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A plasmid-based reverse genetics system for mammalian orthoreoviruses driven by a plasmid-encoded T7 RNA polymerase

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Abstract

Mammalian orthoreoviruses (reoviruses) have served as highly useful models for studies of virus replication and pathogenesis. The development of a plasmid-based reverse genetics system represented a major breakthrough in reovirus research. The current reverse genetics systems for reoviruses rely on the expression of T7 RNA polymerase within cells transfected with reovirus gene-segment cDNA plasmids. In these systems, the T7 RNA polymerase is provided by using a recombinant vaccinia virus expressing T7 RNA polymerase or a cell line constitutively expressing T7 RNA polymerase. Here, we describe an alternative plasmid-based rescue system driven by a plasmid-encoded T7 RNA polymerase, which could increase the flexibility of such reverse genetics systems. Although this approach requires transfection of an additional plasmid, virus recovery was achieved when A549, BHK-21, or L929 cells were co-transfected with a reovirus 10-plasmid set together with a plasmid encoding T7 RNA polymerase. Theoretically, this system offers the possibility to generate reoviruses in any cell line, including those amenable to propagation of viral vectors for clinical use. Thus, this approach will increase the flexibility of reverse genetics for basic studies of reovirus biology and foster development of reoviruses for clinical applications.

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Keywords

Reovirus; Reverse genetics; T7 RNA polymerase; Eukaryotic expression plasmid

Mammalian orthoreoviruses (reoviruses) are prototype members of the family *Reoviridae*. These viruses encapsidate genomes of 10 to 12 segments of double-stranded RNA (dsRNA) in multiple concentric protein shells (Dermody et al., 2013). Members of this family include rotaviruses, the most important pathogens of acute gastroenteritis in young children, and orbiviruses, economically important pathogens of cattle and sheep. Therefore, reoviruses are highly important experimental models for studies of the replication and pathogenesis of multi-segmented dsRNA viruses.

Remarkable progress has been made in an understanding of multiple aspects of virus biology and pathogenesis through application of reverse genetics technology. Such reverse genetics systems allow generation of infectious viruses completely from cloned cDNAs. Indeed, the development of a plasmid-based reverse genetics system is one of the most important technological advances for studies of any virus. Although plasmid-based systems exist for nearly all major groups of DNA and RNA viruses, development of a technology to produce infectious *Reoviridae* viruses from plasmid cDNAs is more challenging, owing in part to the technical complexity of manipulating multi-segmented genomes (Taniguchi and Komoto, 2012). Reoviruses, which contain a 10-segmented dsRNA genome, were the first members of the *Reoviridae* for which a plasmid-based reverse genetics method was developed. The rescue of infectious reovirus from cloned cDNAs was initially established for serotype 3 prototype strain, type 3 Dearing (T3D) (Kobayashi et al., 2007). Subsequently, a similar approach was used to recover serotype 1 prototype strain, type 1 Lang (T1L) (Kobayashi et al., 2010). As with other DNA and RNA viruses, the development of reverse genetics has revolutionized the study of reoviruses by providing a powerful tool for investigating virus replication and disease (Dermody et al., 2013).

The initial reovirus reverse genetics system consists of 10 plasmids, each containing a full-length cDNA copy of a single reovirus gene segment flanked by bacteriophage T7 RNA polymerase promoter and hepatitis delta virus (HDV) ribozyme sequences. These plasmids are presumed to generate 10 transcripts corresponding to native reovirus positive-sense RNAs that serve as templates for translation and dsRNA replication. The reovirus replication cycle can be launched by transfection of the 10 plasmids encoding the genome into cells expressing T7 RNA polymerase. Therefore, reovirus reverse genetics systems rely on the expression of T7 RNA polymerase within cells transfected with reovirus plasmids.

