

Induction of Chromosomal Instability in Colonic Cells by the Human Polyomavirus JC Virus¹

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ABSTRACT

Most colorectal cancers display chromosomal instability, which is characterized by gross chromosomal rearrangements, loss of heterozygosity and aneuploidy. We have previously demonstrated a link between JC virus strains Mad-1 and $\Delta 98$ and colorectal cancer. Others have also associated the virus to the induction of colon cancer and aneuploid brain tumors by producing a highly tumorigenic protein named T antigen (TAG), which binds to β -catenin and inactivates key proteins such as p53. The aim is to demonstrate that JC virus is capable of inducing chromosomal instability in colonic cells. We used the human colon cancer cell line RKO as a model. The cell line has wild-type p53, wild-type β -catenin and APC and is diploid. Neuroblastoma JCI cells, which are infected with the virus, VA13 fibroblasts, which are transformed by the SV40 TAG, were used as positive controls. HCT116, which has mutated β -catenin, and SW480, which is a model of CIN, were also used as controls. The genomes of the Mad-1 and $\Delta 98$ strains were transfected into cells. As negative controls we used pUC or no plasmids. Cells were collected at 0, 7, 14, and 21 days after transfection. PCR was used for the detection of TAG and the regulatory region DNA sequences at different time frames and Southern blot of whole genomic extracts for viral DNA integration into the host genome. Immunofluorescence and Western blot were performed for TAG, viral capsid proteins, and nuclear β -catenin expressions, whereas coimmunoprecipitation was used to detect protein interactions. Karyotype analysis and electron microscopy were performed to seek chromosomal instability and cell abnormalities, respectively. Retention of viral sequences was observed for Mad-1- and $\Delta 98$ -transfected RKO cells at all time frames with PCR only, whereas Southern blot analysis showed nonintegrated sequences at T7 alone. TAG and capsid protein expressions, as well as increased p53 and nuclear β -catenin, were observed between T0 and T7 for Mad-1 and $\Delta 98$ alone. Also, interaction between TAG and both p53 and β -catenin was also observed between T0 and T7. Chromosomal instability, characterized by chromosomal breakage, dicentric chromosomes, and increasing ploidy, was observed at all time frames for Mad-1 and $\Delta 98$, as well as cell abnormalities. In conclusion, we demonstrate that JC virus Mad-1 and $\Delta 98$ are able to induce chromosomal instability in colonic cells with a hit and run mechanism that involves an early interaction with β -catenin and p53.

INTRODUCTION

Human cells experience a constant barrage of mutations. Many of these mutations will be irrelevant unless the function of a critical gene is lost or an oncogene will be activated, thus creating a growth advantage for that cell, with subsequent clonal expansion. Clonal

expansion, *per se*, might be an innocuous occurrence, where it not for the appearance of additional mutations that participate in multistep carcinogenesis. The mutation rate in normal human tissues is relatively low, and many investigators propose that a form of genomic instability would be necessary to accelerate the mutation rate and account for the number of mutations found in a tumor (1).

Two forms of genomic instability have been described in human colorectal cancers. One that has been well studied and characterized over the past 9 years is MSI³ (2), which occurs in ~12–15% of colorectal cancers. However, the majority of colorectal cancers has a different abnormality, named CIN, which results in many duplications, deletions, and rearrangements in the DNA within a neoplastic nucleus (3). Losses of large segments of chromosomes are an efficient means of inactivating tumor suppressor genes (4), however, the mechanism of CIN remains undetermined.

It has been proposed that three genetic events can be sufficient for converting a human embryonic cell to a tumor cell. These events are: a mutant *H-RAS* allele; the ectopic expression of the telomerase catalytic subunit (*hTERT* gene); and the activity of TAG (5). T (transforming) antigen is a multifunctional protein encoded by polyomaviruses. The TAGs encoded by the SV40 and the human polyomaviruses JC and BK share a high degree of homology (6) and are capable of transforming cells both *in vitro* and *in vivo* by interacting with the p53 protein and with cell cycle regulators pRb and Rb-related p107 and p130 (7, 8). JCV has been linked with the development of aneuploid tumors when injected intracranially in mammals (9) and has been found in aneuploid human brain tumors (10). Furthermore, elevated antibody titers to JCV have been linked with aneuploid (“rogue”) lymphocytes in humans (11).

Recently, JCV was demonstrated to be present in most colorectal cancers and the adjacent normal colonic epithelium, with at least a 10-fold higher viral load in cancers (12). We have also found JC viral DNA sequences throughout the gastrointestinal tract in the majority of healthy human subjects (13). Interestingly, a sequence of the Mad-1 variant of JCV lacking a 98-bp repeat in the TCR, named $\Delta 98$, has been found preferentially in colon cancers, which raises the possibility that certain strains may be selectively activated in colonic epithelial cells (14). Also, JCV DNA sequences were isolated from five colon cancer xenografts and from the aneuploid colorectal cancer cell line SW480 (12).

