Published in final edited form as:
Virology. 2010 March 15; 398(2): 194–200. doi:10.1016/j.virol.2009.11.037.

An Improved Reverse Genetics System for Mammalian Orthoreoviruses

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Abstract

Mammalian orthoreoviruses (reoviruses) are highly useful models for studies of double-stranded RNA virus replication and pathogenesis. We previously developed a strategy to recover prototype reovirus strain T3D from cloned cDNAs transfected into murine L929 fibroblast cells. Here, we report the development of a second-generation reovirus reverse genetics system featuring several major improvements: (1) the capacity to rescue prototype reovirus strain T1L, (2) reduction of required plasmids from ten to four, and (3) isolation of recombinant viruses following transfection of baby hamster kidney cells engineered to express bacteriophage T7 RNA polymerase. The efficiency of virus rescue using the 4-plasmid strategy was substantially increased in comparison to the original 10-plasmid system. We observed full compatibility of T1L and T3D rescue vectors when intermixed to produce a panel of T1L × T3D monoreassortant viruses. Improvements to the reovirus reverse genetics system enhance its applicability for studies of reovirus biology and clinical use.

Keywords

reverse genetics; reovirus; dsRNA; T7 RNA polymerase; reassortment

Introduction

One of the most transformative technologic advancements in virology has been the development of reverse genetics systems for nearly all major groups of RNA- and DNA-

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containing viruses. Although the molecular design and methodologic details of these systems vary, their common feature is the availability of cloned cDNAs encoding viral genomes that can be manipulated and subsequently used to isolate viruses containing engineered changes in genomic nucleic acids. RNA virus rescue systems typically entail intracellular transcription of full-length genomic or antigenomic RNAs from plasmids transfected into permissive cells, frequently in conjunction with transient expression of essential components of the viral replicase (Bridgen and Elliott, 1996; Collins et al., 1995; Fodor et al., 1999; Garcin et al., 1995; Lawson et al., 1995; Neumann et al., 1999; Racaniello and Baltimore, 1981; Schneider, Schwemmle, and Staeheli, 2005; Schnell, Mebatsion, and Conzelmann, 1994; Whelan et al., 1995; Yoneda et al., 2006). Furthermore, translational competence of (+)-strand RNA virus genomes has enabled rescue of infectious virus from cells following introduction of *in vitro*-generated genomic RNAs free of viral proteins (Almazan et al., 2000; Coley et al., 2005; Gritsun and Gould, 1995; Kinney et al., 1997; Racaniello and Baltimore, 1981; Rice et al., 1989; Yount et al., 2003; Yun et al., 2003). Development of a fully plasmid-based reverse genetics strategy for double-stranded (ds) RNA viruses of mammalian species, which includ e coltiviruses, orbiviruses, reoviruses, and rotaviruses, has trailed other major systems owing in part to the technical complexities involved in manipulation of a multipartite genome containing ten or more segments. We have developed a reverse genetics system for mammalian reovirus (Kobayashi et al., 2007), a nonenveloped, double-layered particle containing ten dsRNA gene segments (Schiff, Nibert, and Tyler, 2007), and used this system to engineer changes in both structural and nonstructural proteins for studies of reovirus replication and pathogenesis (Boehme and Dermody, In Press; Danthi et al., 2008a; Danthi et al., 2008b; Kirchner et al., 2008; Kobayashi et al., 2007; Kobayashi et al., 2009; Zurney et al., 2009).

The implications of targeted alterations of the reovirus genome are highly significant to current research focused on therapeutic use of reovirus as an oncolytic agent (Kelly et al., 2009) and to conceptualization of reovirus-vectored vaccines. Goals for our continuing work with this technology include performance improvement in the form of increased efficiency of virus recovery, broadened scope of dissectible viral properties and phenotypes, expanded spectrum of non-viral nucleic acids inserted into the viral genome, shortened time to virus recovery, and reduced reagent preparation time and expense. Progress in these key aspects of the rescue system will accelerate basic studies of reovirus biology and development of reovirus for clinical applications.

The reovirus reverse genetics system is based on the serotype 3 prototype strain, type 3 Dearing (T3D) (Kobayashi et al., 2007), and consists of ten plasmids, each containing a full-length reovirus gene-segment cDNA under transcriptional control of the bacteriophage T7 RNA polymerase promoter and fused at the 3’ terminus with hepatitis delta virus (HDV) ribozyme sequences. These plasmids are presumed to generate transcripts identical in sequence to reovirus-generated (+)-strand RNAs that serve as templates for translation and dsRNA synthesis in infected cells. The primary round of reovirus replication is initiated by co-transfection of all ten plasmids into murine L929 (L) cells infected with a replication-defective vaccinia virus expressing T7 RNA polymerase. Following five days of incubation, recombinant strain (rs) viruses are isolated from co-transfection lysates by plaque assay using L cells. The global replication behavior and strain-specific phenotypes of rs viruses studied to date are qualitatively and quantitatively consistent with the natural parental isolates (Danthi et al., 2008a; Kobayashi et al., 2007; Kobayashi et al., 2009). These results demonstrate that the reovirus reverse genetics system is a biologically plausible approach to study viral replication and pathogenesis, optimize viral oncotropism, and develop reovirus as a live, naturally attenuated vaccine vector for diverse pathogenic microorganisms.

Reovirus strain type 1 Lang (T1L) serves as the prototype for serotype 1 reoviruses. Like T3D, T1L infection has been extensively studied using cultured cells and *in vivo*. T1L and T3D

display numerous phenotypic differences including receptor usage, activities of viral replication proteins, regulation of the interferon response to reovirus infection, apoptosis induction, growth in various types of primary and transformed cells, and virulence, dissemination pathways, and cell and tissue tropism in newborn mice (Schiff, Nibert, and Tyler, 2007). Reassortant viruses derived from co-infections of T1L and T3D have been used to define the genetic basis of several of these biologic polymorphisms. Such analyses have provided key insights into viral replication and disease mechanisms. A marker rescue system for T1L does not exist. However, the availability of tools to derive infectious T1L particles starting with plasmid templates would greatly expand the potential utility of reovirus reverse genetics by permitting any desired combinatorial exchange of T1L and T3D gene segments and providing an alternative genetic background to optimize cell targeting and vectoring capacity.

