

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 VSV 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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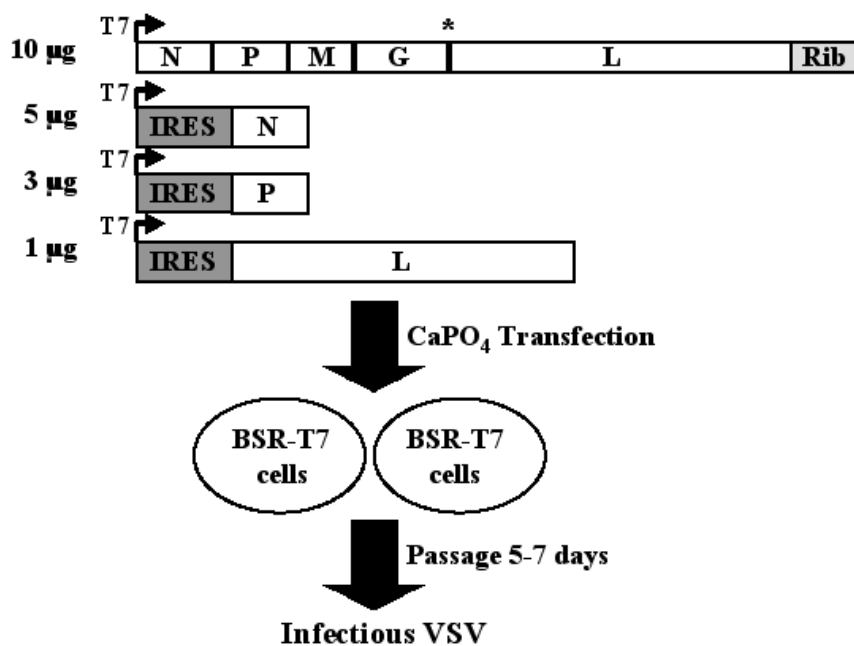


Figure 1. Diagram of VSV reverse-genetics protocol. The indicated amounts of the four plasmids (pT7VSV-FL, pTIT-N, pTIT-P, and pTIT-L) were transfected using CaPO₄ into BSR-T7/5 cells constitutively expressing T7 polymerase. Infectious VSV was recovered after passaging the cells for 5-7 days. Abbreviations: N - nucleoprotein, P - phosphoprotein, M - matrix protein, G - glycoprotein, L - polymerase, IRES - internal ribosomal entry site, and Rib - Hepatitis delta virus ribozyme. The asterisk (*) indicates the position of the unique *NheI* restriction site. Diagram not drawn to scale.

VSV. 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 *AflIII* fragment (PCR-amplified with the primers 5'-CCCCTCATGAAGTGCCTTTTGTACTTAG-3' and 5'-AACAGCTATGACCATGATTACG-3' from BS-N; Lawson, 1995) into pTIT and subsequently integrating a *BglII/XmaI* 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 *NcoI/PstI*-digested fragment into pTIT. Plasmid pTIT-L was constructed by inserting a *NcoI*-digested fragment (PCR-amplified from BS-L using primers 5'-GGGGCCATGGAAGTCCACGATTTTGTAGA-3', and 5'-GTCTTGC TCTGAGAACAGGTTG-3'; Lawson, 1995) into pTIT. Subsequent insertion of a *HpaI/XmaI*-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 8×10^8 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