Recently, JCV TAG has been found expressed in medulloblastomas and colorectal cancers and demonstrated to interact with β -catenin in these tumors (15, 16). β -Catenin is involved in colon carcinogenesis through the WNT signaling pathway after its mutation or mutation of the APC protein (17). Interaction between TAG and β -catenin in these tumors lead to translocation of the latter into the nucleus, thus working

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³The abbreviations used are: CIN, chromosomal instability; JCV, JC virus; CRC, colorectal cancer; MSI, microsatellite instability; TAG, T antigen; TCR, transcriptional control region; TBS, Tris-buffered saline; TBSGBA, tris-buffered saline with gelatin, bovine serum albumin and sodium azide.

as a transcription factor for oncogenes such as c-myc (15). This data support the theory of an involvement of TAg in developing these tumors. Interestingly, viral DNA sequences have also been found in tumors not showing TAg expression, suggesting that the virus may be involved early in carcinogenesis but may be counteradaptive and selected against later in the malignant process for many of the cells and found finally deleted from an actively growing tumor mass. Transformation of the epithelial cells would occur after the early expression of TAg, which is not capable to drive viral replication or virion formation. The early inactivation of tumor suppressors and deregulation of the Wnt pathway would determine transformation. Later on, the uncontrolled cell proliferation would not need the expression of TAg because cells have already been transformed.

Previous data regarding JCV TAg-transforming activity are mostly based on studies performed with expression vectors under the control of the cytomegalovirus or SV40 promoters (18). However, it has been always postulated that JCV-transforming activity, and cellular tropism was limited by the regulatory region sequence (10, 19, 20).

In this study, we investigated JCVs' induction of CIN in colonic cells. We used full-length viral genomes and evaluated the expression of TAg and its interaction with p53 and β -catenin in the RKO cells derived from a human colorectal carcinoma.

MATERIALS AND METHODS

Cell Culture. RKO, HCT116, SW480 human colon carcinoma cell lines were obtained from the American Type Culture Collection (American Type Culture Collection, Manassas VA). RKO is a cell line that displays MSI for the hypermethylation of the hMLH1 promoter and expresses wild-type p53, APC, and β -catenin (21, 22). HCT116 also exhibits microsatellite instability and shows increased nuclear β -catenin levels because of mutation of the gene. SW480 is a prototype example of CIN and contains elevated levels of mutant p53 protein. RKO cells were cultured in DMEM (Invitrogen, Rockville MD), HCT116 in McCoy's 5A, and SW480 in Leibovitz media. Media were supplemented with 10% fetal bovine serum and antibiotics (ampicillin and streptomycin). Human neuroblastoma JCI cells, persistently infected with JCV (23), were used as positive controls. The SV40-transformed human embryo fibroblast VA13 cell line, obtained from American Type Culture Collection, was also used as a positive control for TAg expression. Both control cells were cultured in DMEM (Invitrogen) with 10% fetal bovine serum and antibiotics.

Generation of Full-Length Circular Viral Genomes and Transfection. Full-length Mad-1 and Δ 98 genomes were cloned into pBR322 (24). Full-length viral genomes were obtained after digestion with *Eco*RI (Invitrogen). Digests were electrophoresed on 1.2% agarose gels, the linearized genomes were recovered by slicing the gel bands, and purified DNA was re-ligated with

T4 ligase (Invitrogen). One μ g of DNA was transfected into cells plated on 24-well plates with Lipofectamine 2000 and Plus reagent (Invitrogen) following the manufacturer's suggestion. Untransfected cells and cells transfected with either pUC or pBR322 plasmids were used as negative controls.

Antibodies for Immunoblot, Immunofluorescence, and Immunoprecipitation. The anti-SV40 mouse antibody, Pab416, (Oncogene Research Product, Boston MA), recognizes the NH₂ terminus of large TAg of both SV40 and JCVs (25) and was used for immunoblotting studies and immunofluorescence analysis. Mouse antibody, clone E5 (Santa Cruz Biotechnology, Santa Cruz, CA), recognizes β -catenin [and a second mouse monoclonal antibody was used to detect p53 (Dako, Glostrup, Denmark)]. All of these antibodies were used at a dilution of 1:100. A rabbit antibody that recognizes JCV VP1 capsid protein (26) was used at a dilution of 1:3000.

DNA Extraction of Genomic and Viral DNA. Genomic DNA was extracted from cell lines using the QIAmp DNA minikit for DNA extraction (Qiagen, Hilden, Germany) following the instructions provided by the manufacturer, after suspending the cell pellet in 450 μ l of cold DNA extraction buffer [0.32 M sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 1% Triton X-100]. Episomic DNA was extracted using the Hirt method (27).

Amplification of JCV TAg and TCR Sequences. We used two methods and primers for the amplification of the TAg and TCR of JCV (12, 14). For both amplifications, 100 ng of total DNA were amplified by PCR, and the amplicons were sequenced and aligned with the JCV sequences published previously.