A reduction in the total number of plasmids necessary to initiate the reovirus replication cycle would improve certain practical aspects of the reverse genetics system including effort and costs associated with reagent preparation. A decreased plasmid requirement also theoretically carries the possibility of increased co-transfection efficiency resulting in enhanced virus isolation, which is particularly relevant to recovery of viruses that replicate poorly. Introduction of changes that handicap reovirus replicative fitness is a productive approach to the elucidation of structure-function relationships in viral proteins (Kirchner et al., 2008; Kobayashi et al., 2006; Kobayashi et al., 2009). Therefore, technical enhancements such as construct consolidation that potentially lower the efficiency barrier to virus replication should strengthen system performance and broaden its utility.

In this report, we describe the establishment of reverse genetics for strain T1L and validate performance of this system by isolation of viable rsT1L and rsT3D single-gene reassortant viruses using plasmid vectors. We improved the reovirus reverse genetics system by consolidating viral gene segment cDNAs into four plasmids. Compared to the 10-plasmid system, this strategy permits more efficient virus recovery than the 10-plasmid system from L cells using recombinant vaccinia virus as a source of T7 RNA polymerase. Finally, we show that both T1L and T3D can be recovered following plasmid transfection of baby hamster kidney (BHK) cells in which the T7 RNA polymerase is constitutively expressed. These enhancements to reverse genetics for reovirus represent a significant advance for this technology.

Results

Plasmid-based reverse genetics for reovirus T1L

To generate T1L from cloned cDNAs, plasmids encoding each gene segment were engineered in similar fashion to T3D cDNAs used for reverse genetics (Kobayashi et al., 2007) (Figure 1). Each plasmid contains one full-length gene segment cDNA placed under control of the bacteriophage T7 RNA polymerase promoter and appended with the HDV ribozyme at the 3'terminus. These plasmids are anticipated to generate full-length (+)-sense RNAs containing native 5' and 3' ends following transcription with T7 RNA polymerase. L cells were infected with replication-defective T7 RNA polymerase-expressing vaccinia virus strain rDIs-T7pol 1 h prior to co-transfection with the ten T1L cDNA plasmids. Recombinant viruses were recovered from cell-culture supernatants by plaque assay using L-cell monolayers. Thus, rsT1L can be recovered from plasmid-transfected cells.

Characterization of T1L obtained using reverse genetics

Genomic dsRNA segments of different reovirus strains produce characteristic electrophoretic patterns when resolved using SDS-PAGE (Barton et al., 2001). To confirm that rsT1L contains the correct combination of gene segments, genomic dsRNA isolated from native T1L and rsT1L virions was electrophoresed in an SDS-polyacrylamide gel and visualized by ethidium bromide

staining (Figure 2A). The electropherotype of rsT1L was indistinguishable from that of native strain T1L. To exclude the possibility that rsT1L represents contamination by native T1L, a unique PmlI site in the L3 gene was destroyed by the introduction of two silent point mutations into the L3 rescue plasmid: C to T at nucleotide 2059 and G to C at nucleotide 2062 (Figure 2B). An 1189 bp fragment of the L3 gene was amplified using RT-PCR of viral dsRNA extracted from T1L and rsT1L virions. The L3 gene RT-PCR product derived from T1L was digested with Pm1I to produce 586 bp and 603 bp fragments, whereas the L3 product derived from rsT1L was not (Figure 2C), indicating that rsT1L originated from plasmids. To ascertain whether rsT1L and native T1L have similar replication kinetics, yields of rsT1L and T1L were quantified following infection of L cells at an MOI of 2 PFU/cell (Figure 2D). Titers of rsT1L and T1L were virtually identical at all time points tested.

Reoviruses form characteristic cytoplasmic inclusions in infected cells (Becker, Peters, and Dermody, 2003; Kobayashi et al., 2009; Miller et al., 2004). These inclusions are thought to represent sites of viral genome replication and vary in morphology dependent on the infecting strain. T1L forms filamentous inclusions, whereas the inclusions formed by T3D are globular (Parker et al., 2002). To determine whether rsT1L inclusions are similar to those produced by T1L, L cells were infected with each virus, fixed at 24 h post infection, and imaged using confocal microscopy (Figure 2E). Inclusions formed by rsT1L and T1L were indistinguishable and displayed filamentous morphology. Taken together, these data demonstrate comparable replication characteristics of rsT1L and native T1L.

Generation of an improved reverse genetics system using four plasmid vectors

To reduce the number of plasmids required for reverse genetics, two or four T1L gene transcription cassettes encoding reovirus cDNAs flanked by the T7 RNA polymerase promoter and HDV ribozyme were combined in a single plasmid (Figure 3A). Specifically, L1 and M2 (pT7-L1-M2T1L), L2 and M3 (pT7-L2-M3T1L), L3 and S3 (pT7-L3-S3T1L), and S1, S2, S4, and M1 (pT7-S1-S2-S4-M1T1L) transcription cassettes were jointly introduced into the rescue backbone plasmid using PCR to generate requisite restriction enzyme recognition sites compatible with multiplex cloning (Figure 3A).

To assess rescue efficiency of the 4-plasmid system, L cells were infected with vaccinia virus strain rDIs-T7pol 1 h prior to transfection with the 4- or 10-plasmid vector set, and viral titers in transfected cell lysates were determined at 24 and 48 h post-transfection by plaque assay using L-cell monolayers. Viral titers following transfection with ten plasmids encoding the T1L genome were 10 and 1000 PFU/ml at 24 and 48 h, respectively. However, viral titers following transfection with t he 4-plasmid T1L cDNA vector set were markedly higher at both 24 (1000 PFU/ml) and 48 (10,000 PFU/ml) h post-transfection (Figure 3B). Thus, transfection with four plasmids substantially enhances rescue efficiency.

A 4-plasmid reverse genetics system also was developed for T3D. Using this strategy, L1 and S1 (pT7-L1-S1T3D), L2 and M3 (pT7-L2-M3T3D), L3 and M1 (pT7-L3-M1T3D), and M2, S2, S3, and S4 (pT7-M2-S2-S3-S4T3D) gene cassettes were introduced into individual plasmids (Figure 3C). To compare the efficiency of the T3D rescue systems, the T3D 4-plasmid and 10-plasmid vector sets were independently transfected into rDIs-T7pol-infected L cells, and viral titers in culture lysates were determined by plaque assay at 24 and 48 hr. Viral titers in cultures transfected with ten plasmids were below the limit of detection at 24 h and ~100 PFU/ml at 48 h. In contrast, L-cell cultures transfected with four plasmids produced ~100 PFU/ml of virus at 24 h and ~1000 PFU/ml at 48 h (Figure 3D). From these data, we conclude that both T1L and T3D can be recovered using four plasmids and that a reduction in plasmid number substantially improves rescue efficiency.

