JVI Journals.ASM.org

Engineering Recombinant Orsay Virus Directly in the Metazoan Host *Caenorhabditis elegans*

Hongbing Jiang, Carl J. Franz, David Wang

Departments of Molecular Microbiology and Pathology & Immunology, Washington University in St. Louis School of Medicine, St. Louis, Missouri, USA

ABSTRACT

The recent identification of Orsay virus, the first virus that is capable of naturally infecting *Caenorhabditis elegans*, provides a unique opportunity to explore host-virus interaction studies in this invaluable model organism. A key feature of this system is the robust genetic tractability of the host, *C. elegans*, which would ideally be complemented by the ability to genetically manipulate Orsay virus in parallel. To this end, we developed a plasmid-based reverse genetics system for Orsay virus by creating transgenic *C. elegans* strains harboring Orsay virus cDNAs. Both wild-type and mutant Orsay viruses, including a FLAG epitope-tagged recombinant Orsay virus, were generated by use of the reverse genetics system. This is the first plasmid-based virus reverse genetics system in the metazoan *C. elegans*. The Orsay virus reverse genetics we established will serve as a fundamental tool in host-virus interaction studies in the model organism *C. elegans*.

IMPORTANCE

To date, Orsay virus is the first and the only identified virus capable of naturally infecting *Caenorhabditis elegans*. *C. elegans* is a simple multicellular model organism that mimics many fundamental features of human biology and has been used to define many biological properties conserved through evolution. Thus, the Orsay virus-*C. elegans* infection system provides a unique opportunity to study host-virus interactions. In order to take maximal advantage of this system, the ability to genetically engineer mutant forms of Orsay virus would be highly desirable. Most efforts to engineer viruses have been done with cultured cells. Here we describe the creation of mutant viruses directly in the multicellular organism *C. elegans* without the use of cell culture. We engineered a virus expressing a genetically tagged protein that could be detected in *C. elegans*. This provides proof of concept for modifying Orsay virus, which will greatly facilitate studies in this experimental system.

Orsay virus, which was recently discovered in a wild *Caenorhabditis elegans* strain, is the only known virus capable of naturally infecting the model organism *C. elegans* (1). Orsay virus has a bipartite positive-sense RNA genome and is most closely related to nodaviruses (1, 2). The Orsay virus RNA1 genome segment, which is ~3.4 kb in length, encodes an RNA-dependent RNA polymerase (RdRP), while the Orsay virus RNA2 genome segment, which is ~2.5 kb, encodes the viral capsid protein and a novel capsid-delta fusion protein of unknown function that is expressed by a ribosomal frameshifting mechanism (3).

As many fundamental biological discoveries have been made in the *C. elegans* model, the ability of Orsay virus to infect *C. elegans* provides a unique and powerful experimental system to define multiple aspects of host-virus interactions. Prior to the discovery of Orsay virus, only limited studies with *C. elegans* using a Flock House virus replicon (4), vesicular stomatitis virus infection of primary nematode cells (5, 6), or artificial infection of *C. elegans* by vaccinia virus (7) have been reported. Those studies defined antiviral roles for the RNA interference (RNAi) pathway and apoptosis genes in *C. elegans*. To date, analysis of Orsay virus infection has confirmed a role for the RNAi pathway, and dicer-related helicase 1 (*drh-1*) in particular, in antiviral defense in *C. elegans* (8, 9). Additional efforts to define novel pathways of antiviral immunity using Orsay virus will capitalize on the robust host genetics available in *C. elegans*. As most viruses encode proteins that antagonize host antiviral defenses, it would be ideal if the Orsay virus genome could be manipulated to identify such proteins. The Orsay-*C. elegans* system could also exploit another unique property of *C. elegans*, its transparency, which could be leveraged to directly visualize Orsay virus infection and spread *in vivo*, provided a suitable reporter virus (e.g., fluorescently tagged) could be created. Furthermore, the ability to genetically define a function for the novel capsid-delta fusion protein would be enhanced by creating mutant viruses that cannot express the capsid-delta fusion protein. Thus, engineering Orsay virus would greatly enhance studies in the Orsay-*C. elegans* infection system.

For many viruses, reverse genetics have been developed in order to be able to create mutant viruses (10–15). Reverse genetics systems for multiple nodaviruses, including Flock House virus, Pariacoto virus, and a fish-infecting betanodavirus, have been described previously (16–20). In these examples, a viral cDNA construct driven by a T7 promoter is transfected into mammalian cells that express a helper T7 polymerase. All of these systems rely upon mammalian cell cultures for the generation of recombinant virus. However, no cell culture system capable of propagating Orsay virus has been identified, and therefore, an alternative approach is required to develop reverse genetics for Orsay virus.