The T7 RNA polymerase is provided by one of two methods (Boehme et al., 2011): infection of murine L929 fibroblast cells with a recombinant vaccinia virus expressing T7 RNA polymerase (rDIs-T7pol; Ishii et al., 2002) or use of baby hamster kidney (BHK) cells constitutively expressing T7 RNA polymerase under control of a cytomegalovirus (CMV) promoter (BHK-T7 cells; Buchholz et al., 1999). Although both rDIs-T7pol- and BHK-T7-based reverse genetics systems are capable of generating reoviruses from plasmids, these methods might be limited by the toxicity of rDIs-T7pol in some cell types or a requirement to use cells other than BHK-T7 cells for plasmid-based rescue.

In our efforts to increase the flexibility of reovirus reverse genetics, we developed an alternative plasmid-based reverse genetics system driven by a plasmid-encoded T7 RNA polymerase, thus eliminating the requirement for either rDIs-T7pol or BHK-T7 cells. The main goal of our study was to develop a new reverse genetics system for the recovery of genetically modified reovirus from any cell line and not necessarily for improvement in the efficiency of virus recovery. Once a modified virus can be recovered, even at low efficiency, it can be propagated to high titers in other appropriate types of cells.

A strategy involving a T7 RNA polymerase-expressing plasmid as a source of T7 RNA polymerase has been successfully employed in rescue systems for several RNA viruses (Neumann et al., 2002; Herfst et al., 2004; Sanchez and de la Torre, 2006; Witko et al., 2006; Kaur et al., 2008). Since this method requires an additional plasmid for transfection, recovery of modified virus may be less efficient in experiments using cell types that have low transfection efficiencies. However, we thought that a T7 RNA polymerase-expressing plasmid would support reovirus rescue if an efficient expression vector was used and optimal conditions were defined for introduction of this plasmid into cells.

A eukaryotic expression plasmid has been constructed to express T7 RNA polymerase under control of a strong CMV early enhancer/chicken β -actin (CAG) promoter (pC-T7pol; Neumann et al., 2002). To determine whether use of pC-T7pol allows recovery of reovirus, monolayers of L929 cells (3×10^6 cells) seeded into 60-mm dishes (BD Falcon) were co-transfected with 10 plasmids encoding the genome of strain T3D together with pC-T7pol using 3.3 μ l of FuGENE HD transfection reagent (Promega) per μ g of plasmid DNA (Fig. 1). A total of 17.75 μ g T3D plasmid was used, as previously described (Boehme et al., 2011). To systematically determine the optimal quantity of pC-T7pol for co-transfection, various amounts of this plasmid (3, 4, 5, and 6 μ g) were combined with 17.75 μ g of the T3D plasmids and introduced into L929 cells in triplicate. For comparison, we also used the original reovirus reverse genetics system with rDIs-T7pol as a positive control (Kobayashi et al., 2007). After 5 days of incubation, the transfected L929 cells were lysed by freeze-thaw, and viral titers in cell lysates were determined by a plaque assay (Urasawa et al., 1982) using L929 cells (Fig. 2A). The pC-T7pol-based reverse genetics system successfully yielded recombinant strain (rs) T3D viruses, but the titers were low (~ 10 PFU/ml) (Fig. 2B), and the efficiency was not uniform (66, 100, 66, and 66% for 3, 4, 5, and 6 μ g of pC-T7pol, respectively). In contrast, the original rDIs-T7pol-based system was robust and yielded rsT3D viruses at titers of $\sim 10^3$ PFU/ml (data not shown), in line with previous observations (Kobayashi et al., 2007). Although the consistent virus recovery with this approach using 4 μ g of pC-T7pol was encouraging, the virus titers were lower than those with the rDIs-T7pol-based rescue system.