Evaluation of Viral Replication and Integration of the JCV Genome into the Host Genome. To evaluate the integration of viral DNA, 20 μ g of genomic DNA were digested with 200 units of *Eco*RI. Samples were electrophoresed in a 0.7% agarose overnight and blotted onto a nylon membrane. Digested and undigested Mad-1 was used as size marker. DNA samples were probed with full-length viral DNA labeled with ³²P using the Bioprime random primer kit (Invitrogen).

Cytogenetics. After reaching 50–60% confluency, cells were resuspended in appropriate medium with 0.1 μ g/ml Colcemid (Sigma Chemical Co., St. Louis MO) and incubated at 37°C for 4 h. After collection, mitotic cells were lysed in freshly prepared 75 mM KCl and fixed twice with a solution of 3:1 methanol:acetic acid. After the last centrifugation in fixative solution, chromosomal spreads were obtained by dripping a single drop of solution from a Pasteur pipette held 10 cm above a glass slide previously prepared with 95% ethanol. Slides were then baked at 65°C for 4 h to harden the chromatin and stored at –20°C until they were examined.

Metaphases were stained with Giemsa (Carlo Erba, Rodano, Italy) and visualized under a microscope at a \times 60 magnification. One hundred metaphases on each slide were screened for aneuploidy and gross chromosomal abnormalities.

Protein Extraction and Western Blot. Cells were grown at 80–90% confluency, washed in cold PBS, and solubilized at 4°C for 20 min in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 2.5 mM MgCl₂, 0.5% Triton

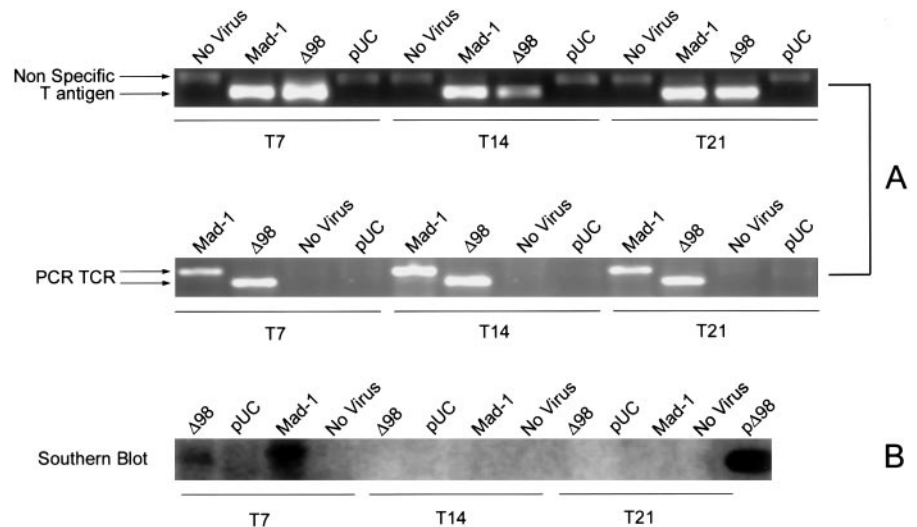


Fig. 1. A, Amplification of JCV TAg and TCR sequences from transfected RKO cells. Viral DNA sequences were detected in Mad-1- and Δ 98-transfected RKO cells at all time points. B, Southern blot analysis of 20 μ g of genomic DNA extracted at days 7, 14 and 21 after transfection and digested with *Eco*RI. DNA sequences were not hybridized after 7 days posttransfection. The positions of the DNA on the gel are consistent with linearized full-length viral genomes.

X-100, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitor. The lysates were sonicated, then centrifuged at 4°C for 10 min at 15,000 rpm, and the protein concentration of the clear supernatant was determined using a BCA assay kit (Pierce, Rockford IL). Detergent-soluble protein extracts were boiled at 95°C for 10 min in SDS-loading buffer [65 mM Tris-HCl (pH 7.5), 65 mM 2-mercaptoethanol, 1% SDS, 10% glycerol, and 0.003% bromophenol blue] and electrophoresed on a 12% polyacrylamide gel. Each lane was loaded with 100 µg of protein and blotted onto nitrocellulose membranes (Hybond C Extra, Amersham Pharmacia, Little Chalfont, United Kingdom). Membranes were blocked in 5% nonfat dry milk for 50 min, washed in PBS, and incubated with the appropriate antibody overnight at 4°C. Protein quantities were normalized against actin levels using a mouse antiactin antibody (Sigma Chemical Co.). After repeated washing, the membranes were incubated with antimouse horseradish peroxidase-conjugated secondary antibodies using the EnVision dextran polymer visualization system (Dako). After 40 min, membranes were washed, and autoradiographies were obtained by enhanced chemiluminescence. Digital images of autoradiographies were acquired with a scanner (Fluor-S Multimager; Bio-Rad, Hercules CA), and signals were quantified using a specific densitometric software (Quantity-one; Bio-Rad) in absorbance units (A) after light calibration with a reference autoradiography.

