Vaccinia Virus-Free Recovery of Vesicular Stomatitis Virus

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Abstract

The advent of reverse-genetics represents a powerful new approach to elucidate aspects of negative-sense RNA virus replication. The reverse-genetics system established previously for vesicular stomatitis virus (VSV) required four plasmids encoding the nucleoprotein (N), phosphoprotein (P), polymerase (L), and the full-length, anti-genomic RNA. Transcription to yield the antigenomic RNA as well as the N, P, and L, mRNAs was initiated by bacteriophage T7 polymerase expressed from a recombinant Vaccinia virus. In this report, we describe the successful recovery of infectious VSV in the absence of Vaccinia virus. The N, P, and L genes of VSV were inserted downstream of both the T7 promoter and an internal ribosomal entry site (IRES element). T7 polymerase was expressed constitutively from BSR-T7/5 cells. RT-PCR was used to confirm that the recovered VSV was derived from transfected DNA. Virion protein profile, CPE in tissue culture, and virus titer of the recombinant virus were indistinguishable from those of parental VSV. Thus, the need for Vaccinia virus is eliminated with this system, making it an attractive, alternative approach for the recovery of infectious VSV from DNA.

Because of the negative-sense nature of their RNA genomes, the ability to utilize standard molecular biological techniques to study the replicative cycles of negative-sense RNA viruses was once impossible. However, major breakthroughs in reverse-genetics technology using influenza and rabies viruses allowed investigators for the first time to genetically manipulate the genomes of these important human and animal pathogens (Luytjes et al., 1989; Schnell et al., 1994). Reverse-genetics systems now exist for numerous nonsegmented and segmented, negative-sense RNA viruses (Schnell et al., 1994; Garcin et al., 1995; Lawson et al., 1995; Radecke et al., 1995; Whelan et al., 1995; Bridgen and Elliott, 1996; Palese et al., 1996; He et al., 1997; Hoffman and Banerjee, 1997; Jin et al., 1998; Leyrer et al., 1998; Buchholz et al., 1999; Fodor et al., 1999; Neumann et al., 1999; Romer-Oberdorfer et al., 1999). The approach most often used for the recovery of recombinant virus from DNA involves the use of bacteriophage T7 polymerase expressed from a recombinant Vaccinia virus (VvT7; Fuerst et al., 1986) [for review see Pekosz et al., 1999]. The T7 polymerase is required to initiate transcription of the full-length viral genome (or antigenome) and of the viral mRNAs required for subsequent replication and amplification of the genomic RNA. For example, the four plasmids required for the recovery of infectious VSV include: T7VSV-FL (encoding the full-length anti-genome of VSV), T7VSV-N (encoding the nucleoprotein), T7VSV-P (encoding the phosphoprotein), and T7VSV-L (encoding the RNA-dependent RNA polymerase) (Lawson et al., 1995; Whelan et al., 1995). While the use of VvT7 has facilitated the consistent recovery of VSV, as well as other negative-sense RNA viruses, the need for additional biosafety precautions when using VvT7, and the need to eventually inhibit and completely remove the contaminating VvT7 during the recovery process represent inconvenient aspects of this approach. To alleviate or eliminate these inconveniences, investigators have used modified Vaccinia virus Ankara (MVA), which is growth-restricted in specific cell lines (He et al., 1997; Leyrer et al., 1998). Alternatively, the use of a cell line to constitutively express T7 polymerase represents another method to avoid the use of VvT7. Indeed, the use of a cell line expressing T7 polymerase was first used successfully for the recovery of infectious measles virus from DNA (Radecke et al., 1995). More recently, a BHK-21 cell clone, BSR-T7/5, was generated and shown to express bacteriophage T7 polymerase after numerous passages in tissue culture. BSR-T7/5 cells have been used for the successful recovery of Newcastle disease, bovine respiratory syncytial, and rabies viruses (Buchholz et al., 1999; Finke and Conzelmann, 1999; Romer-Oberdorfer et al., 1999). To overcome the loss of 5' mRNA capping that was provided by VvT7 enzymes, the viral N, P, and L genes must be inserted downstream of an internal ribosomal entry site (IRES element) to allow for the N, P, and L mRNAs to be efficiently translated in transfected cells.

In this report we describe the successful use of BSR-T7/5 cells to establish a Vaccinia virus-free recovery system for VSV. Recombinant VSV was recovered from BSR-T7/5 cells (kindly provided by Drs. Conzelmann and Finke) transfected with pT7VSV-FL (kindly provided by Dr. J. Rose), pTIT-N, pTIT-P, and pTIT-L. RT-PCR and restriction endonuclease digestion were used to demonstrate that the recovered virus was derived from the transfected DNA plasmids. In addition, the recovered virus was shown to possess properties indistinguishable from those of parental
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Thus, this Vaccinia virus-free rescue system represents a safe, alternative approach for recovery of VSV from DNA.