Generation of rsT1L × rsT3D monoreassortant viruses

Using a combination of the 4-plasmid and 10-plasmid rescue systems, we generated a panel of 20 rsT1L × rsT3D monoreassortants viruses. These viruses contain nine genes from one parental strain and one gene from the other. Genotypes of recovered viruses were confirmed using a combination of gene segment electrophoretic mobility and sequence analysis (data not shown). The electrophoretic pattern of a representative monoreassortant, rsT1L-T3M1, which contains the T3D M1 gene segment in an otherwise T1L background, clearly shows co-migration of the M1 RNA with that of T3D (Figure 4). Conversely, the M1 RNA of the reciprocal reassortant virus, rsT3D-T1M1, co-migrates with T1L M1. The generation of T1L × T3D monoreassortant viruses demonstrates the versatility of the reverse genetics system to potentially rescue viruses with any desired genetic combination, including monoreassortants, which have traditionally been the most difficult to obtain by co-infection of cells with T1L and T3D.

Generation of rs viruses using BHK-T7 cells

BHK cells have been engineered to stably express T7 RNA polymerase (BHK-T7) under control of the cytomegalovirus promoter (Buchholz, Finke, and Conzelmann, 1999). To determine whether these cells allow recovery of viable reovirus following plasmid transfection, thus eliminating the requirement for rDIs-T7pol, we transfected BHK-T7 cells with the T1L or T3D 4-plasmid vector sets in the absence of rDIS-T7pol and determined viral titers in transfected cell lysates by plaque assay at 48 h post-transfection (Figure 5). We found that transfection with either T1L or T3D cDNAs led to substantial viral titers at 48 h post-transfection. From these results, we conclude that BHK-T7 cells can be used as an alternative to rDIs-T7pol-infected L cells for the recovery of reovirus using plasmid vectors.

Discussion

The prototype strain for type 1 reovirus, T1L has been studied extensively in replication and pathogenesis models. Therefore, a primary goal of this study was to generate a reverse genetics system for T1L. As demonstrated for strain T3D (Boehme and Dermody, In Press; Danthi et al., 2008a; Danthi et al., 2008b; Kirchner et al., 2008; Kobayashi et al., 2007; Kobayashi et al., 2009; Zurney et al., 2009), development of a reverse genetics system for T1L affords an opportunity to leverage an extensive knowledge base of virus structure and biology toward further acceleration of basic and applied reovirus research. Here, we show production of T1L virions from cloned cDNAs. Similar to rsT3D and native T3D, the replication characteristics of rsT1L reflect native T1L. Growth kinetics, total yields, inclusion morphology, and genomic electrophoretic signatures were indistinguishable between T1L and T3D. These results further validate retention of native viral properties by plasmid-derived viruses. Moreover, development of the T1L rescue system will expand the utility of reverse genetics for studies of reovirus biology.

Analysis of a large panel of T1L × T3D reassortant viruses has shown that reovirus gene segments display nonrandom segregation upon co-infection of L cells by the parental strains (Nibert, Margraf, and Coombs, 1996). This observation suggests there may be interactions among viral proteins, RNAs, or both that influence reovirus evolution. Additionally, compensatory mutations that promote viral replication efficiency may be necessary to stabilize certain genetic combinations (Roner et al., 1995). Prior to this study, it was unclear whether generation of all 20 single-gene T1L × T3D reassortant viruses would be feasible due to uncertainties about the replicative fitness of gene constellations not previously isolated in T1L × T3D reassortant panels. Development of a fully plasmid-based rescue system for T1L will now allow systematic characterization of gene segment co-evolution and may facilitate

isolation of viruses with replication impediments or combinations of T1L and T3D genes normally disfavored in mixed infections (Nibert, Margraf, and Coombs, 1996).

Establishment of reverse genetics for reovirus has allowed engineering of mutations into structural and nonstructural reovirus proteins and provided an opportunity to observe the effects of these mutations on viral replication and pathogenesis in the context of infection. However, the current system is limited by the inefficiency of transfection of ten plasmids (Kobayashi et al., 2007). Therefore, a second goal of this study was to improve viral yields by reducing the number of plasmids required for virus recovery. This goal was accomplished by combining two or four transcriptional cassettes in a single plasmid, reducing the total number of plasmids from ten to four. Compared to the first-generation marker-rescue system, the 4-plasmid strategy enhanced efficiency of virus recovery by reducing time to virus isolation and supporting increased total yields. The improved reverse genetics system also offers the economic advantage of requiring fewer reagents and less preparation time. Interestingly, recovery of T1L appears to be more efficient than T3D using either the 4- or 10-plasmid reverse genetics system. While the simplest explanation is that T1L exhibits an intrinsic replication advantage over T3D in cell lines used for virus rescue, another possibility is that different gene arrangements between the T1L and T3D 4-plasmid systems contribute to higher yields of T1L. Whether genetic linkage within the rescue constructs affects virus production is unknown.

The 4-plasmid reverse genetics system reported here could conceivably enhance efficiency of virus recovery by three non-mutually exclusive mechanisms. First, since fewer plasmids are required to simultaneously enter a cell, the probability is increased that a full set of viral RNAs will accumulate to a critical threshold for replication initiation. While plasmids are provided in vast excess, there may be a limit to the amount of plasmid that can enter a single cell. Second, it is possible that only a small fraction of transfected plasmids avoid degradation and remain competent for transcription. By decreasing the number of plasmids from ten to four, the likelihood that a single cell will contain a sufficient number of intact transcription cassettes is enhanced. Third, it is possible that constraining transcription and translation of individual viral RNAs to the same intracellular microenvironment, as might be anticipated for multicistronic vectors, facilitates essential protein-protein or protein-RNA interactions required for virus recovery.

Technical complexities associated with manipulation of multicistronic vectors may limit their use for certain applications. However, the 10-plasmid system remains a useful alterative for demanding cloning tasks. Tetracistronic vectors can be reduced to tricistronic constructs by simple endonucleolytic excision of a gene segment cDNA and then complemented by co-transfection with the corresponding single gene vector containing the desired mutations. We have found that the 4- and 10-plasmid vector systems are fully compatible for virus rescue. Hence, the monocistronic vectors remain important adjuncts to the 4-plasmid strategy by combining enhanced flexibility with gains in efficiency.