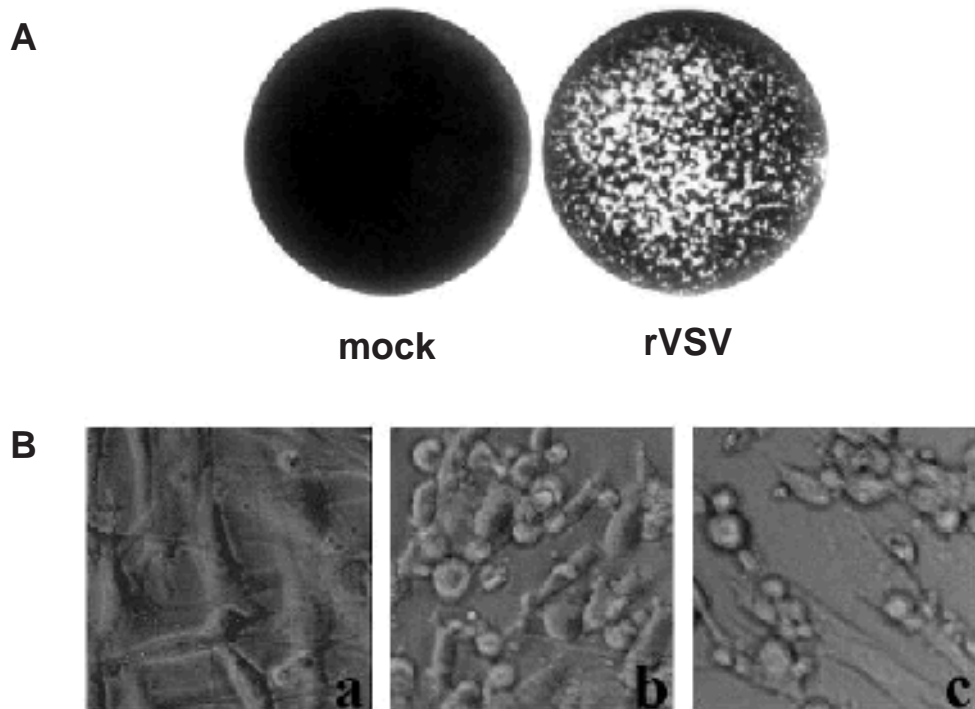


Figure 2. CPE induced by parental and recombinant VSV. A) Plaque assay of mock-infected (mock), or recombinant VSV (rVSV) infected BSR-T7/5 cells. The rVSV dish shown represents a 10^{-5} dilution of virus. B) Light microscopic analysis of CPE from: a) mock-infected, b) parental VSV-infected, and c) rVSV-infected BHK-21 cells.

were readily observed for both virus preparations at approximately 8 hours postinfection (Figure 2B). As expected, mock-infected cells exhibited no CPE (Figure 2B). To formally prove that the recovered virus was indeed

derived from pT7VSV-FL DNA, RT-PCR and restriction endonuclease digestion with *NheI* were performed. The genome encoded by pT7VSV-FL was engineered to contain a unique *NheI* 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 (CATTCAAGACGC TGCTTCGCAACTTCC forward) and (CATGATGTTAA CATCTCAAGA 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 *NheI* restriction endonuclease, and analyzed by agarose gel electrophoresis (Figure 3B). Following digestion with *NheI*, the 622 bp PCR product was cleaved into two fragments indicating that the recovered virus was indeed derived from the input pT7VSV-FL DNA (Figure 3B, lane 2).

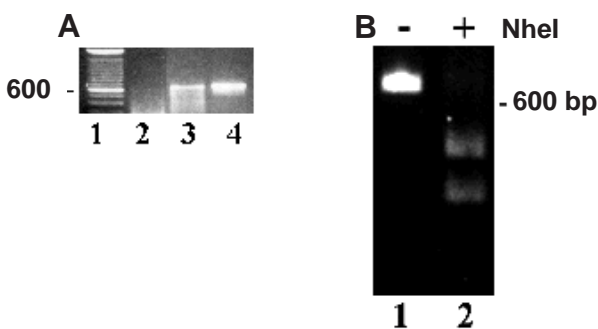


Figure 3. RT-PCR and restriction endonuclease analysis of rVSV. A) Total RNA from rVSV-infected BHK-21 cells was used as template for RT-PCR using VSV specific primers (as described in the text). The expected PCR product of 622 bp was obtained (lanes 3 and 4). Lanes 3 and 4 differ only in the amount of magnesium sulfate added during PCR amplification. The 622 bp PCR product was not detected in the absence of reverse transcriptase (negative control, lane 2). Molecular weight standards are shown in lane 1. B) A portion of the 622 bp PCR product was incubated in the absence (lane 1), or presence (lane 2) of the *NheI* restriction endonuclease. Cleavage of the PCR product into two smaller fragments was readily observed in the presence of *NheI* (lane 2).

