Construction of a Novel JCV/SV40 Hybrid Virus (JCSV) Reveals a Role for the JC Virus Capsid in Viral Tropism

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JC virus (JCV) is a common human polyomavirus that infects greater than 70% of the general population worldwide. JCV is also the causative agent of progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease of the CNS. Currently, little is known about the mechanisms that restrict JCV tropism to a few human cell types and tissues. In vivo, JCV can be detected in oligodendrocytes and astrocytes in the CNS of patients with PML. The virus can also be detected in kidney, tonsil, and B lymphocytes of patients both with and without PML. In vitro, JCV can only be propagated in cultures of human fetal glial cells or in cell lines derived from this tissue. In contrast, the closely related monkey polyomavirus, SV40, has a broad tropism for primate cells, including those cells that are also susceptible to infection by JCV. We hypothesized that one potential block to infection is at the level of virus entry. To examine this, we constructed a JCV/SV40 chimeric viral genome that contains the regulatory region and the early genes of SV40 and the late structural genes of JCV. The hybrid virus (JCSV) induced SV40-like cytopathic effect in human glial cells and hemagglutinated human type O red blood cells similar to JCV. More importantly, the hybrid virus maintained the host range of JCV, suggesting that interactions between the virus capsid and host cell receptors contribute to JCV tropism. © 2002 Elsevier Science (USA)

Key Words: Polyomavirus; JCV; tropism; glial cells; virus receptors.

INTRODUCTION

The human polyomavirus JC virus (JCV) is the causative agent of the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML). Seronepidemiological studies have shown that greater than 70% of the human population worldwide is infected by JCV, with seroconversion typically occurring in early childhood (Padgett and Walker, 1973). Affecting mainly immunocompromised patients, PML was formerly considered a rare disease; however, the frequency of disease has dramatically risen in association with increasing HIV infection. It is estimated that 85% of PML cases have AIDS as the underlying immune deficit and that 2–4% of AIDS patients will develop this rapidly fatal, opportunistic disease (Berger and Concha, 1995). There is currently no effective treatment for PML, although highly active antiretroviral therapy (HAART) has been shown to improve symptoms of PML in some patients (Clifford et al., 1999).

PML develops from lytic infection of myelin-producing oligodendrocytes in the CNS. In vivo, JCV can also be found in B cells in lymphoid organs and it has been hypothesized that these tissues may be a reservoir of latent JCV genomes and that B cells may traffic JCV to the CNS (Major et al., 1992; Monaco et al., 1996, 1998; Tornatore et al., 1992; Wei et al., 2000). In vitro, the tropism of JCV is restricted to human glial cells and to a limited extent, human B cell lines. In our laboratory we use the SV40 T antigen transformed SVG-A cell line to propagate JCV. This cell line was derived by limited dilution cloning of the parental SVG cell line described by Major et al. (1985). SVG-A cells support JCV infection and replication in vitro to levels comparable to primary human fetal glial cells.

The mechanisms restricting the tropism of JCV to these few cell types is not known. SV40, in comparison, can infect a broad range of cells of primate origin, including human and monkey cell lines such as SVG-A (human fetal glial), CV-1 (monkey kidney), and BSC-1 (monkey kidney) (Tooze, 1973). The broad tropism of SV40 is consistent with the fact that SV40 utilizes the ubiquitously expressed major histocompatibility complex (MHC) class I protein as a receptor for virus entry (Atwood and Norkin, 1989; Breau et al., 1992; Stang et al., 1997). In contrast, JCV does not use MHC class I proteins as a receptor but rather uses an N-linked glycoprotein containing α 2–6 linked sialic acid as one component of its receptor (Liu et al., 1998). The proteinaceous component of the receptor has not been identified.

To assess the contribution of viral structural proteins to host cell tropism, we constructed a novel JCV/SV40
chimeric virus (JCSV) containing the regulatory and early regions of SV40 and the late capsid coding region of JCV. We asked whether this chimeric virus would maintain the host range of JCV or whether its host range would be expanded to cells that are susceptible to SV40 infection but not to JCV infection. The chimeric virus displayed physical and biological properties of both SV40 and JCV but maintained the host range of JCV. These data clearly demonstrate that early events in the life cycle of JCV contribute to virus tropism.