Immunofluorescence. Cells were grown to ~50% confluence on coverslips, fixed with 2% paraformaldehyde in appropriate media 37°C for 30 min, rinsed with TBS, then washed with TBS-0.1% saponin. Cells were blocked

with TBSGBA-0.1% saponin for 15 min and incubated at 4°C with the appropriate primary antibody in TBSGBA. After incubation, cells were washed three times for 20 min in TBS-0.01% saponin and incubated for 2 h at room temperature in the dark with Alexa Fluor 488 goat antimouse and/or Texas Red goat antimouse antibody (Molecular Probes, Eugene OR) at a dilution of 1:100 in TBSGBA. For VP1 expression, a Texas Red antirabbit antibody was used (Molecular Probes). After washes, cells were visualized using an epifluorescent microscope.

Immunoprecipitation. Five hundred µg of total protein extract were preincubated with either anti-β-catenin or anti-p53 monoclonal antibody in the presence of protein A-agarose. Immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blot using anti-TAg antibody.

Electron Microscopy. Transfected and nontransfected cells were harvested at T7, T14, and T21 posttreatment. Cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), postfixed in 0.1 M OsO₄ 1% in cacodylate buffer, dehydrated, and embedded in Araldite. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Philips 410 Transmission Electron microscope.

RESULTS

The JCV genome consists of early and late coding regions separated by two noncoding regions at both the 5' and 3' ends. The