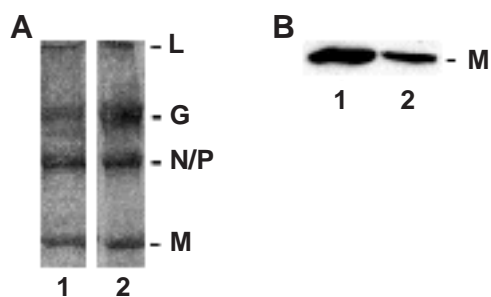


Figure 4. Parental VSV and rVSV virion proteins. A) Parental VSV and rVSV virions were purified from infected BHK-21 cells, and the virion proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue. Identical virion protein profiles were observed for both parental VSV (lane 1) and rVSV (lane 2). B) Western blot analysis of parental VSV- and rVSV-infected cells extracts. Equivalent amounts of extracts from parental and rVSV-infected BHK-21 cells were blotted onto 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).

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 filters and reacted with an anti-VSV M monoclonal antibody (kindly provided by Dr. Z. Ye; Figure 4B). The anti-VSV M monoclonal antibody reacted with the 30 kDa M protein present in both virion preparations (Figure 4B).

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-T7/5 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).

The elimination of VvT7 makes this system an attractive, alternative approach for the recovery of

recombinant VSV. While this system does represent an alternative to the VvT7 system, an extensive comparison of both systems for their efficiency of virus rescue has yet to be performed. It is likely that further manipulation of the recovery conditions described in this report may serve to optimize the efficiency of VSV recovery. VSV remains an important model system for negative-sense RNA viruses, and this plasmid-based rescue system should facilitate further investigations into the molecular aspects of VSV replication. Indeed, the ability to recover infectious VSV from DNA has provided investigators with a powerful tool of unlimited potential for deciphering many aspects of the VSV replication cycle (Lawson *et al.*, 1995; Whelan *et al.*, 1995). For example, reverse-genetics has been used recently to identify genomic RNA sequences that are critical for VSV gene regulation (Wertz *et al.*, 1994; Whelan and Wertz, 1999a; Whelan and Wertz, 1999b). Moreover, in an elegant series of experiments, reverse-genetics was used to demonstrate that the gene order of VSV was important not only for gene expression, but also for pathogenesis of VSV in an animal host (Wertz *et al.*, 1998).

The potential use of VSV as a vector for vaccine development represents another important use of the VSV reverse-genetics system. Foreign genes have been inserted into the genome of VSV, and their subsequent expression has been monitored both *in vitro* and *in vivo* (Schnell *et al.*, 1996; Johnson *et al.*, 1997; Kretzschmar *et al.*, 1997; Schnell *et al.*, 1997). The use of both VSV and rabies virus as potential vectors was illustrated elegantly in a series of experiments involving CD4, the cellular receptor for HIV-1 (Mebatsion and Conzelmann, 1996; Johnson *et al.*, 1997; Mebatsion *et al.*, 1997; Schnell *et al.*, 1997).

We are currently utilizing the reverse-genetics system described in this report to dissect the role of the M protein of VSV in virus budding (Craven *et al.*, 1999; Harty *et al.*, 1999). This system provides us with a straightforward approach to generate and recover VSV with specifically engineered mutations. Similar VvT7-free transfection systems are likely to be developed for other negative-sense RNA viruses, as they alleviate the obstacles encountered when working with Vaccinia virus.

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