RESULTS

Production of infectious virus from the chimeric genome

To determine the contribution of the viral coat proteins of JCV to the host cell tropism of this virus, we exchanged the late region of SV40 for that of JCV (Fig. 1). Once the chimeric viral genome was constructed, we separated the viral DNA from plasmid sequences by digesting with BamHI and gel purifying the viral genome fragment. This linear DNA (10 μg) fragment was then transfected into SVG-A cells (~8 × 10⁶ cells/75 cm² flask) by calcium phosphate-mediated transfection. To determine whether the viral genome was being expressed, the transfected cells were plated onto coverslips in six-well dishes. One week posttransfection, the cells were fixed and stained for VP1 expression with the monoclonal antibody PAB597. Immunofluorescent detection revealed V antigen expression, indicating transcription of the hybrid genome (Fig. 2, pJCSV-2.5.1).

To demonstrate that infectious virions were being produced, lysates of the transfected cells were used to infect fresh SVG-A cells. Positive staining for V antigen was observed, indicating that viable, infectious virus was present, hereafter referred to as JCSV (Fig. 2, JCSV). Infection of SVG-A cells was blocked by anti-JCV antisera, indicating that the source of infectious material in the lysate was virus and not viral DNA (not shown).

Biological characterization of JCSV

We compared the cytopathic effects induced in SVG-A cells by infection with equivalent amounts of SV40, JCSV, and JCV (Mad-1 SVEΔ). Note that SV40 is known to induce extensive cytoplasmatic vacuolization in infected cells. In contrast, infection of cells with JCV induces a necrotic phenotype that takes several weeks to develop. Interestingly, the cytopathic effect (CPE) induced by infection of SVG-A cells by the hybrid virus, JCSV, was similar to SV40-induced CPE (Fig. 3, compare A and C). Infection of SVG-A cells with the Mad-1SVEΔ strain of JCV induced the expected necrotic phenotype (Fig. 3B).

We next compared the abilities of JCSV, JCV (Mad-1 SVEΔ), and SV40 to hemagglutinate human type O red blood cells. Both JCSV and JCV (Mad-1 SVEΔ) hemagglutinated red blood cells in a dose-dependent manner (Fig. 3). As expected, SV40 did not hemagglutinate human type O red blood cells (Fig. 3).

Infection of SVG-A, CV-1, and COS-7 cells with SV40, JCSV, and JCV (Mad-1SVEΔ)

The SVG-A cell line was derived from primary human fetal glial cells transformed by origin-defective SV40. SVG-A cells are permissive to both JCV and SV40 infection. CV-1 cells, an African monkey kidney cell line, are permissive for the lytic growth of SV40 but are nonpermissive for JCV infection. As COS-7 cells are also transformed with SV40 T antigen, we used them to control for any effects that SV40 T antigen expression might have on JCV tropism. Note that COS-7 cells have been reported to have low level susceptibility to JCV (Hara et al., 1998).

SVG-A, CV-1, and COS-7 cells were plated in six-well dishes and infected with 25 HAU of JCSV, JCV (Mad-1 SVEΔ), or with 1.2 × 10⁶ PFU of SV40, and scored for either T-antigen (CV1) or V-antigen (SV40 and JCSV) expression at 3 days postinfection. SVG-A cells were efficiently infected by SV40, JCSV, and JCV (Mad-1 SVEΔ) (Fig. 4). The results show that equivalent amounts of JCSV and JCV (Mad-1 SVEΔ) led to comparable levels of infection in SVG-A cells, 32 and 40%, respectively (Fig. 4). In contrast, CV-1 and COS-7 cells were not efficiently infected by either JCV or JCSV (Fig. 4). As expected, they were highly susceptible to infection with SV40 (Fig. 4).