In our effort to improve reovirus reverse genetics, we found that BHK-T7 cells, which constitutively express T7 RNA polymerase (Buchholz, Finke, and Conzelmann, 1999), allow recovery of both rsT1L and rsT3D. The capacity of BHK-T7 to support virus rescue obviates the requirement for rDIs-T7pol and thereby greatly simplifies the rescue protocol. BHK-T7 cells are easier to maintain and propagate than rDIs-T7pol and thus provide an additional improvement to the reovirus rescue system.

Our progress with reovirus reverse genetics in many respects parallels the evolution of reverse genetics technology for influenza virus, which also has been modified to improve efficiency. Early reverse genetics systems for influenza virus required co-transfection of 12–16 plasmids encoding viral mRNAs or proteins (Neumann et al., 1999). Subsequently, the number of

required plasmids was reduced to eight; each containing a viral gene flanked by both pol I and pol II promoters that drive viral genomic and messenger RNA expression, from the same cDNA (Hoffmann et al., 2000). As these systems were practical only in cell types supporting high transfection efficiencies, further improvements were achieved by combining eight RNA pol I transcription cassettes for viral RNA synthesis into a single plasmid and condensing three of the four protein expression cassettes onto one plasmid. The resulting three-plasmid system increases efficiency of virus production in Vero cells, which are amenable to propagation of influenza vaccines for use in humans (Neumann et al., 2005). Hence, reverse genetics of viruses containing segmented genomes appears to be inherently inefficient, but this problem is surmountable through plasmid reduction.

We have established an improved reverse genetics system of reovirus that allows recovery of prototype strain T1L from plasmid vectors and reduces the number of plasmids for both the T1L and T3D systems. These enhancements have increased the flexibility and efficiency of reverse genetics for basic studies of reovirus biology and will accelerate development of reovirus for clinical and technological purposes.

Materials and methods

Cells and viruses

L cells were grown in Joklik's modified Eagle's minimal essential medium (Irvine Scientific) supplemented to contain 5% fetal calf serum (Gibco), 2 mM L-glutamine, 100 U of penicillin G/ml, 100 μg of streptomycin/ml, and 0.25 μg amphotericin B/ml (Gibco). BHK-T7 cells were grown in Dulbecco's modified Eagle's minimal essential medium (Invitrogen) supplemented to contain 5% fetal calf serum, 2 mM L-glutamine, 2% MEM Amino Acid Solution (Invitrogen), and 1 mg/ml geneticin (Invitrogen). Reovirus strains T1L and T3D are laboratory stocks originally obtained from the laboratory of Dr. Bernard Fields. Strain rsT3D was produced by reverse genetics (Kobayashi et al., 2007). Virus was purified from L cells by CsCl-gradient centrifugation (Furlong, Nibert, and Fields, 1988).Viral titers were determined by plaque assay using L cell monolayers as described (Virgin et al., 1988). Attenuated vaccinia virus strain rDIs-T7pol expressing T7 RNA polymerase (Ishii et al., 2002) was propagated in chicken embryo fibroblasts.

Plasmid construction

T1L plasmid cDNAs for rescue of recombinant infectious reovirus were constructed using RT-PCR, viral genomic RNA, and gene-specific primer sets (Table 1). RT-PCR products were cloned into the SmaI-RsrII (L1, L2, and S3), SmaI-BseRI (S2), EcoRV-RsrII (M3), or EcoRV-BesRI (S4) sites of p3E5EGFP (Watanabe et al., 2004), resulting in pT7-L1T1L, pT7-L2T1L, pT7-M3T1L, pT7-S2T1L, pT7-S3T1L, and pT7-S4T1L. The T1L L3 gene cDNA, amplified from viral genomic RNA with specific primers (Table 1), was inserted into the EcoRI-RsrII site of pT7-L3T3D (Kobayashi et al., 2007), thereby replacing the T3D L3 cDNA and generating pT7-L3T1L. pT7-M2T1L was constructed by insertion into the p3E5EGFP RsrII site of RT-PCR amplification products generated with viral genomic RNA and specific primers (Table 1). The resulting construct containing the cloned T1L M2 gene was digested with SmaI and AvrII, followed by self ligation to remove GFP-encoding sequences and the Ebola virus leader and trailer sequences. pT7-M1T1L and pBacT7-S1T1L, encoding T1L M1 and S1 genes, respectively, were described previously (Kobayashi et al., 2007;Kobayashi et al., 2009).

Recombinant reovirus generation from four plasmids was facilitated by combining two or four viral gene transcription cassettes encoding T1L and T3D reovirus cDNAs flanked by the T7 RNA polymerase promoter (5') and HDV ribozyme (3') onto one plasmid. Bicistronic reovirus

cDNA constructs pT7-L1-M2T1L (T1L L1 and M2), pT7-L2-M3T1L (T1L L2 and M3), pT7-L3-S3T1L (T1L L3 and S3), pT7-L1-S1T3D (T3D L1 and S1), pT7-L2-M3T3D (T3D L2 and M3), and pT7-L3-M1 (T3D L3 and M1) were generated by cloning PCR-amplified T1L M2, T1L M3, T1L S3, T3D S1, T3D M3, and T3D M1 gene transcription cassettes into the SphI site of pT7-L1T1L, NheI site of pT7-L2T1L, HindIII site of pT7-L3T1L, HindIII site of pT7-L1T3D, NheI site of pT7-L2T3D, and HindIII site of pT7-L3T3D, respectively. Tetracistronic reovirus cDNA constructs, pT7-S1-S2-S4-M1T1L (T1L S1, S2, S4, and M1) and pT7-S2-S3-S4-M2T3D (T3D S2, S3, S4, and M2) were generated by cloning PCR-amplified T1L S3, T1L S4, T1L M1, T3D S3, T3D S4, and T3D M2 gene transcription cassettes into the XbaI (T1L S1), SalI (T1L S4), or NheI (T1L M1) sites of pT7-S2T1L or SalI-HindIII (T3D S3), HindIII-NheI (T3D S4), or NheI (T3D M2) sites of pT7-S2T3D.