To establish a Vaccinia virus-free transfection system for VSV, the N, P, and L genes were first subcloned downstream of the T7 promoter and an IRES element to allow for cap-independent translation of VSV proteins in T7 RNA polymerase-expressing cells. The previously described pTIT vector, which contains the encephalomyocarditis virus (EMCV) IRES element was used (kindly provided by Drs. Conzelmann and Finke; Finke and Conzelmann, 1999). Briefly, pTIT-N was constructed by inserting a 200 basepair $\text{Afl} III$ fragment (PCR-amplified with the primers 5′-CCCCTCATGAAGTGCCTTTTGTA CTTAG-3′ and 5′-AACAGCTATGACCATGATTACG-3′ from BS-N; Lawson, 1995) into pTIT and subsequently integrating a $\text{Bgl} II/\text{Xma} I$ fragment from pT7T-N. Plasmid pTIT-P was constructed by PCR-amplifying the coding region of VSV P from BS-P (Lawson, 1995) using the primers 5′-GGGGCCATGGATAATCTCACAAAAGTCC-3′ and 5′-AACAGCTATGACCATGATTACG-3′ and inserting this $\text{Nco} I/\text{Pst} I$-digested fragment into pTIT. Plasmid pTIT-L was constructed by inserting a $\text{Nco} I$-digested fragment (PCR-amplified from BS-L using primers 5′-GGGGCCATGGAAGTCCACGATTTTGAGA-3′, and 5′-GTCTTGTTGAGAACAGGTTG-3′; Lawson, 1995) into pTIT. Subsequent insertion of a $\text{Hpa} I/\text{Xma} I$-digested fragment from PT7T-L resulted in pTIT-L.

The newly constructed pTIT-N, pTIT-P, and pTIT-L plasmids were employed in transfection experiments along with pT7VSV-FL to recover infectious VSV. Briefly, two 100mm dishes of BSR-T7/5 cells at approximately 75% confluency were transfected with 10.0µg VSV-FL, 5.0µg of pTIT-N, 3.0µg of pTIT-P, and 1.0µg of pTIT-L using a mammalian calcium phosphate transfection kit (Stratagene). At 24 hours posttransfection, the cells were trypsinized, pooled, pelleted for 10 minutes at 3000 rpm, and split into six 6-well plates. After 48 hours in culture the cell monolayers were confluent. The cells were again trypsinized, pooled, pelleted as before, and split into three T175cm flasks. After 48 hours in culture, CPE was observed in one of the three T175 cm flasks. Virus-containing supernatant was then harvested, clarified, and stored at -80°C.

Titration of the recovered virus was performed using BSR-T7/5 cells. Cells were grown in 35mm dishes to approximately 90% confluency, and serial dilutions of the recovered virus were used to infect the cells for 1 hour at 37°C. The inoculum was then removed, and 3.0 ml/dish of DMEM containing 1.0% methylcellulose (Sigma) was used to overlay the infected cell monolayers. The recombinant virus produced visible plaques overnight. The methylcellulose overlay was removed, the cells were washed with 1X phosphate buffered saline (PBS), and the monolayer was stained with crystal violet (Figure 2A). The recombinant virus titer was determined to be $8 \times 10^8$ PFU/ml. The ability to form plaques rapidly and to achieve titers of approximately $10^9$ PFU/ml represent characteristics of VSV.