To determine whether the reduced efficiency of JCSV infection of CV-1 and COS-7 cells was due to a block at entry or at a postentry step, we transfected the cells with DNA isolated from SV40, JCSV, and JCV (Mad-1 SVEΔ) virions. At three days posttransfection the cells were fixed and assayed for T antigen (CV1) or V antigen (COS-7) expression. Transfection of CV1 cells with DNA from each of the three viruses led to T antigen expression in a minority of the cells (Fig. 5). The percentage of cells expressing T antigen following transfection was consistent with the poor transfection efficiencies measured in these cells (not shown). In contrast to CV1 cells transfection efficiency in COS-7 cells is very high. Transfection of these cells with each of the three viral DNAs led to high levels of viral antigen expression (Fig. 5). Although it is difficult to make definitive conclusions regarding the susceptibility of CV1 cells to infection with virus or viral DNA due to poor transfection efficiency, the results in COS-7 cells clearly demonstrate that a major block to infection in COS-7 cells is at the level of viral entry as JCV and JCSV viral DNA but not virions led to efficient viral gene expression in these cells.

DISCUSSION

The ability to generate pseudotyped and chimeric viral genomes has played a major role in allowing us to understand how early events, such as host cell receptor
recognition, contribute to viral tropism. The human polyomavirus, JCV, has a very restricted cell tropism which is due in part to molecular events downstream of viral entry (Feigenbaum et al., 1987; Raj and Khalili, 1995; Sumner et al., 1996; Tornatore et al., 1994; Vacante et al., 1989). The generation of chimeric viral genomes has helped to elucidate some of these properties. For example, early work swapping the regulatory and T antigen coding regions of
JCV and SV40 found that the host range of JCV was not expanded by the substitution of SV40 regulatory elements into a JCV backbone (Chuke et al., 1986). SV40 DNA containing JCV regulatory sequences, however, were able to replicate in CV-1 cells and produce lytically active virus upon transfection. This indicated that SV40 large T antigen could efficiently interact with the JCV transcription signals but that the JCV T antigen was not fully capable of interacting with SV40 regulatory signals.

The Mad-1 SVEΔ/H9004 strain of JCV was originally developed to address the role of the regulatory region in restricting virus tropism (Vacante et al., 1989). The virus was constructed by inserting the entire SV40 regulatory region into the JCV Mad-1 backbone. Following transfection of this hybrid DNA into glial cells, a virus emerged that had deleted portions of both the SV40 and the JCV regulatory region. This chimeric virus was found to have expanded its host range to human embryonic kidney cells and to both fetal and adult rhesus monkey glial cells (Vacante et al., 1989). The chimeric virus did not infect the WI-38 line of human diploid lung fibroblasts or the BSC-1 line of African green monkey kidney cells, indicating that either the hybrid regulatory region was not permissive in these cell types or the virus failed to enter these cells (Vacante et al., 1989).

We have made extensive use of the Mad-1 SVEΔ strain of JCV to characterize the early events of JCV infection, including virus–host cell receptor interactions (Liu et al., 1998; Pho et al., 2000; Wei et al., 2000). However, as the virus has a hybrid regulatory region and is still restricted for infection of some cell types, it has not been possible to completely dissect contributions to tropism made by the capsid from contributions made by the regulatory elements. To address this, we constructed a novel virus by inserting the entire late region of JCV into the SV40 backbone. Sequencing was performed at each step dur-

FIG. 2. Expression and production of JCSV by transfection of SVG-A cells. SVG-A cells were either mock transfected or transfected with 10 µg of pICSV-2.5.1 DNA excised from the plasmid pBRICSV-2.5.1 (as indicated). At 1 week posttransfection cells were plated onto coverslips, fixed in acetone, and assayed for the V antigen expression by indirect immunofluorescence assay. Lysates of these transfected cells (JCSV) were then used to infect SVG-A cells. At 3 days postinfection the cells were assayed for V antigen expression.
ing the construction of this virus to be sure that we were not introducing additional, unexpected mutations and alterations in the hybrid genome. Once the virus was constructed, we asked whether it would maintain the host range of JCV or whether its host range would be expanded to those cells that are also susceptible to SV40 infection. Our initial characterization of the virus revealed that JCSV had a true hybrid phenotype. Infection of SVG-A cells with JCSV led to SV40-like CPE, a characteristic that is not shared by either wild-type Mad-1 or Mad-1SVEΔ. Similar to JCV, however, the hybrid was capable of hemagglutinating red blood cells. When SVG-A, CV-1, and COS-7 cells were challenged with JCSV only, SVG-A cells were efficiently infected. In contrast, each of these cell types expressed viral antigens following transfection with viral DNA. Our results do not differ significantly from a recent report demonstrating infection of a wide variety of cell types by JCV (Suzuki et al., 2001). This report used a highly sensitive PCR-based approach to demonstrate that input viral DNA could be amplified from nuclear fractions following incubation with JCV. As the percentages of infected cells were not documented in this report, it is likely that the positive PCR results derive from a minority of cells that did become infected.