Plasmid transfection and recovery of recombinant virus using L cells

Monolayers of L cells at 90% confluency (approximately 3×10^6 cells) seeded in 60 mm dishes (Costar) were infected with rDIs-T7pol at an MOI of approximately 0.5 $TCID_{50}$. At 1 h post-infection, cells were co-transfected with plasmids representing the cloned reovirus genome using 3 μl of TransIT-LT1 transfection reagent (Mirus) per microgram of plasmid DNA. The amount of each plasmid used for transfection was as follows: pT7-L1T1L, 2 μg; pT7-L2T1L, 2 μg; pT7-L3T1L, 2 μg; pT7-M1T1L, 1.75 μg; pT7-M2T1L, 1.75 μg; pT7-M3T1L, 1.75 μg; pT7-S1T1L, 2 μg; pT7-S2T1L, 1.5 μg; pT7-S3T1L, 1.5 μg; pT7-S4T1L, 1.5 μg; pT7-L1-M2T1L, 4.5 μg; pT7-L2-M3T1L, 4.5 μg; pT7-L3-M3T1L, 4.5 μg; pT7-S1-S2-S4-M1T1L, 4.5 μg; pT7-L1-S1T3D, 4.5 μg; pT7-L2-M3T3D, 4.5 μg; pT7-L3-M1T3D, 4.5 μg; and pT7-M2-S2-S3-S4T3D, 4.5 μg. Quantities of T3D monocistronic plasmid were used as previously described (Kobayashi et al., 2007). Following 1–5 d of incubation, recombinant virus was isolated from transfected cells by plaque purification using L cells (Virgin et al., 1988).

SDS-PAGE analysis of viral gene segments

Purified virions (5×10^{10} particles) were loaded into wells of 10% SDS-polyacrylamide gels. Gels were electrophoresed for approximately 600 mAmp-hours. Gene segments were visualized by ethidium bromide staining.

Quantification of virus infectivity

Monolayers of L cells (5×10^5 cells) seeded in 24-well plates (Costar) were adsorbed with virus at an MOI of 2 PFU/cell. After 1 h adsorption at room temperature, the inoculum was removed, cells were washed with PBS, and fresh medium was added. Cells were incubated at 37°C for various intervals, and viral titers in cell lysates were determined by plaque assay using L cells (Virgin et al., 1988).

Immunofluorescence detection of reovirus infection

L cells were plated on glass coverslips in 24-well plates (Costar) and adsorbed at an MOI of 2 PFU/cell. Following incubation at 37°C for 24 h, cells were fixed and stained with rabbit μNS antiserum followed by fluorophore-conjugated, goat anti-rabbit secondary antibody (Invitrogen). Images were acquired using a Zeiss LSM 510 META inverted confocal system (Carl Zeiss) with a Zeiss inverted Axiovert 200M microscope.

Plasmid transfection and recovery of recombinant virus using BHK-T7 cells

Monolayers of BHK-T7 cells at 90% confluency (approximately 3×10^6 cells) seeded in 60 mm dishes were co-transfected with plasmids representing the cloned reovirus genome using 3 μl of TransIT-LT1 transfection reagent (Mirus) per microgram of plasmid DNA. The amount of each plasmid used for transfection was identical to that described for L cell transfection.

Following 1–5 d of incubation, recombinant virus was isolated from transfected cells by plaque purification using monolayers of L cells (Virgin et al., 1988).

Acknowledgments

We thank Yoshihiro Kawaoka for plasmid p3E5EGFP, Tatsuo Miyamura and Koji Ishii for vaccinia virus rDIs-T7pol, and Ulla Buchholz for BHK-T7 cells. This research was supported by a fellowship from the Naito Foundation (T.K.), K08 AI62862 (J.D.C.), R01 AI32539, and the Elizabeth B. Lamb Center for Pediatric Research.

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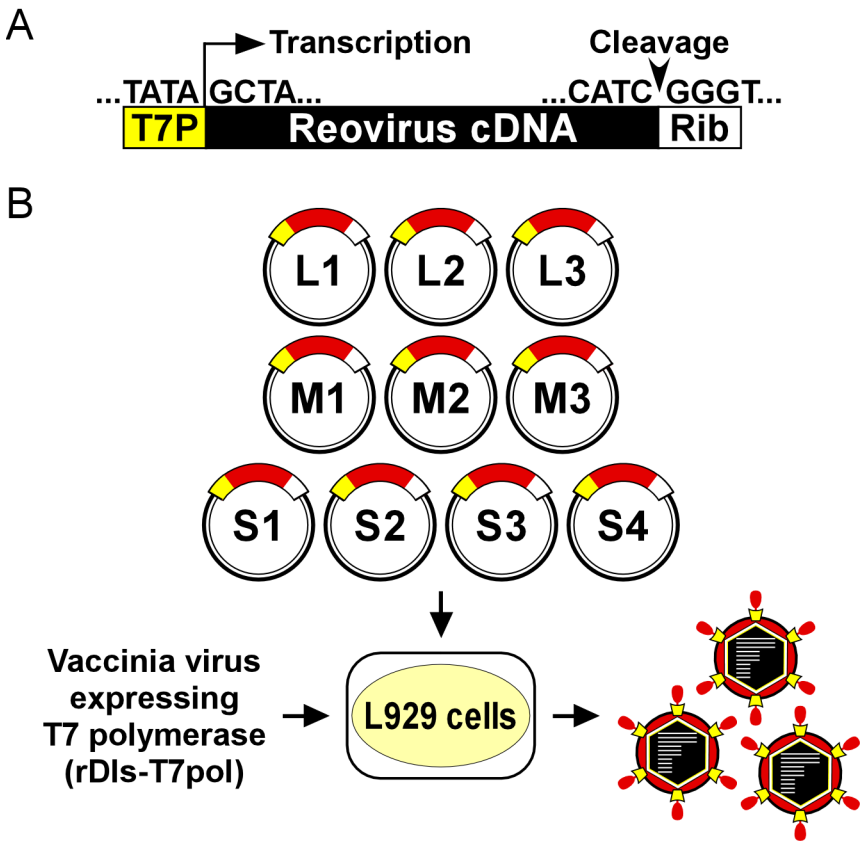

Fig 1. Experimental strategy to generate reovirus type 1 Lang (T1L) from cloned cDNAs

(A) Rescue plasmids containing T1L gene-segment cDNAs. Vectors contain cDNAs representing each of the ten full-length T1L gene segments. Reovirus cDNAs are flanked by the bacteriophage T7 RNA polymerase promoter (T7P) and the antigenomic hepatitis delta virus (HDV) ribozyme (Rib). (B) Reverse genetics procedure. The ten T1L cDNA constructs are transfected into murine L cells that express T7 RNA polymerase from recombinant vaccinia virus strain DIs-T7pol. Plasmid-derived transcripts correspond to viral mRNAs with native 5' termini. The 3' termini of nascent transcripts are fused to HDV ribozyme sequences, which generate authentic 3'ends through autocatalytic cleavage. After five days of incubation, transfected cells are lysed by freeze-thaw, and recombinant-strain (rs) viruses are isolated from lysates by plaque assay using L cells.