Received 5 June 2014 **Accepted** 25 July 2014

Published ahead of print 30 July 2014

Editor: K. Kirkegaard

Address correspondence to David Wang, davewang@borcim.wustl.edu.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.01630-14.

doi:10.1128/JVI.01630-14

TABLE 1 Primers used in this study

Primer	Sequence (5′–3′)
HIPR1F1	GAATGCGGGTATATAAGGAAAGCGGGCTCAGAGGAAGCCACAATTGTCGACTTGATACC
HIPR1F2	AATGCATCTAGGACCTTCTAGAACATTCTGTAAGGCTGCAGAATGCGGGTATATAAGG
RzRev1-1	AGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCACCGTCCCGTGGG
RzRev1-2	AGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCACGGCCAAAGAGGTTA
RzRev2	GGACGTGCGTCCTCCTTCGGATGCCCAGGTCGGACCGCGAG
RzRev3-1	TCCCGGGCCGCGGCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCC
HIPR2F	GCTGCAGAATGCGGGTATATAAGGAAAGCGGGCTCAGAGGAAGCCTAAATCGGTTAACCGAATTA
RzRev3-2	AGGATCCCCGCGGCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCC
RNA1muX	GGCTCGAGGG**C**CT**C**GA**T**CAATACCAATCTTGGAAGCG[a]
R2MF	GTTCCAATTGGGAATGCCTTCTGAAG
R2MR	GTCCTTGTAGTCAGCGGCCGCTCCGGAGTCGTTGCGGGTAGCCAAGACG
R2FLAGF	GCGGCCGCTGACTACAAGGACGACGATGACAAGTAGAGCCGCGAGACATCAACATGC
HIPR2R	AGGATCCCCGCGGCTCCCTTA

[a] The mutated nucleotides are in bold type.

Foreign genes can be readily introduced and expressed in *C. elegans* by methodologies such as microinjection or gene bombardment (21–23). Foreign genetic material is usually preserved as an extrachromosomal DNA array and can be inherited by the next generation in a semistable manner (23, 24). Addition of a phenotypic selection marker (either a fluorescent or a behavioral marker) facilitates the maintenance of animals carrying the extrachromosomal arrays, or alternatively, the plasmids can be stably integrated into the *C. elegans* genome by X-ray or gamma ray irradiation (21). Using this approach, a transgenic Flock House virus replicon system has been previously created for *C. elegans* that supports Flock House virus RNA replication but not viral capsid assembly or spread (4). This system has been used to identify a role for RNAi in antiviral defense in *C. elegans* (4, 25), for transgenerational inheritance of small virus-derived RNAs (26), and to identify suppressors of RNA silencing (27). More recently, a transgenic system for generating recombinant Sindbis virus in *Drosophila* was described (28), providing the first example of engineering infectious virus particles in a metazoan host.

Here, we describe the first plasmid-based virus reverse genetics system in *C. elegans*. By generating transgenic animals that stably maintain plasmids encoding Orsay virus using standard nematode manipulations, we bypassed the need for traditional cell culture. Viable virus production was demonstrated by *de novo* infection of *C. elegans* with viral filtrates produced from the transgenic animals. In addition to recombinant wild-type Orsay virus, mutant viruses carrying either multiple synonymous mutations or a FLAG epitope tag fused to the Orsay alpha-delta fusion protein were generated. This novel reverse genetics system provides a route to engineer defined mutations in the Orsay genome that will greatly facilitate studies of both the virus life cycle and its interactions with the host.

MATERIALS AND METHODS

***C. elegans* strains.** Laboratory *Caenorhabditis elegans* Bristol N2 strain and the RNAi-deficient mutant *rde-1* strain (WM27) were maintained by feeding with *Escherichia coli* bacterial strain OP50 at 20°C on nematode growth medium (NGM) plates (29). Serial passage of both *C. elegans* N2 and the *rde-1* strain was done by transferring 1 cm^2 of agar with mixed-stage animals onto a fresh OP50-seeded NGM plate.

Plasmid construction. (i) pHIP_RNA1 cloning. The complete Orsay virus RNA1 segment was cloned into plasmid pPD49.83 driven by a *C. elegans* heat shock promoter (30). First, the hepatitis D virus (HDV) ribozyme sequence (31) was introduced at the 3′ end by step extension PCR with three overlapping primers (Table 1; see also Fig. S1 in the supplemental material). A multistep cloning strategy was used to insert this fragment into plasmid pPD49.83 as follows. A partial sequence of the heat shock promoter along with the NsiI site was introduced during step extension PCR amplification and was then cloned into a Topo-TA cloning vector (Invitrogen). The resulting Topo-RNA1 was digested by NsiI and XmaI. Two fragments were generated from the Topo-RNA1 genome besides the vector due to the presence of a second NsiI site in the Orsay virus RNA1 genome. One of the digested fragments flanked by NsiI and XmaI was subcloned into pPD49.83 vector, which was also digested by NsiI and XmaI restriction enzymes. The resulting intermediate plasmids were further singly digested by NsiI and ligated to the second delta-NsiI fragment digested from the aforementioned Topo-RNA1. The orientation of insertion was checked by restriction enzyme digestion and verified by Sanger sequencing. The final plasmid construct was designated pHIP_RNA1.

(ii) pHIP_RNA2 cloning. HDV ribozyme was introduced at the 3′ end of the Orsay virus RNA2 genome by step extension PCR. A strategy similar to that for cloning Orsay virus RNA1 was implemented (Table 1; see also Fig. S1 in the supplemental material). Briefly, part of the heat shock promoter sequence along with the PstI site was introduced into the RNA2 genome by PCR amplification and was then cloned into the Topo-TA vector (Invitrogen). The resulting Topo-RNA2 was digested by PstI and BamHI. The pPD49.83 vector was also digested by the two restriction enzymes PstI and BamHI, and a 335-bp fragment was removed from the vector, as there are two PstI sites on the vector sequence. Orsay virus RNA2 with HDV ribozyme and partial promoter sequence digested from Topo-RNA2 was ligated with pPD49.83 without the 335-bp fragment. The resulting plasmids were then singly digested by PstI, and the 335-bp fragment was then inserted. The orientation of insertion was checked by restriction enzyme digestion and verified by Sanger sequencing. The final plasmid construct was designated pHIP_RNA2.