FIG. 3. Biological characterization of JCSV. SVG-A cells were infected with either JCSV (A), the Mad-1 SVEΔ strain of JCV (B), or with SV40 (C). The cultures were monitored weekly for signs of cytopathic effect (CPE). SVG-A cells infected with either JCSV or SV40 developed extensive CPE with SV40 similar to vacuolization of the cytoplasm within 5 days postinfection (A and C, arrows). In contrast, SVG-A cells infected with JCV (Mad-1 SVEΔ) developed CPE with a necrotic phenotype by 2 weeks postinfection, which is characteristic of JCV infection (B). Hemagglutination assay. Twofold serial dilutions of JCSV, JCV (M1-SVEΔ), or SV40 in PBS were made in 96-well dishes and a 0.5% suspension of human type O red blood cells was added. Following incubation at 4°C for 2 h, hemagglutination activity was scored. JCSV had a hemagglutination titer of 128 HAU/50 μl and JCV (M1-SVEΔ) had a hemagglutination titer of >4096 HAU/50 μl. SV40 does not hemagglutinate. Control = no virus added.
We also observe low levels of infectivity in both CV1 and COS-7 cells (typically 1–3%). This low level of infectivity could be due to either low level expression of JCV receptors on CV-1 and COS-7 cells or entry by an alternative less efficient mechanism. We are currently exploring these possibilities. This new hybrid virus will be an invaluable tool for dissecting the role that receptors and other early events play in determining the tropism of this important human polyomavirus.

MATERIALS AND METHODS

Cells, viruses, and antibodies

SVG-A (SV40 transformed human fetal glial cell line), CV-1 (African green monkey kidney cell line), and COS-7 (SV40 transformed CV1) cells were propagated in Eagle’s minimum essential medium (EMEM, Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Mediatech) and 1% penicillin/streptomycin (P/S, HyClone) (complete media). Cells were maintained as a monolayer at 37°C in a humidified atmosphere of 5% CO₂.

The Mad-1 SVEΔ (M1-SVEΔ) strain of JCV and the wild-type 777 strain of SV40 were used in these experiments. M1-SVEΔ is a hybrid strain of JCV described previously that contains portions of the SV40 regulatory region (Vacante et al., 1989). The remaining genome is contributed completely by the Mad-1 strain of JCV. Viral DNA from both viruses were obtained by lysing CsCl₂-purified virus in 1% SDS followed by phenol/chloroform extraction and ethanol precipitation. The numbering systems used for the JCV and SV40 genomes are those established by Frisque and Fiers, respectively (Fiers et al., 1984).

For immunodetection of V antigen, PAB597, a mouse monoclonal antibody raised against SV40 V-antigen that cross-reacts with JCV, was used. A mouse monoclonal antibody against SV40 T antigen (Calbiochem) was used to detect SV40 T antigen expression in CV-1 cells. T antigen expression was not assessed in SVG-A or COS-7 cells because these cells constitutively express SV40 T antigen as a result of SV40 transformation. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (GaM-FITC) was used as a secondary antibody to detect positive expression of V or T antigens.

Construction of JCV-SV40 chimeric genome

To construct the JCV-SV40 hybrid genome, the late region of JCV was cloned by PCR and used to replace the SV40 late region, as shown in Fig. 1. Briefly, the late region of JCV was PCR amplified using primers JC275-Kpn and JC2533-Bam/Sal. JC275-Kpn contains an engineered KpnI site at the 5’ end placed 2 bp upstream of the start of Ag. JC2533-Bam/Sal contains adjacent engineered BamHI and SalI sites at the 3’ end directly following the stop codon of VP1. The template for PCR was M1-SVEΔ viral DNA. PCR was performed with 0.2 μg template, 6 mM MgSO₄, 1.5 mM dNTPs, 1 μg of each primer, and 125 U Platinum Pfx DNA polymerase (Gibco-BRL), using the following protocol: 94°C 2 min, then 95°C 15 s, 50°C 30 s, 72°C 3 min for 30 cycles, followed by a final 72°C extension for 7 min. The PCR fragment was then A-tailed and subcloned into the T-tailed cloning vector pGEM-T (Promega). DNA was isolated from potential positive clones using the QIAprep Spin Miniprep Kit (Qiagen) and analyzed for insert by restriction digest using KpnI and SalI. Inserts from positive clones were then completely sequenced in both directions to ensure that no mutations had been introduced by the polymerase. In one clone, pGEMJC(Vp)-2.5, no errors were found except for loss of the SalI site in the insert. This error could be compensated for by using the SalI site in the pGEM-T vector. pGEMJC(Vp)-2.5 was used to construct the chimeric genome.