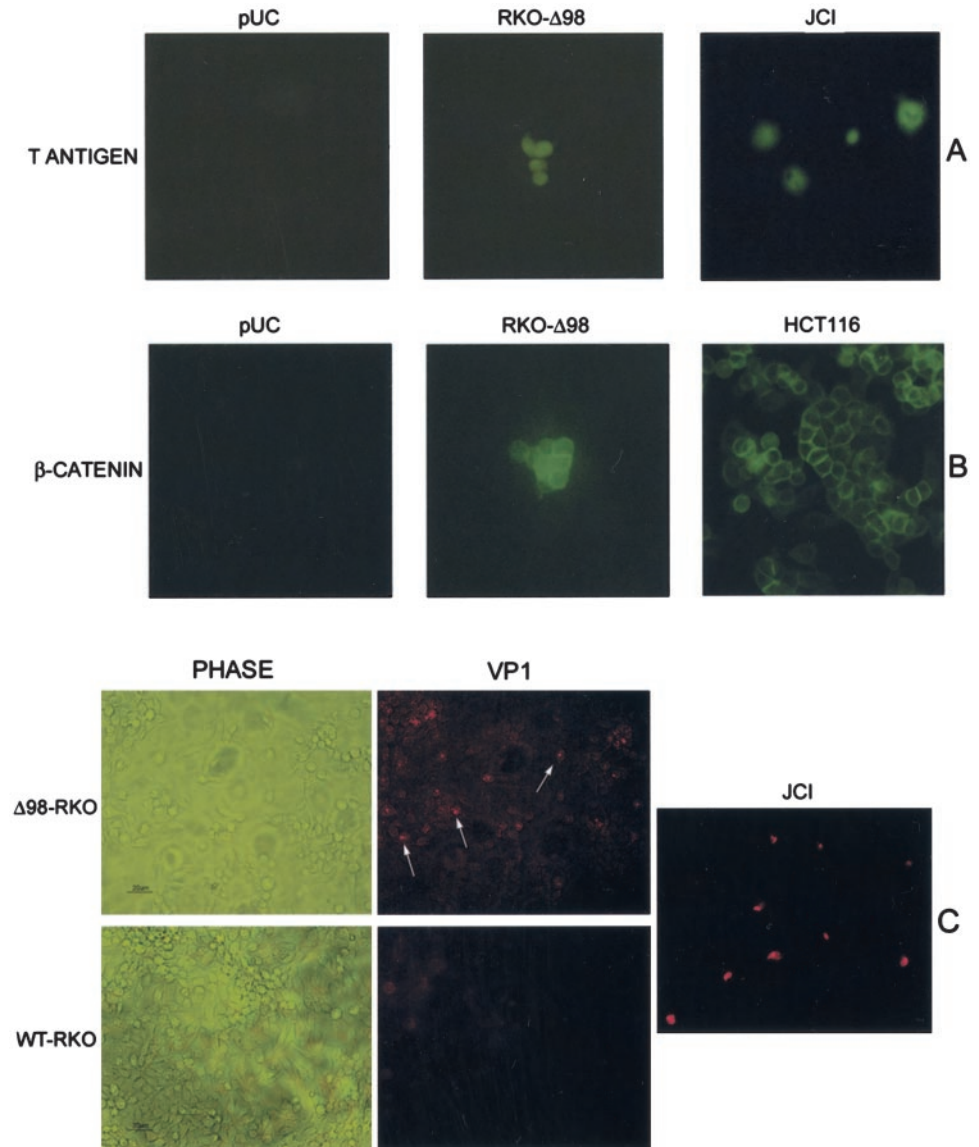


Fig. 2. A, TAg expression at 7 days after transfection in RKO cells transfected with Δ98 JCV or pUC plasmid (negative control). The neuroblastoma cell line, JCI, is persistently infected with JCV Mad-1 and was used as positive control. B, increased nuclear β-catenin in Δ98-transfected RKO cells (Mad-1-transfected cells not shown). Cells transfected with pUC (left panel) or left untransfected were negative for nuclear β-catenin. HCT116 cells were used as a positive control (right panel). C, expression of viral capsid protein in the nucleus of untransfected and JCV DNA-transfected RKO cells. Viral protein was detected in Δ98- and Mad-1 (data not shown for Mad-1)-transfected cells but not in pUC or untransfected RKO cells. JCI cells were used as a positive control.

5'-noncoding sequence is the TCR, which contains the promoter and enhancer for early and late transcription. JCV can be classified into two forms: the archetype (CY) and tandem repeat variants. Among the tandem repeat variants of JCV, the best studied is Mad-1. The regulatory region of Mad-1 is characterized by two 98-bp repeats. In a previous study, we found a Mad-1 variant characterized by a single 98-bp sequence without any archetypal features that we named Δ98 (14, 24).

We transfected the intact genomes of the Mad-1 and Δ98 variants into the RKO cell line. We evaluated these cells for the presence and integration state of JCV TAg coding and TCR sequences by PCR and Southern blot analyses. By PCR analysis, we found DNA sequences all time points in Mad-1- and Δ98-transfected-RKO cells, (T7; Fig. 1A). Because PCR is a sensitive method to amplify small amount of nucleic acids, we wanted to detect viral DNAs by Southern blot analysis using whole DNA extracts from the transfected cells. In particular, we looked for viral DNA integration into the host genome and/or free circular DNA by digesting 20 μg of DNA with the appropriate restriction enzyme and hybridizing with the full-length

viral genome used as a probe. All viral DNAs were detectable in the transfected cells at T7, but no viral sequences were detected at T14 or T21 (Fig. 1B). The migration of the bands on the gel was consistent with the pattern expected for a free viral genome, suggesting that the DNA was not integrated.

JCV TAg and VP1 Expression in Colonic Cells. Transfected and nontransfected RKO cells were examined for TAg and capsid protein expression. TAg expression was observed by immunofluorescence in Δ98/Mad-1-transfected cells at T7 (Fig. 2A), suggesting that Δ98 and Mad-1 are capable of producing the protein in epithelial cell, whereas the protein was not detectable at T14 and T21 (data not shown). All negative controls did not express TAg at any time point. We also found that in Δ98-RKO and Mad-1-RKO cells, VP1 capsid protein was expressed at T7 (Fig. 2C, data not shown for Mad-1 RKO), indicating that the virus replicates at early stages, although not efficiently because lytic effects were not observed. None of the other cells expressed VP1 protein. This data were confirmed by immunoblotting (Fig. 3C).

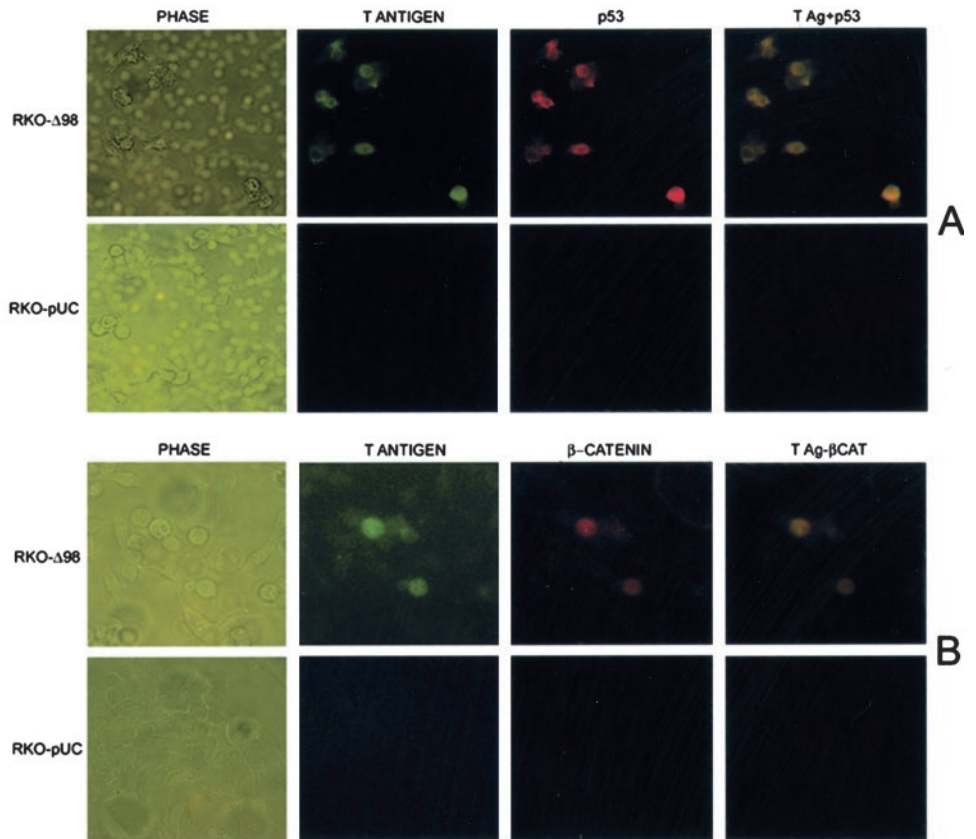
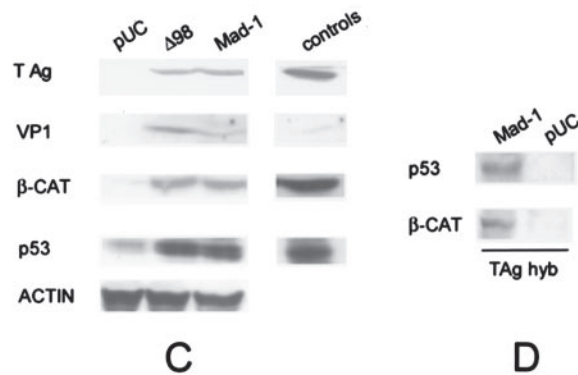