(iii) pHIP_RNA1MUT cloning. For generation of a genetic marker in the Orsay virus genome, a fragment of Orsay virus RNA1 was PCR amplified with a forward primer, RNA1muX, which has the designated mutations (C, C, and T), and a reverse primer, HIPR1R. The PCR product was further digested with XhoI and XmaI and then ligated into pHIP_RNA1 digested with the same two restriction enzymes. The final plasmid was sequence confirmed by Sanger sequencing and was designated pHIP_RNA1MUT.

(iv) pHIP_RNA2FLAG cloning. A sequence encoding a short peptide linker (SGAAA) followed by the FLAG peptide (DYKDDDDK) was introduced into the 3′ end of the alpha-delta coding region of Orsay virus RNA2 by overlapping PCR. Briefly, a C-terminal 1-kb fragment of the Orsay virus RNA2 alpha-delta open reading frame (ORF) was generated by PCR with a forward primer, R2MF, and a reverse primer, R2MR. A 3′

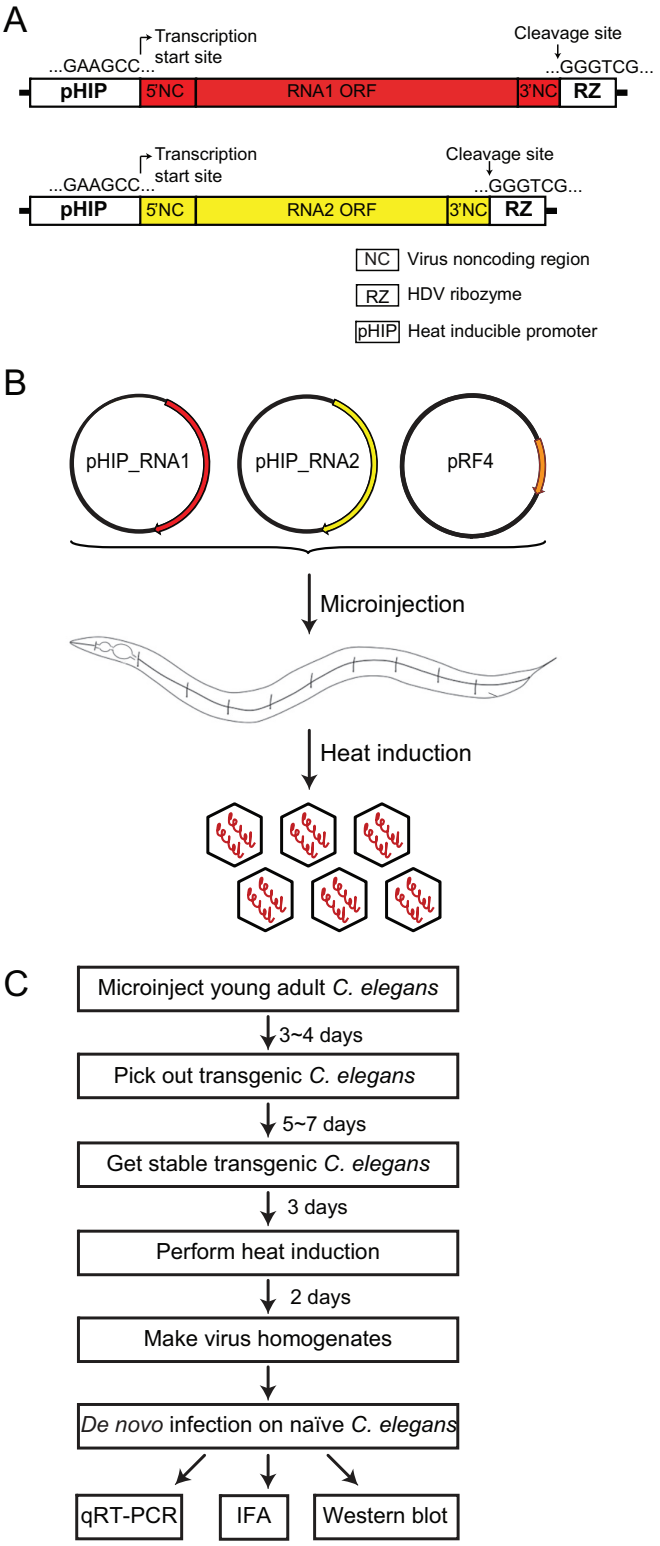

FIG 1 Design and work flow of Orsay virus reverse genetics. (A) Design of the Orsay virus reverse genetics constructs. Orsay virus cDNAs for the entire RNA1 and RNA2 genome segments were each cloned into a heat-inducible promoter (pHIP)-driven plasmid and juxtaposed at the 3′ end to the self-cleaving hepatitis D virus ribozyme (RZ) to generate pHIP_RNA1 and pHIP_RNA2, respectively. Arrows indicate the transcription start site and the ribozyme cleavage site. (B) Experimental strategy of the Orsay virus reverse genetics. Recombinant viral plasmids were microinjected into *C. elegans* to establish transgenic animals. Viruses were then produced from transgenic animals following heat induction. (C) Work flow describing Orsay virus reverse genetics. IFA, immunofluorescence assay.

fragment of pHIP_RNA2 was generated by PCR amplification with a forward primer, R2FLAGF, which bears the FLAG sequence, and a reverse primer, HIPR2R. The two fragments were then annealed together and amplified by primers R2MF and HIPR2R. The resulting fragment was digested by MfeI and BamHI and then cloned into the pHIP_RNA2 plasmid, which was digested by the same two restriction enzymes. The resulting plasmid is designated pHIP_RNA2FLAG. All primer sequences used in this study are listed in Table 1.