The recombinant virus-induced CPE was virtually indistinguishable from that induced by parental VSV (Figure 2B). BHK-21 cells infected with either parental VSV or recombinant virus were observed by light microscopy (Figure 2B). CPE typical of VSV including rounded cells
derived from pT7VSV-FL DNA, RT-PCR and restriction endonuclease digestion with \(\text{Nhe} I\) were performed. The genome encoded by pT7VSV-FL was engineered to contain a unique \(\text{Nhe} I\) site that is not present in the genome of parental VSV (Lawson et al., 1995). BHK-21 cells grown in 35 mm plates were infected with either parental VSV, or recombinant virus at an MOI of 1.0. Total cellular RNA was isolated at approximately 8 hours postinfection using either the Qiagen RNeasy mini kit, or the Trizol reagent (Gibco BRL) and recommended protocols. First strand DNA synthesis and subsequent amplification were performed using VSV-specific primers (CATTCAAGACGCTGCTTCGCAACTTCC forward) and (CATGATGTTACATCTCAAGA reverse) and either an enzyme mixture (Omniscript RT, Sensiscript RT, and HotStarTaq DNA polymerase), or AMV RT alone. A PCR product of the expected size (622 bp) was obtained using the VSV-specific forward and reverse primers and total RNA isolated from BHK-21 cells infected with recombinant virus (Fig 3A, lanes 3 and 4). In contrast, the 622 bp PCR product was not observed in the absence of reverse transcriptase (Figure 3A, lane 2). The resultant PCR product was gel-purified, digested with \(\text{Nhe} I\) restriction endonuclease, and analyzed by agarose gel electrophoresis (Figure 3B). Following digestion with \(\text{Nhe} I\), the 622 bp PCR product was cleaved into two smaller fragments indicating that the recovered virus was indeed derived from the input pT7VSV-FL DNA, RT-PCR and restriction endonuclease digestion with \(\text{Nhe} I\) were performed. The genome encoded by pT7VSV-FL was engineered to contain a unique \(\text{Nhe} I\) site that is not present in the genome of parental VSV (Lawson et al., 1995). BHK-21 cells grown in 35 mm plates were infected with either parental VSV, or recombinant virus at an MOI of 1.0. Total cellular RNA was isolated at approximately 8 hours postinfection using either the Qiagen RNeasy mini kit, or the Trizol reagent (Gibco BRL) and recommended protocols. First strand DNA synthesis and subsequent amplification were performed using VSV-specific primers (CATTCAAGACGCTGCTTCGCAACTTCC forward) and (CATGATGTTACATCTCAAGA reverse) and either an enzyme mixture (Omniscript RT, Sensiscript RT, and HotStarTaq DNA polymerase), or AMV RT alone. A PCR product of the expected size (622 bp) was obtained using the VSV-specific forward and reverse primers and total RNA isolated from BHK-21 cells infected with recombinant virus (Fig 3A, lanes 3 and 4). In contrast, the 622 bp PCR product was not observed in the absence of reverse transcriptase (Figure 3A, lane 2). The resultant PCR product was gel-purified, digested with \(\text{Nhe} I\) restriction endonuclease, and analyzed by agarose gel electrophoresis (Figure 3B). Following digestion with \(\text{Nhe} I\), the 622 bp PCR product was cleaved into two smaller fragments indicating that the recovered virus was indeed derived from the input pT7VSV-FL DNA (Figure 3B, lane 2).
As a final analysis, the virion proteins of the recombinant virus were compared to those of parental VSV by staining with Coomassie brilliant blue and by Western blot analysis. To ensure that the virion proteins of the recombinant virus were identical in size to those of parental VSV, both recombinant and parental virions were harvested and purified from infected WI-38 cells. Infected cell supernatants were clarified by centrifugation at 1,200 RPM for 10 minutes, and the virions were then pelleted through a 20% sucrose cushion. Virion pellets were suspended in 1X TE buffer, and aliquots of parental and recombinant virions were analyzed by SDS-PAGE (Figure 4). As expected, the virion protein profile for both parental and recombinant VSV were identical as determined by Coomassie blue staining of the five virion proteins (Figure 4A). In addition to viewing the overall protein profile by Coomassie blue staining, the virion proteins were transferred to nitrocellulose and reacted with a MAb (1:5,000) against the VSV (Indiana) M protein. The MAb reacted with M protein present in both parental VSV-infected cell extract (lane 1) and in rVSV-infected cell extract (lane 2).

In this report, we describe the establishment of a Vaccinia virus-free transfection system for the recovery of infectious VSV entirely from plasmid DNA. The essential components of the Vaccinia virus-free system include: the pT7VSV-FL plasmid encoding the full-length antigenome of VSV (Lawson et al., 1995), BSR-T7/T5 cells which constitutively express T7 polymerase (Buchholz et al., 1999; Finke and Conzelmann, 1999; Romer-Oberdorfer et al., 1999), pTIT-N, pTIT-P, and pTIT-L plasmids. The resultant recombinant virus was shown to originate from the input plasmid DNA and to possess characteristics indistinguishable from those of parental VSV. Although in this report we describe only the recovery of wild type VSV, this system has also been used recently to recover an M gene mutant of VSV (Harty et al., unpublished data).

Acknowledgements

We wish to thank Drs. J. Rose, K-K. Conzelmann, S. Finke, and Z. Ye for generously providing us with reagents. We also thank J. Paragas for his astute comments and suggestions.

References