Fig. 3. Dual immunofluorescence detects TAg-p53 (A) and TAg-β catenin (B) interactions. TAg expression is observed in Δ98-transfected RKO cells and colocalizes with nuclear p53 and β-catenin, whereas negative control show no increase of TAg and β-catenin/p53. Western blot analysis (C) and coimmunoprecipitation (D) at day 7 after transfection. Expression of TAg, VP1, β-catenin, and p53 increased in Δ98- and Mad-1-transfected RKO cells. The expression of these proteins decreases after 7 days posttransfection (data not shown). Coimmunoprecipitation results reveal interactions between TAg and β-catenin and TAg and p53.



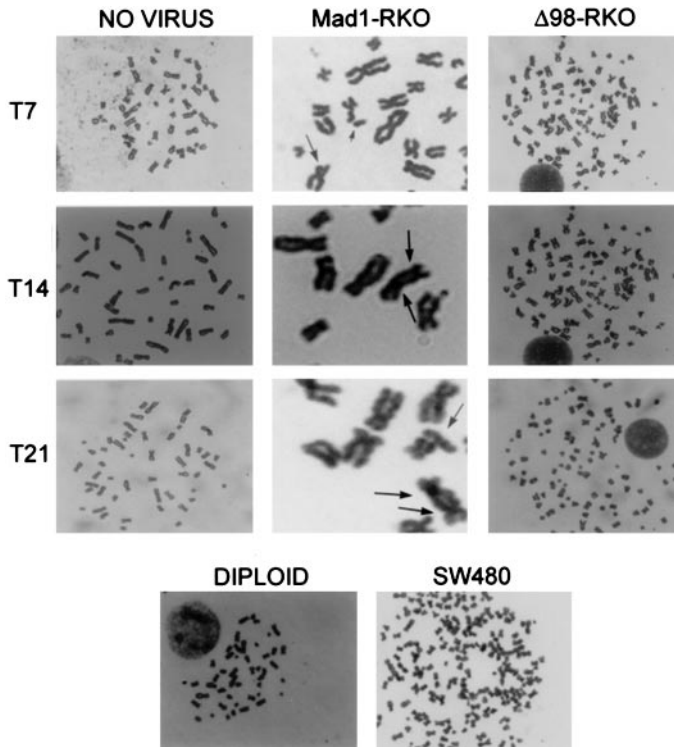


Fig. 4. Chromosomal instability in JCV DNA-transfected cells. Chromosomal instability, characterized by chromosomal breakages, dicentric chromosomes, increasing chromosomal number, and other gross abnormalities, was seen to increase in $\Delta 98$ - and Mad-1-transfected cells between day 7 and 21 after transfection. These abnormalities were not observed in untransfected or pUC-transfected cells. Arrows show chromosomal breakages and dicentric chromosomes. Human fibroblasts were used as a diploid control, whereas SW480 was used as a prototype of chromosomal instability.

JCV Induces Nuclear β -Catenin Expression in RKO. RKO cells usually show no nuclear expression of β -catenin because the gene and the APC protein are not mutated in this system. This indicates that the RKO line represents the best colonic epithelial cell, among cancerous cells, to test for the increase of β -catenin induced by JCV TAG. In our experiments, we found nuclear β -catenin accumulated in $\Delta 98$ -RKO (Fig. 2B) and Mad1-RKO cells (data not shown). Moreover dual immunofluorescence shows colocalization of β -catenin in cells in which TAG was also expressed (Fig. 3B). This data were confirmed by coimmunoprecipitation (Fig. 3D), supporting previous reports that TAG interacts with β -catenin (15, 16). Negative control transfected and nontransfected cells resulted in no nuclear increase of β -catenin.