Microinjection and transgenic lines. One day prior to the microinjection experiment, L4 *C. elegans* larvae were picked onto a fresh OP50-seeded NGM plate. For microinjection, two viral cDNA-carrying plasmids (pHIP_RNA1 and pHIP_RNA2 or mutant forms of these plasmids) were mixed with the comicroinjection marker plasmid pRF4 (rol-6) (32) at final concentrations of 50 ng/μl, 50 ng/μl, and 120 ng/μl, respectively. *C. elegans* N2 or *rde-1* young adults were injected by using a microinjection system (Zeiss). Sets of three microinjected animals were placed on OP50-seeded 6-cm NGM plates and maintained at 20°C. F1 animals displaying a roller phenotype were individually transferred onto new OP50-seeded 6-cm NGM plates 3 to 5 days after injection. F1 plates that produce F2 roller animals were considered to contain a stable transgenic line. The stable transgenic lines were maintained and passaged by picking 5 roller transgenic progeny animals each time onto a new OP50-seeded 6-cm NGM plate.

Heat induction and transgenic virus filtrate preparation. Heat induction was performed on stable transgenic animals. Five F2 transgenic roller animals were transferred to a new OP50-seeded 6-cm NGM plate and maintained for 5 days at 20°C to generate a mixed population. For heat induction, animals were incubated at 33°C for 2 h and then at 23°C for 2 days. Animals on each 6-cm NGM plate were harvested by washing off the plates with 1 ml of M9 buffer, pelleted by spinning at 3,000 rpm for 1 min, and concentrated to 0.5 ml of M9 buffer. The concentrated animals were resuspended and transferred to a homogenizing tube. Animals were mixed with 0.5 ml of 1.0-mm silicon beads (zirconia/silica) and then homogenized by 2 cycles of shaking at 6,000 rpm for 1 min in a tissue homogenizer (MagNALyzer; Roche) and cooling on ice for 2 min. The resulting homogenate was centrifuged at maximum speed with a benchtop Eppendorf centrifuge for 5 min. The supernatant was collected and filtered through a 0.22-μm filter (Millipore) and stored at −80°C.

Virus infection and real-time qRT-PCR quantification of virus replication. To quantify Orsay virus production from transgenic *C. elegans*, we developed a standard 6-well format infection. Briefly, a synchronized population of *C. elegans* embryos was generated by bleaching, which kills all of the adult animals. Five hundred *C. elegans* embryos were added per well of a 6-well plate seeded with a 20-μl lawn of OP50 bacteria. Sixteen hours later, 20 μl of transgenic Orsay virus filtrate was added to the bacterial lawn of each well. Infected *C. elegans* animals were harvested 3 days after infection. Animals were washed off the plates using 1 ml of M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 ml of 1 M $MgSO_4$, H_2O to 1 liter) and then pelleted by centrifugation at 3,000 rpm for 2 min with a benchtop Eppendorf centrifuge. M9 buffer was removed by pipetting. The *C. elegans* pellet was resuspended with 350 μl of TRIzol (Invitrogen) and frozen in liquid nitrogen. RNA extraction and DNase treatment in the middle of RNA extraction were performed using a Zymo Direct-zol RNA extraction kit (Zymo Research) by following the manufacturer's protocol. RNA was eluted in 30 μl of RNase- and DNase-free water. One-step real-time quantitative reverse transcription-PCR (qRT-PCR) was performed with Orsay virus RNA1 or RNA2 probe in a 1:100 dilution of viral RNA as described previously (3).