We thought it possible that the substantial difference in virus titers recovered using the pC-T7pol- and rDIs-T7pol-based rescue systems might be attributable to activation of the interferon (IFN) response in L929 cells leading to impaired virus replication, presumably caused by the transfection procedure involving a large quantity of plasmid DNA (Rautsi et al., 2007). In support of this idea, L929 cells are capable of producing IFNs (Takano-Maruyama et al., 2006). On the other hand, for the original rDIs-T7pol-based rescue system,

the IFN response in L929 cells might be blunted by the rDIs-T7pol infection, as vaccinia virus encodes several proteins that efficiently block IFN action (Seet et al., 2003).

To circumvent the potential involvement of IFN action in the pC-T7pol-based reovirus reverse genetics system, we used native BHK cells, as these cells do not express IFNs (Takano-Maruyama et al., 2006) (Fig. 1). Monolayers of BHK-21 cells (JCRB9020; Health Science Research Resources Bank) (2.5×10^6 cells) seeded into 60-mm dishes were co-transfected with 17.75 μ g of the T3D 10-plasmid set together with 4 μ g of pC-T7pol using 2.5 μ l of FuGENE HD transfection reagent per μ g of plasmid DNA in triplicate. For comparison, we also transfected the T3D 10-plasmid set into BHK-21 cells stably expressing T7 RNA polymerase (BHK/T7-9 cells), which were established from BHK-21 cells transfected with pC-T7pol (Ito et al., 2003). After 5 days of incubation, lysates of transfected BHK-21 cells were subjected to a plaque assay to detect infectious reovirus (Fig. 2A). Despite the fact that the transfection efficiency of BHK-21 and L929 cells is similar (60–70%), we found that co-transfection of the T3D 10-plasmid set and pC-T7pol into BHK-21 cells led to a substantial virus yield, with titers approaching 10^3 PFU/ml in every transfection (Fig. 2B). This efficiency of recovery is comparable to rescue methods using BHK/T7-9 cells (10^3 PFU/ml) (data not shown) or rDIs-T7pol (10^3 PFU/ml). We have consistently rescued rsT3D viruses at 10^3 PFU/ml in multiple independent attempts, demonstrating the efficiency of the pC-T7pol-based rescue system involving the BHK-21 cell line. Thus, although we have not biochemically characterized the IFN response of L929 cells co-transfected with the reovirus 10-plasmid set and pC-T7pol, efficient virus recovery was achieved by using cells lacking an IFN response in the pC-T7pol-based rescue system.

To confirm that the rsT3D viruses recovered following plasmid transfection contain the correct combination of gene segments, rescued viruses were propagated in L929 cells, and virion dsRNAs were extracted and analyzed by polyacrylamide gel electrophoresis (PAGE) (Komoto et al., 2006), followed by ethidium bromide staining to visualize viral gene segments. Profiles of viral dsRNAs from native T3D virus (VR-824; American Type Culture Collection) used as a reference (lane 1) and rsT3D viruses rescued with the pC-T7pol-based rescue system using L929 cells (lane 2) and BHK-21 cells (lane 3) are shown in Fig. 2C. The dsRNA migration patterns of both rsT3D viruses were identical to that of native T3D virus. Furthermore, both rsT3D viruses retained a specific mutation in their L1 genes as a rescue-specific gene marker (Kobayashi et al., 2007) (data not shown), indicating that the rescued reoviruses originated from plasmid DNAs.

To test whether plasmid-based delivery of T7 polymerase would allow recovery of reovirus from cells not previously used for reovirus reverse genetics, we determined whether reovirus could be rescued from plasmid-transfected A549 cells (human lung carcinoma cell line), in which T3D virus replicates less efficiently than in L929 cells (>10-fold-lower titer) (Berard et al., 2012). Although virus could not be directly rescued from this IFN-competent cell line following co-transfection with the reovirus 10-plasmid set and pC-T7pol, we were able to recover infectious virus when L929 cells were overlaid onto the transfected A549 cells 2 days after transfection. However, the titers were low (~ 10 PFU/ml) (Fig. 3). The successful rescue of rsT3D virus from co-cultured A549 cells showed that a cell line less susceptible to reovirus infection can be made amenable to plasmid-based recovery of reovirus when

incubated with L929 cells. These findings provide evidence that the pC-T7pol-based rescue system can be employed using cell types other than L929 and BHK-21 cells.