JCV TAG Binds p53 in Transfected RKO Cells. p53 is a tumor suppressor protein that regulates cellular proliferation by influencing apoptosis, cell cycle progression, and angiogenesis (for review, see Ref. 28). Previous studies have demonstrated that TAG binds and inactivates p53 (8). Furthermore, binding of TAG to p53 has been linked to the development of aneuploid tumors. p53 overexpression in colorectal cancers may result from mutations of the p53 gene, however, some cancers express elevated levels of nonmutated but inactive p53 (28). We examined transfected RKO cells for elevated amounts of p53 and for p53-TAG interactions. Immunofluorescence and Western blot analyses showed increases in p53 expression in $\Delta 98$ - and Mad-1-transfected cells (Fig. 3C). p53 levels did not increase in the T14 and T21 samples. Dual immunostaining (Fig. 3A) and coimmunoprecipitation (Fig. 3D) demonstrated an interaction between TAG and p53 in virus-transfected cells.

CIN in JCV-Transfected Cells. To demonstrate that JCV is capable of inducing CIN, we collected metaphase chromosome spreads

at T7, T14, and T21 and looked for chromosomal abnormalities by scanning 100 metaphase spreads/transfected cell culture (Fig. 4). We found evidence of CIN, characterized by chromosomal breakages, dicentric chromosomes, and increasing chromosome numbers in $\Delta 98$ - and Mad-1-transfected RKO, whereas all of the negative controls resembled the native RKO cells. Interestingly, CIN was already present at T7 and continued to increase through T7 and T21, although no traces of viral proteins were found (Table 1).

Cellular Morphological Changes in JCV-Transfected Cells. Finally, we looked for cellular morphological changes induced by JCV. No nuclear changes or viral capsids were found in any of the transfected cells. After 7 days posttransfection, morphological changes were visible in Mad-1- and $\Delta 98$ -transfected cells. These changes were mostly abnormalities of the cellular membrane with increasing number of microvilli and few pseudopodial cytoplasmic projections (Fig. 5A). More importantly, at T14 and T21, membrane abnormalities increased and cells started to lose cell-cell contact with enlargement of intercellular gaps (Fig. 5B), indicating that early interaction with β -catenin may modify the architectural pattern of both cytoskeleton and intercellular interactions.

DISCUSSION

In this study, we have demonstrated that: (a) JCV Mad-1 and $\Delta 98$ variants produce TAG in epithelial cells; (b) both variants replicate in RKO cells, albeit inefficiently; (c) TAG binds to β -catenin and p53; (d) TAG-positive cells become chromosomally unstable, and this instability increases after progressive loss of viral genomes and protein; and finally, (e) transfected cells accumulate morphological changes and lose cell-cell contact (Table 2).

Recently, TAG expression and interactions with β -catenin have

Table 1 Cells with chromosomal instability after transfection

	Cells with chromosomal aberrations		
	T7	T14	T21
pUC-RKO/WT-RKO	0/100	0/100	0/100
$\Delta 98$ -RKO	3/100	5/100	11/100
Mad1-RKO	2/100	3/100	7/100

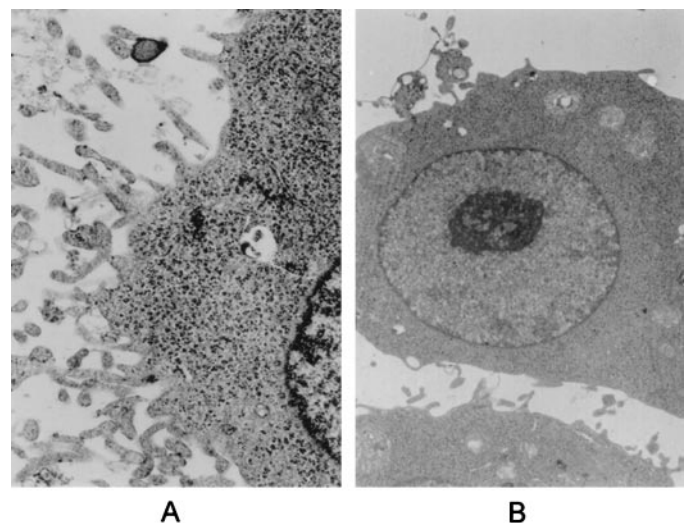


Fig. 5. Morphological changes in RKO cells transfected with JCV DNA. After 7 days posttransfection, morphological changes were visible in Mad-1- and $\Delta 98$ -transfected cells. These changes were primarily abnormalities of the cellular membrane with increasing numbers of microvilli and pseudopodial projections (A). At days 14 and 21 after transfection, cells began to lose contact with one another, and enlargement of intercellular gaps was apparent (B).