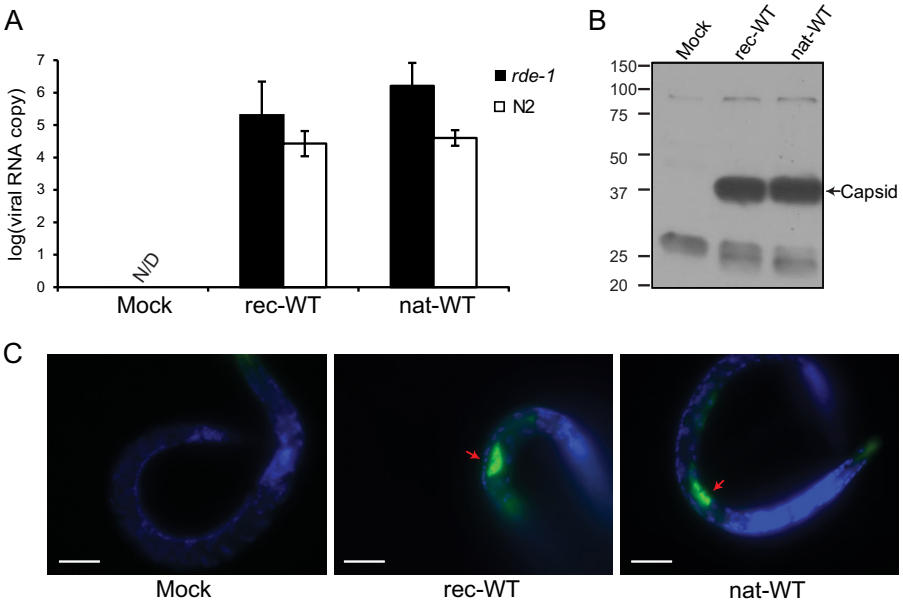

FIG 2 *De novo* infection of *C. elegans* by virus produced from transgenic animals. (A) Real-time qRT-PCR quantification of both recombinant wild-type Orsay virus (rec-WT) and naturally occurring Orsay virus (nat-WT) replication in *C. elegans* N2 and *rde-1* strains. A nontransgenic *C. elegans* N2 preparation was used as a mock control. N/D, viral RNA was not detected. Error bars indicate standard deviations from three independent infection experiments. (B) Western blot with anti-Orsay virus capsid antibody. Arrow indicates the virus capsid band. (C) Immunofluorescence staining of recombinant and natural Orsay virus replication in infected *C. elegans* with anti-Orsay virus capsid antibody. Arrows indicate the infected intestinal cells. Scale bars represent 20 μm.

Western blotting. *C. elegans* was infected with transgenic filtrate as described above. The infected animals in a 6-well plate were passaged to expand by transferring 1 cm^2 of agar to a 10-cm NGM plate seeded with OP50. Approaching starvation, infected *C. elegans* animals were harvested and homogenized. Western blotting was performed as previously described (3). Briefly, the infected homogenates were mixed with 6× sample loading buffer (0.35 M Tris-HCl, 10% SDS, 0.6 M dithiothreitol [DTT], 0.012% bromphenol blue, 30% glycerol) and boiled for 5 min. Protein samples were loaded into an 8.6-cm-long precast SDS-PAGE gel (Any kD Mini-PROTEAN TGX gel; Bio-Rad), and the gel was run at 30 mA for 1 h. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen) and blotted with specific viral capsid peptide-derived antibody.

Immunofluorescence assay. (i) Epifluorescence analysis of rec-WT and nat-WT infection (see Fig. 2). Immunofluorescence staining for Orsay virus capsid was performed as described before (33). The initial recombinant wild type Orsay virus (rec-WT)-infected and the natural Orsay virus (nat-WT)-infected *rde-1* animals were passaged by transfer from a 6-well NGM plate to a 10-cm OP50-seeded NGM plate. After 4 days, the infected *C. elegans* animals were fixed in Bouin's fixative and quickly frozen in liquid nitrogen. Immunofluorescence staining was performed by incubation with a rabbit anti-capsid primary antibody followed by Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (Invitrogen). Images for epifluorescence were acquired with a Zeiss Axioskop Mot Plus fluorescence microscope.

TABLE 2 Transgenic *C. elegans* strains generated in this study

Construct	Strain name or genotype	No. of transgenic strains	No. of virus-producing strains
rec-WT1	N2	3	1
rec-WT2	N2	4	3
rec-MUT	N2	7	3
rec-FLAG	*rde-1*	5	1

(ii) Confocal microscopy of rec-FLAG and rec-WT infection (see Fig. 4). *rde-1* animals were synchronized by bleaching, and 2,000 embryos were seeded in each well of a 6-well plate with a 20 μl of an OP50 bacterial lawn. *C. elegans* animals were infected 16 h later with 20 μl of rec-WT or recombinant FLAG-tagged Orsay virus (rec-FLAG) virus filtrate prepared as described above. Twenty-four hours after infection, the infected *C. elegans* animals were fixed with Bouin's fixative and frozen in liquid nitrogen. Double immunofluorescence staining was performed with an anti-FLAG M2 monoclonal antibody (Sigma) and viral protein-specific antibodies (rabbit-derived anti-capsid antibody or anti-delta antibody). Secondary antibodies were Alexa Fluor 488-labeled goat anti-mouse antibody or Alexa Fluor 568-labeled goat anti-rabbit antibody (Invitrogen). Nuclear staining was performed with TO-PRO3 (Invitrogen) for 20 min. Confocal images were acquired with a Zeiss LSM510 Meta laser scanning confocal microscope (Carl Zeiss Inc., Thornwood, NY).

RESULTS AND DISCUSSION

Experimental strategy for development of Orsay virus reverse genetics. The overall strategy for generating recombinant Orsay virus entailed engineering plasmids that contain cDNAs of Orsay virus RNA1 and RNA2 and then microinjecting the plasmids together to create transgenic animals harboring extrachromosomal arrays of the two plasmids, which should then initiate host cell transcription from the plasmids. In theory, translation of the DNA templated transcripts would lead to expression of the Orsay virus RdRP, which would then trigger authentic viral replication and subsequent capsid expression and viral packaging. To evaluate the production of viable virus, the animals were then homogenized and filtered through a 0.22-μm membrane; the resulting filtrate was then used for *de novo* infection of naive animals and subsequently assayed by qRT-PCR, Western blotting, or immunofluorescence assay (Fig. 1).