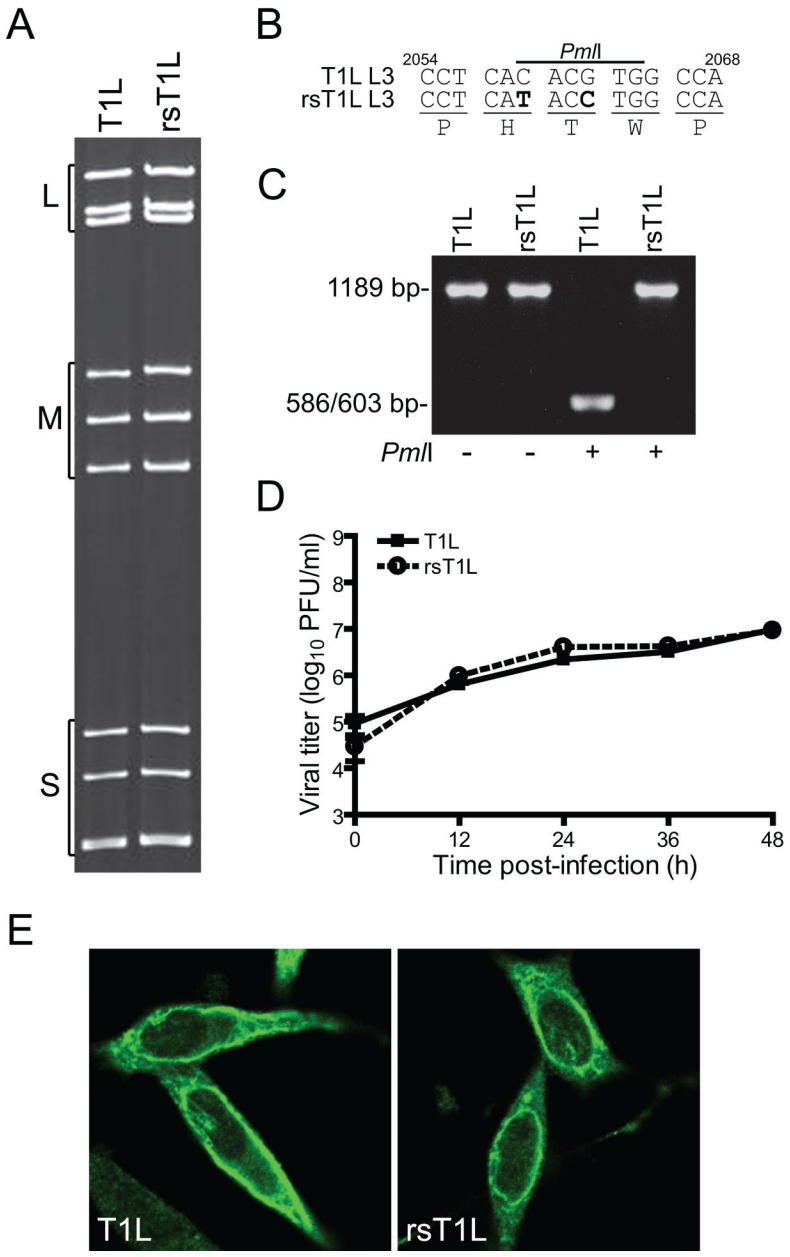

Fig 2. Characterization of T1L viruses derived using reverse genetics

(A) Electropherotypes of native T1L and plasmid-derived rsT1L. Purified virions were electrophoresed in an SDS-polyacrylamide gel, followed by ethidium bromide staining to visualize viral gene segments. Size classes of gene segments (L, M, S) are indicated. (B) Novel mutations in the L3 gene of rs viruses. The C→T change at nucleotide 2059 and G→C change at nucleotide 2062 eliminate a unique PmlI site. (C) Digestion of the L3 gene RT-PCR products of T1L and rsT1L with PmlI to confirm the presence or absence of a unique restriction site. Size markers are indicated. (D) Growth of T1L and rsT1L. L cells were adsorbed with T1L or rsT1L at an MOI of 2 PFU/cell, and viral titers in cell lysates were determined by plaque assay at the times shown. Results are presented as mean viral titers for triplicate experiments. Error bars indicate SD. (E) Immunofluorescence analysis of cells infected with T1L and rsT1L. L cells were adsorbed with either T1L or rsT1L and stained at 24 h post-infection with μNS-

specific antiserum followed by a fluorophore-conjugated, goat-anti rabbit secondary antibody to visualize reovirus inclusions. Representative images of T1L- and rsT1L-infected cells are shown.