Table 2. The effect on JCV on RKO cells

	7 days				14 days				21 days			
	WT ^a	Mad-1	Δ98	pUC	WT	Mad-1	Δ98	pUC	WT	Mad-1	Δ98	pUC
PCR (TAg and TCR)	–	Y	Y	–	–	Y	Y	–	–	Y	Y	–
Southern blot	–	Y	Y	–	–	–	–	–	–	–	–	–
TAg expression	–	Y	Y	–	–	–	–	–	–	–	–	–
VPI expression	–	Y	Y	–	–	–	–	–	–	–	–	–
p53 expression	–	Y	Y	–	–	–	–	–	–	–	–	–
Nuclear β-catenin	–	Y	Y	–	–	–	–	–	–	–	–	–
Chr. aberrations	–	+	+	–	–	++	++	–	–	+++	+++	–

^a WT, no virus; Y, yes; chr. aberr.: chromosomal aberrations.

been observed in colorectal cancers, extending previous results that indicate JCV is present in most colorectal cancers (12–14, 16).

TAg is an oncogenic protein capable of transforming mammalian cells by interacting with cellular proteins such as p53 and the Rb family proteins (5). TAg also has ATPase and helicase activities, the latter of which may eventually contribute to chromosomal breakage and recombination. In our study, we found the rate of CIN increased in Mad-1- and Δ98-transfected cells over time. As previously demonstrated, this effect is achieved through the early interaction and blocking of p53: the cell with achieved chromosomal damage would replicate and accumulate chromosomal aberrations. Furthermore, although our data are preliminary, looking up to 21 days after transfection, the early interaction with β-catenin would result in aberrations of the cytoskeleton and cellular interactions.

In cells transformed by TAg, stable marker chromosomes evolve after crisis, indicating the selection of clones with *in vitro* growth advantages, suggesting that cells become TAg independent after passage (29). Canaani *et al.* (30) have reported that SV40 TAg-immortalized cells had a stable uniform karyotype during serial passage. TAg is known for its ability to induce transformation after causing chromosomal damages (31). Li *et al.* (32) have shown that SV40-TAg alone is able to destabilize the karyotype and generate aneuploidy. In polyomavirus-transformed cells, transformation characteristically occurs after the viral DNA is retained as an integration event, although in some instances episomal DNA might persist (33–35). The entire viral genome is often not retained because viral replication is not required for maintenance of the tumor (for review, see Ref. 35).

We found that cells start to lose viral DNA shortly after being transfected. The DNA is only detectable by PCR after 14 and 21 days posttransfection, suggesting a very low initial replication activity. This, in turn, indicates that colonic cells are semi/nonpermissive for the virus and thus susceptible to transformation rather than to lytic effects.

A form of genomic instability appears to be necessary in carcinogenesis. A normal cell must generate a sufficient amount of genomic diversity to overcome the nuclear omeostasis that makes malignant transformation unlikely. CIN is particularly useful in providing the second hit at a tumor suppressor gene locus because chromosomal losses can be large, and events that delete genes unambiguously can inactivate cell growth controls. Many of the tumor suppressor genes that are inactivated in colorectal cancer such as *APC* or *p53* appear to be lost through a point mutation to one allele, followed by a second deletional event (commonly termed loss of heterozygosity) to the other allele (4). Once a critical number of genetic misadventures have accumulated in a cell, clonal expansion occurs and a neoplasm can evolve. At some point in the evolution of a tumor mass, the ideal number of genetic rearrangements will occur that support malignant growth, and a relative degree of genomic stability would be favored. At this point, cells that lose the TAg might experience a growth advantage and would tend to overgrow persistently infected cells.

For many years, JCV has been studied for its potential to induce

tumors when injected into mammals. In fact, a number of experiments involving primates showed that after intracranial inoculation of the virus, several animals developed aneuploid brain tumors with low expression of the early gene and no viral replication (36). In more recent years, JCV DNA sequences were amplified from many human neoplasms, including central nervous system and colon tumors. Recently, JCV DNA sequences were found highly prevalent in primary colon cancers (88%) and a somewhat lower frequency in a small series of xenografts (50%; Ref. 12). Furthermore, as reported by Enam *et al.* (16), colonic tumors contain expression of viral TAg. However, it is interesting that viral DNA has always been detected more frequently than TAg expression in both central nervous system and colonic tumors. This suggests that either in some samples, the viral copy number is too low to determine expression of the early gene or, alternatively, that the growing tumor tends to lose viral sequences as shown by our *in vitro* data. To support this, data on adenomatous polyps, the early precursors of colon cancers, are needed. This would clarify the role of TAg expression in early stages of carcinogenesis (which is characterized by the increasing accumulation of nuclear β-catenin), rather than in tumors that are already transformed. It is possible that the accumulation of genetic alterations during multistep carcinogenesis, which favor malignant transformation, may eventually render a transforming virus unnecessary; alternatively, there may even be selection against persistent infection after a threshold genetic alteration has been reached. Thus, it is not difficult to imagine that an essential event that favors the initiation of tumorigenesis in the colon may be subject to pressures that force it to “hit and run.”

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