Specifically, cDNA sequences of the Orsay virus RNA1 and RNA2 were cloned into a *C. elegans* expression vector driven by a

heat-inducible promoter. One reason we chose this promoter is that it is known to be preferentially active in the *C. elegans* pharyngeal and intestinal tissue (30), which coincides with the Orsay virus *in vivo* tissue tropism (33). Prior studies that developed cell culture-based reverse genetics systems for influenza A virus and Pariacoto virus, an insect nodavirus, demonstrated the strict requirement for maintaining authentic termini of the genome segments for optimal viral replication (10, 11, 16). Therefore, we introduced a hepatitis D virus ribozyme at the 3′ end of the RNA1 segment, and at the 5′ end, the Orsay virus cDNA was cloned immediately 3′ to the heat-inducible promoter transcriptional start site (Fig. 1A). The transcript produced from this construct following ribozyme cleavage should, in theory, be identical in sequence to authentic Orsay virus genomic RNAs.

Wild-type Orsay virus produced from transgenic *C. elegans* is infectious. To demonstrate that the transgenic *C. elegans* reverse genetics system works in principle, we focused first on generating recombinant wild-type Orsay virus. Plasmids pHIP_RNA1 and pHIP_RNA2 were microinjected along with pRF4, a "roller" phenotypic marker plasmid, and three stable F2 transgenic animals were identified by screening for the roller phenotype. The stable transgenic animals were then heat shocked to induce the expression of Orsay virus RNA. Two days post-heat shock, expression of both Orsay virus RNA1 and RNA2 was detected by real-time qRT-PCR in all three lines (data not shown).

Virus filtrates were generated from each of these lines, and following infection of N2 and *rde-1* strains of *C. elegans*, one of the three filtrates yielded viable virus that behaved as natural Orsay virus (nat-WT). By qRT-PCR, the recombinant wild-type Orsay virus (rec-WT) produced from transgenic animals replicated to higher levels in the RNAi-deficient *C. elegans rde-1* strain than in the N2 strain, as seen with natural nat-WT infection (Fig. 2A). Patterns of viral capsid protein expression as assessed by both Western blotting (Fig. 2B) and immunofluorescence assay (Fig. 2C) were similar for both natural and recombinant Orsay virus. In a second independent experiment, three out of four stable transgenic strains that we generated produced recombinant wild-type Orsay virus (Table 2). Overall, the process of microinjection and identification of progeny producing virus requires about 15 days.

To evaluate the stability of virus production in the transgenic *C. elegans* harboring pHIP_RNA1 and pHIP_RNA2, we conducted 7 serial passages of the transgenic strain. Filtrates generated following heat induction of passages 5, 6, and 7 each contained viable virus as defined by *de novo* infection and qRT-PCR (see Fig. S2A in the supplemental material). In addition, an aliquot of the F4 that had been frozen in a −80°C freezer, upon revival and heat shock of its F5 progeny, also yielded viable virus (see Fig. S2A). These results demonstrate the generation of recombinant wild-type Orsay virus through a plasmid-based reverse genetics system in the metazoan host *C. elegans* and the stability of the transgenic strains.

A genetic marker can be introduced into Orsay virus genome by reverse genetics. We next created a plasmid containing a series of three synonymous point mutations that were in the wobble position of three consecutive codons (pHIP-RNA1MUT) in the middle of the Orsay virus RdRP to demonstrate that recombinant viruses with defined mutations could be generated (Fig. 3A). Transgenic *C. elegans* strains were created by microinjecting pHIP_RNA1MUT and pHIP_RNA2, and viral filtrates were prepared as before. Infection of the *rde-1* strain with mutant viruses