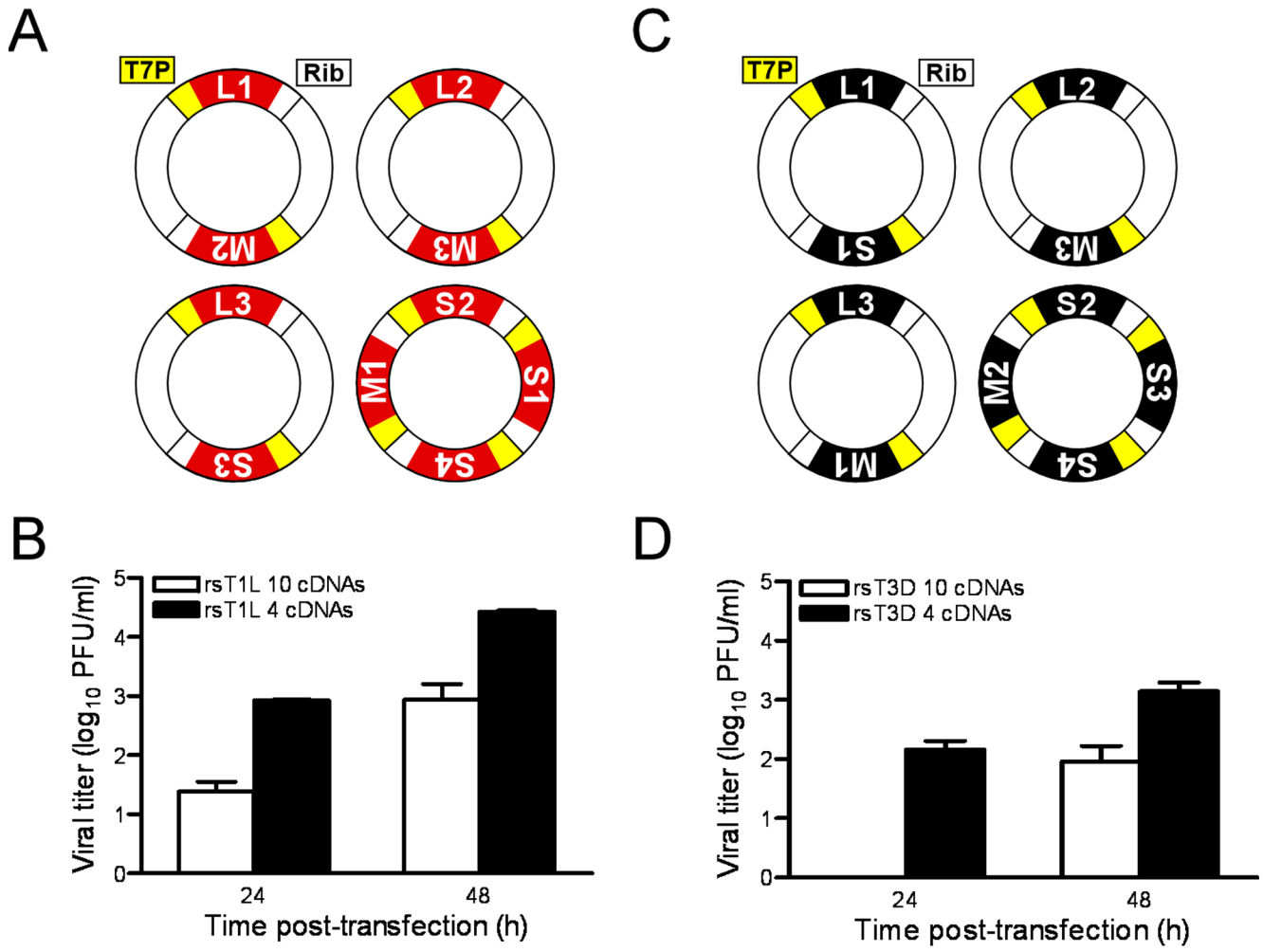

Fig 3. Improved reverse genetics for reovirus strains T1L and T3D

(A, C) Two or four gene transcription cassettes encoding reovirus cDNAs flanked by the T7 RNA polymerase promoter and HDV ribozyme sequences were combined into single plasmids, creating four plasmids for the T1L and T3D reverse genetics systems. (B, D) L cells expressing T7 polymerase were co-transfected with either four or ten rescue plasmids corresponding to the T1L (B) and T3D (D) genomes. Following 24 or 48 h incubation, transfected cells were lysed by freeze-thaw, and viral titers in cell lysates were determined by plaque assay. Results are presented as mean viral titers for triplicate experiments. Error bars indicate SD.

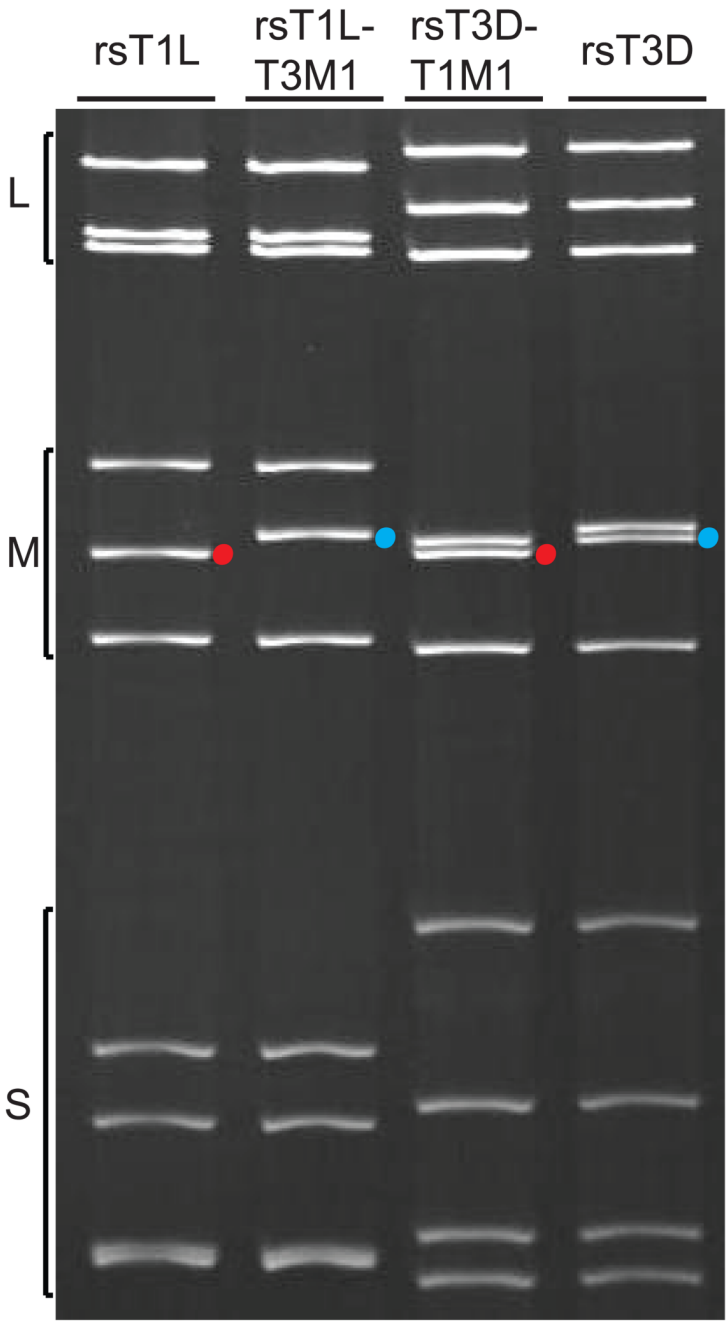

Fig 4. Representative electropherotype of recombinant monoreassortant viruses

Purified virions of rsT1L, rsT3D, and T1L × T3D monoreassortants containing reciprocal exchanges of the M1 gene, rsT1L-T3M1 and rsT3D-T1M1, were electrophoresed in an SDS-polyacrylamide gel, followed by ethidium bromide staining to visualize viral gene segments. Size classes of gene segments (L, M, S) are shown. Positions of the T1L and T3D M1 genes are indicated by red and blue dots, respectively.

