbers ranging from $1.8 \times 10^5$ to $1.8 \times 10^9$ copies of JCV (Mad1) DNA.

Correlation Coefficient: 1.00
PCR Efficiency: 100.0%

Figure 7B. Linearity of SYBR Green-Based Real-Time Quantitative PCR. Linear regression plot was generated for SYBR Green-based real-time quantitative PCR of linear JCV (Mad1) DNA dilutions. The linear JCV(Mad1)DNA copy equivalents on the horizontal axis are plotted against the Ct (Threshold cycle) on the vertical axis.
**Figure 7C. Dissociation plot.** Dissociation plot of amplified known standards and 44 samples of JCV DNA extracted from infected or transfected U87MG glioblastoma and PHFG cells. Temperature on the x-axis is plotted against the first derivative of the measured fluorescence \([d(F)/dT]\) on the y axis. The melt curve analysis was done in the range of 55 - 95°C, and single peaks of different amplicons were observed at 81°C.
1. U87MG glioblastoma cell line infected with 500 HA of JCV Mad 1A
2. PHFG cells infected with 500 HA of JCV Mad 1A
3. U87MG glioblastoma cell line transfected with 500 ng JCV DNA type 1A
4. U87MG glioblastoma cell line infected with lysate obtained from U87MG cells day 14 p.t.
5. PHFG cells infected with lysate obtained from U87MG cells day 14 p.t.

**Figure 7D. Estimated Quantity of JCV In Infected or Transfected U87MG Glioblastoma Cells With JCV (Mad1) DNA or Molecular Clone of JCV (Mad1).** JCV copy numbers in experimental samples were calculated by generating a standard curve (correlation of coefficient 1.00) with a previously diluted JCV (Mad1) DNA, as mentioned above. The above graph depicts virus copy numbers on days 0, 3, 5, 8, and 14, post-infection or -transfection. All samples were tested in duplicate.
References