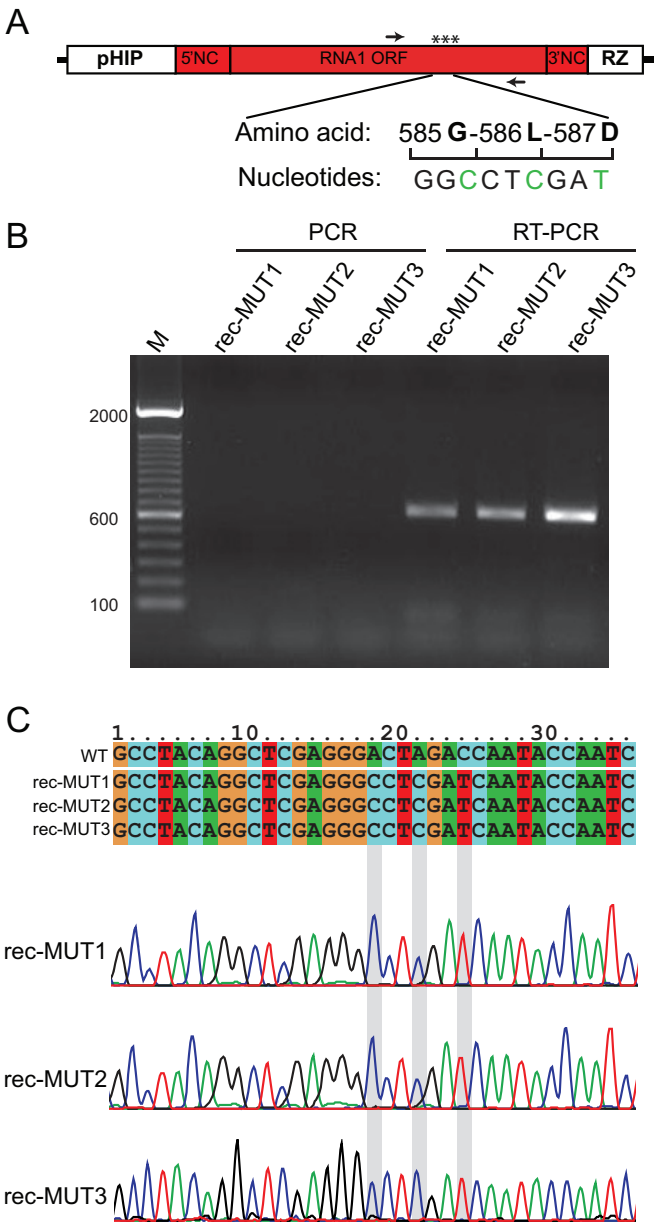

FIG 3 Introduction of a genetic marker into the Orsay virus genome. (A) Design of an Orsay virus genetic marker in its RNA1 genome segment. Arrows indicate the position where primers were used to perform RT-PCR. Letters in green highlight the three synonymous point mutations introduced, with asterisks indicating their positions in the genome. (B) RT-PCR and PCR spanning the mutated locus. RNA was extracted from three *C. elegans* cultures infected by three independent mutant virus filtrates. The expected PCR band is ~600 bp. M, molecular size marker. (C) The genetic marker was preserved in the recombinant virus as shown by the Sanger sequencing chromatogram of the three mutant viruses. Aligned boxes indicate the genetic marker.

(rec-MUT1, rec-MUT2, and rec-MUT3) produced from three independent transgenic strains showed levels of replication similar to that with rec-WT infection in the *rde-1* strain (data not shown). Confirming that these viruses were recombinant in origin, RT-PCR of RNA extracted from the *de novo*-infected *rde-1 C. elegans* generated the expected band (Fig. 3B), and sequencing of the amplicon yielded the three synonymous mutations (Fig. 3C). As a