Results presented in this report establish an alternative reovirus reverse genetics system driven by a plasmid-based T7 RNA polymerase. Although this new system requires the transfection of an additional plasmid encoding T7 RNA polymerase (pC-T7pol), virus recovery was achieved using L929, BHK-21, and A549 cells. Theoretically, this system offers the possibility to produce infectious reovirus from a variety of cell lines including those appropriate for propagation of viral vectors for clinical use in humans (Neumann et al., 2005).

In summary, we have developed an alternative plasmid-based rescue system for reovirus that is driven by a plasmid-encoded T7 RNA polymerase, thus eliminating the requirement for rDIs-T7pol or BHK-T7 cells. This approach should increase the flexibility of reovirus reverse genetics systems for basic research on reovirus biology and the development of reovirus for clinical applications.

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Fig. 1.

Experimental strategy of a plasmid-based reovirus reverse genetics system driven by a plasmid-encoded T7 RNA polymerase. A monolayer of L929 or BHK-21 cells is co-transfected with a T3D 10-plasmid set together with pC-T7pol. Each of the 10 T3D plasmids contains a full-length T3D gene segment cDNA, flanked by T7 RNA polymerase promoter and HDV ribozyme sequences. In cells co-transfected with a total of 11 plasmids, T7 RNA polymerase is expressed under control of a strong CAG promoter from pC-T7pol, which induces expression of transcripts corresponding to the native reovirus positive-sense RNAs from the 10 T3D plasmids. After 5 days of incubation, recombinant reoviruses (rsT3D) are recovered by a plaque assay using L929 cells. P_{T7} , Rib, and P_{CAG} denote T7 RNA polymerase promoter, HDV ribozyme, and CAG promoter, respectively.

Fig. 2.

Rescue and characterization of rsT3D viruses generated with the pC-T7pol-based reverse genetics system. (A) Plaque formation by rsT3D viruses generated with the pC-T7pol-based system using L929 cells (middle panel) and BHK-21 cells (right panel). The transfected L929 and BHK-21 cells were lysed by freeze-thaw cycles and directly inoculated onto L929 cells for plaque assay. After 5 days of incubation without protease in the primary overlay medium (0.7% agarose), infected cells were stained with secondary overlay medium containing 0.7% agarose and 0.005% neutral red. Transfections without T3D plasmids or pC-T7pol were used as negative controls. The result of a rescue experiment without pC-T7pol is shown as a representative negative control (left panel). (B) Comparison of virus recovery with the pC-T7pol-based system using L929 and BHK-21 cells. Virus titers in cell lysates of transfected L929 and BHK-21 cells were determined by a plaque assay. The data are presented as the mean virus titers for three independent cell cultures. Error bars indicate standard deviations. (C) PAGE of dsRNAs extracted from rescued rsT3D viruses. Lane 1, dsRNAs from the native T3D virus as a reference; lanes 2 and 3, dsRNAs from rsT3D viruses rescued with the pC-T7pol-based system using L929 cells (lane 2) and BHK-21 cells (lane 3). The order of the genomic dsRNA segments of strain T3D (L1, L2, L3, M1, M2, M3, S1, S2, S3, and S4) is indicated.

Fig. 3.

Plaque formation by rsT3D virus generated with the pC-T7pol-based system using A549 cells. Two days after transfection, L929 cells were overlaid onto the transfected A549 cells. Three days later, the co-cultures were lysed by freeze-thaw cycles and directly inoculated onto L929 cells for plaque assay (right panel). The result of rescue experiment without the additional co-culture step with L929 cells is also shown (middle panel). Transfection without pC-T7pol was used as a representative negative control (left panel).