DNA transfection

All transfections were done by calcium phosphate-mediated transfection. Cells were seeded so that they reached a density of approximately 70% confluence prior to transfection. For a 75-cm² flask of cells, viral DNA was added to 2.0 ml HEPES buffer followed by the addition of 125 μl of 2 M CaCl₂. For six-well dishes, 200 μl HEPES buffer and 12.5 μl 2 M CaCl₂ were used. The mixture was then added directly to cells washed with HEPES buffer and incubated at room temperature for 20 min with occasional rocking. Complete media were then added to the cells and incubated at 37°C in a 5% CO₂ humidified atmosphere for 4 h. Cells were then rinsed two times with complete media, allowed to incubate at 37°C, 5% CO₂ in complete media, and monitored for CPE.

Virus infection

Virus infections were carried out under Biosafety Level 2 safety precautions. Cells were seeded so that they reached a density of approximately 70% confluence prior to infection. Virus was diluted appropriately in EMEM supplemented with 2% FBS/1% P/S in a volume sufficient to cover the cells. Virus was allowed to adhere to cells for 1 h at 37°C, 5% CO₂ with occasional rocking. Complete media were then added and the infected cells were incubated at 37°C, 5% CO₂ in a humidified incubator.

Virus stock preparation

Twenty 175-cm² tissue culture flasks were seeded with SVG-A cells at a density of approximately 50% confluence prior to infection. Virus inoculum was prepared by diluting the virus 1:60 in EMEM/2% FBS/1% P/S in a volume of 5 ml per flask. Media were aspirated from the cells followed by addition of virus inoculum directly to the cells for 1 h at 37°C, 5% CO₂ with occasional rocking.
Complete media (25 ml) were then added to the culture and incubated at 37°C, 5% CO₂ in a humidified atmosphere. Media were replaced once a week by aspirating old media and adding 25 ml of fresh complete media. When significant CPE was observed, cells were harvested by scraping cells off the flask and pelleting at

FIG. 4. Infection of SVG-A, CV-1, and COS-7 cells with SV40, JCSV, and JCV (Mad-1 SVEΔ). SVG-A, CV-1, and COS-7 cells were infected with 25 HAU of JCSV, JCV (Mad-1 SVEΔ), or with 1.2 × 10⁶ PFU of SV40. Cells were scored at 3 days postinfection for V-antigen expression (SVG-A and COS-7 cells) or T antigen expression (CV-1 cells) by indirect immunofluorescent assay. The percentage of infected cells is indicated. JCSV and JCV (Mad-1 SVEΔ) infected SVG-A cells with similar efficiency. JCSV also infected CV-1 and COS-7 cells but at much reduced efficiency when compared to infection of SVG-A cells (32% vs 1% for CV1 and 3% for COS-7). JCV (Mad-1 SVEΔ) did not infect CV-1 cells but did infect COS-7 cells, again with reduced efficiency (32% vs 2%). SV40 infected all three cell types efficiently. Magnification, ×100.

FIG. 5. Transfection of CV-1 and COS-7 cells with viral DNA. CV-1 and COS-7 cells were transfected with 1.5 μg of SV40, JCSV, or JCV (Mad-1 SVEΔ) viral DNA and scored for T antigen (CV1) or V antigen (COS-7) expression at 3 days posttransfection. The percentage of T or V antigen expressing cells is indicated. Magnification, ×100.
3000 g for 20 min at 4°C. The pellet was then suspended in 1/10 the volume of supernatant. The remaining supernatant was discarded since virus remains highly cell-associated. The cells were subjected to three freeze-thaw cycles (−80°C to −37°C) with vortexing. Deoxycholate was then added to a final concentration of 0.25% (v/v) and incubated at 37°C for 30 min to release virus. Cellular debris was pelleted by centrifugation at 12,000 g for 30 min at 4°C. The virus-containing supernatant was saved, aliquoted, and stored at −80°C.