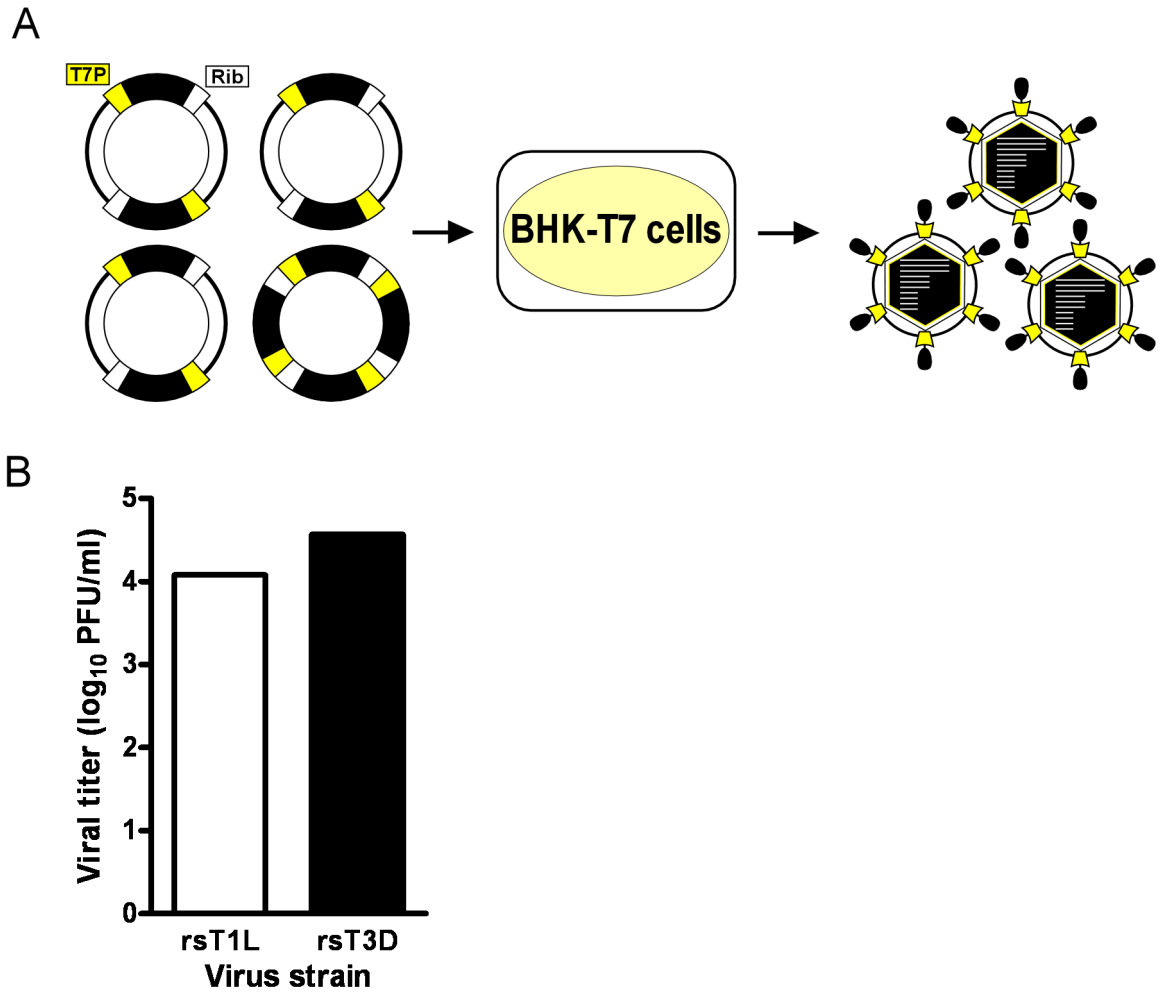

Fig 5. Recombinant virus generated using BHK-T7 cells

(A) Schematic of reovirus reverse genetics system using BHK-T7 cells. (B) BHK-T7 cells were co-transfected with four rescue plasmids corresponding to the T1L and T3D genomes. Following 48 h incubation, transfected cells were lysed by freeze-thaw, and viral titers in cell lysates were determined by plaque assay.

Table 1

Oligodeoxynucleotide primers used for plasmid construction

Plasmid DNA		Nucleotide sequence[a]
pT7-L1T1L	Forward	5'-TAACCCGGGTAATACGACTCACTATAGCTACACGTTCCACGACAATGTCATCCA-3'
	Reverse	5'-GGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCGATGAGTTGACGCACCACGACCCAT-3'
pT7-L2T1L	Forward	5'-TAACCCGGGTAATACGACTCACTATAGCTATTGGCGCAATGGCGAACGTTTGGG-3'
	Reverse	5'-GGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCGATGAATTAGGCACGCTCACGAGGG-3'
pT7-L3T1L	Forward	5'-TAAGAATTCTAATACGACTCACTATAGCTAATCGTCAGGATGAAGCGGATTCCA-3'
	Reverse	5'-GGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCGATGAATCGGCCCAACTAGCATCGA-3'
pT7-M2T1L	Forward	5'-CCTCGCGGTCCGACCTGGGGTTAATACGACTCACTATAGCTAATCTGCTGACCGTCACTCT-3'
	Reverse	5'-GGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCGATGATGTGCCTGCACCCTTAACC-3'
pT7-M3T1L	Forward	5'-TAAGATATCTAATACGACTCACTATAGCTAAAGTGACCGTGGTCATGGCTTCAT-3'
	Reverse	5'-GGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCGATGAATAGGGGTCGGGAAGGCTTA-3'
pT7-S2T1L	Forward	5'-TAACCCGGGTAATACGACTCACTATAGCTATTCGCTGGTCAGTTATGGCT-3'
	Reverse	5'-ACCGCGAGGAGGTGGAGATGCCATGCCGACCCGATGAATGTGTGGTCAGTCGTGAGGGGTGTG-3'
pT7-S3T1L	Forward	5'-TAACCCGGGTAATACGACTCACTATAGCTAAAGTCACGCCTGTTGTCGTCAC -3'
	Reverse	5'-GGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCGATGATTAGGCGCCACCCACCACC-3'
pT7-S4T1L	Forward	5'-TAAGATATCTAATACGACTCACTATAGCTATTTTTGCCTCTTCCCAAACG-3'
	Reverse	5'-CGCGAGGAGGTGGAGATGCCATGCCGACCCGATGAATGGAGCCTGTCCCACGTC-3'

[a]The T7 RNA polymerase promoter sequence is underlined.