Virus was concentrated by centrifugation through a 20% sucrose solution in Buffer A (10 mM Tris–HCl pH 8.0, 50 mM NaCl, 0.01 mM CaCl₂, 1% Triton X-100) at 35,000 rpm, 15°C for 3 h. The pellet was then resuspended in Buffer A and centrifuged through a premade CsCl step gradient (1.35, 1.32, 1.29, 1.26, 1.23 g/ml) at 35,000 rpm, 15°C overnight. The virus band was removed and dialyzed against Buffer A without Triton X-100 overnight at 4°C.

Viral DNA was isolated by lysing the CsCl-purified virus in 1% SDS at 50°C for 1 h followed by 2× phenol extraction, 2× phenol/chloroform extraction, 1× chloroform extraction, and ethanol precipitation.

Hemagglutination assay

Human type O red blood cells (RBC) were diluted to 0.5% in Alsever’s solution (0.1 M d-glucose, 0.027 M sodium citrate, 0.07 M NaCl, pH 6.5) and used in the HA reaction as previously described (Liu and Atwood, 2001). Briefly, twofold dilutions of each virus to be titrated were made in 96-well U-bottomed dishes. A 50 μl of suspension of RBCs was then added to each well and the plates were incubated at 4°C for 2–4 h. The last dilution to fully hemagglutinate RBCs was used to determine the titer of the virus.

Indirect immunofluorescence assay

Cells were seeded onto glass coverslips in six-well tissue culture dishes so that they reached a density of approximately 70% confluence. Cells were then infected as described, using an appropriate dilution of virus in a volume of 100 μl. Three days postinfection, the media were aspirated and cells were washed twice in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.2; PBS). Cells were then permeabilized and fixed to coverslips in ice-cold acetone for 10 min and allowed to air dry. Cells were probed with primary antibody (PABS97, undiluted, or mouse αSV40-T, diluted 1:50 in PBS) for 30 min at 37°C, washed two times with PBS, and probed with the secondary antibody goat α mouse-FITC (diluted 1:50 in PBS) for an additional 30 min at 37°C. Coverslips were then washed two times with PBS, counterstained with Evans blue, washed two times with PBS, and mounted onto slides with 90% glycerol in PBS. Stained cells were visualized under a Zeiss epifluorescence microscope.

Sequencing and primers

All sequencing was performed by the Brown University Sequencing Core Facility. Sequencing reactions contained 5 μg of DNA and 3.2 pmol primer in a volume of 10 μl of water. Primers (Gibco-BRL) were designed based on the published sequences of the Mad-1 strain of JCV, strain 777 of SV40, pGEM-T vector, and pBR322 vector. Numbering in primer names indicates the 5′ position of primer. For and Rev (forward and reverse) indicate the direction of sequencing. The primers used in these experiments are as follows (5′ → 3′):

JCV275-Kpn: GGTACCCCATGTTTCTTGCCAGCTGTGTCGTAAA
JCV2533-Bam/Sal: GTCGACGGATCCTTACAGCATTTTGTCGTC
JCV265 For: CACCCAGCGTGCATGTTTCTTGCC
JCV776 For: GTTCAACGACTAAGGTG
JCV1369 For: GCACACGAGGTTGCCAATAAG
JCV1982 For: CCAAGAATGTCACAGTGCAATC
JCV986 Rev: CCCCATGTCTAGGATCTAATAG
JCV1568 Rev: CCCAGTTTTAACCTTCTAGAAC
JCV2180 Rev: GCAACTCGTTGTCAGT
SV164 For: GGGACCTTTCCACACCTGTTTGTGCTGAC
pGEM2956 For: ACATTGTAACACGCGGCCAGTG
pGEM140 Rev: TAGGTGACACTATAGAATACTCAAG
pBR762 Rev: CCTACGAGTTGCATGATAAAGAAGAC

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