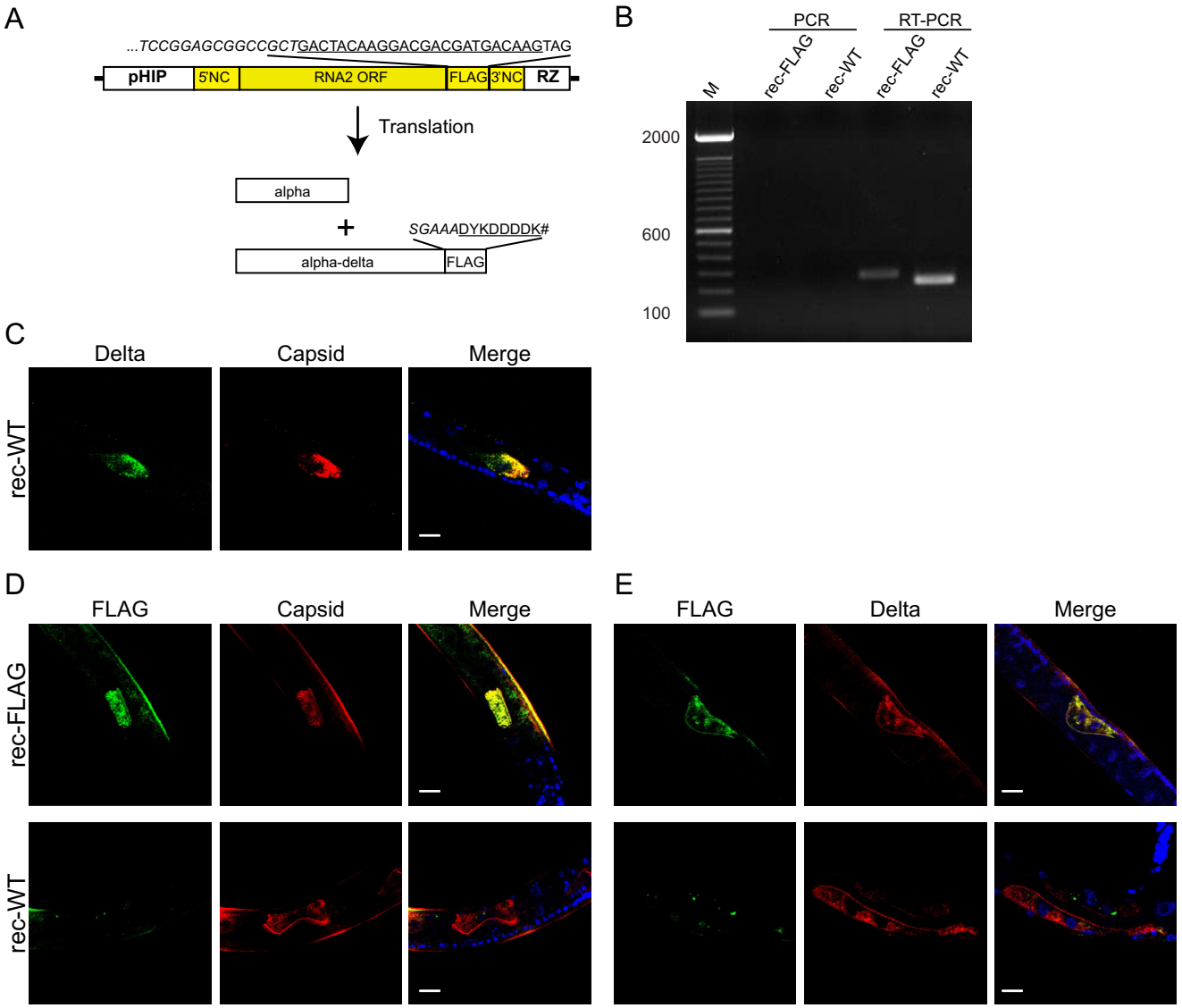

FIG 4 Generation and verification of a FLAG epitope-tagged Orsay virus. (A) A schematic design of FLAG-tagged Orsay virus RNA2 genome segment. Both the genome construct and expected proteins are shown. The nucleotide and amino acid sequences of FLAG peptide tag are underlined, and those for the linker peptide are italicized. #, stop codon. (B) RT-PCR and direct PCR were performed on the FLAG-tagged virus-infected *C. elegans*. (C) Double immunofluorescence staining of rec-WT-infected *C. elegans* with anti-Orsay capsid and anti-Orsay delta peptide antibodies. Scale bars represent 10 μm. (D) Double immunofluorescence staining of rec-FLAG- and rec-WT-infected *C. elegans* with anti-Orsay capsid peptide antibody and monoclonal anti-FLAG antibody. (E) Double immunofluorescence staining of rec-FLAG- and rec-WT-infected *C. elegans* with anti-Orsay delta peptide antibody and monoclonal anti-FLAG antibody.

control, PCR amplification in the absence of RT yielded no bands (Fig. 3B), eliminating any possibility of residual carryover of pHIP_RNA1MUT plasmid from the transgenic animal filtrate (Fig. 3B). Furthermore, serial passage of the rec-MUT virus-infected animals showed the presence of the three synonymous mutations in the recombinant virus genome at the third passage by sequencing (data not shown). These results clearly demonstrate the ability to generate mutants in the Orsay virus genome using this plasmid-based reverse genetics system.

Generation of a FLAG epitope-tagged Orsay virus by reverse genetics. To further demonstrate the feasibility of genetic engineering of Orsay virus, we next created a plasmid (pHIP_RNA2FLAG) that encoded an epitope tag (FLAG: DYKD DDDK) with a 5-amino-acid linker sequence, SGAAA (Fig. 4A). We have previously demonstrated that the Orsay virus RNA2 segment encodes the capsid protein (alpha) and an alpha-delta fusion protein of unknown function that is expressed by a ribosomal frameshifting mechanism (3). Thus, the FLAG tag was designed to be expressed at the C terminus of the alpha-delta fusion protein (Fig. 4A). Following generation of transgenic animals and virus filtrate preparation, *de novo* infection of *rde-1 C. elegans* demonstrated replication of the FLAG-tagged Orsay virus (data not shown). RT-PCR primers that span the FLAG sequence insertion site were designed. A band with higher molecular weight was obtained with RNA extracted from rec-FLAG-infected animals than with RNA from the corresponding rec-WT-infected *C. elegans*

(Fig. 4B, compare lane 3 to lane 4). No bands were obtained from either sample in the absence of reverse transcription. The presence of the FLAG sequence was confirmed by Sanger sequencing of the RT-PCR amplicon (data not shown).

To evaluate the expression of the FLAG epitope, the rec-FLAG virus was used to infect the *rde-1* strain. We have previously reported that infection by Orsay virus primarily leads to a detectable level of viral capsid protein in one or more intestinal cells (33). More recently, we determined that the delta protein is expressed primarily as a fusion with the capsid protein (3). Double immunofluorescence staining with antibodies raised against peptides from the capsid and delta regions led to costaining of the same cells with the same subcellular patterns (Fig. 4C). Thus, we anticipated that double staining of animals infected by rec-FLAG with anti-FLAG and either anti-capsid or anti-delta should similarly colocalize. As expected, staining with both anti-FLAG monoclonal antibody and the anti-Orsay capsid antibody of rec-FLAG-infected animals yielded colocalization of the capsid and FLAG peptide within the same intestinal cells (Fig. 4D). In addition, similar results were obtained when rec-FLAG-infected animals were costained with anti-FLAG antibody and an anti-delta peptide antibody (Fig. 4E). In each case, double staining of animals infected with rec-WT yielded only capsid or delta protein staining; no FLAG staining was observed. Based on these results, we conclude that it is possible to use this reverse genetics system to create recombinant forms of Orsay virus that express mutant or tagged proteins.

Because the presence of the FLAG epitope physically alters the alpha-delta fusion protein, we assessed the stability of the rec-FLAG virus. Infected animals were serially propagated by transfer every 4 days for 6 passages. At each passage, RNA was extracted and RT-PCR using primers flanking the FLAG insertion confirmed the presence of the FLAG coding sequence. No evidence of loss of the FLAG sequence or reversion to wild type was observed during this time frame (see Fig. S2B in the supplemental material; other data not shown).

In summary, we have demonstrated the feasibility of using a transgenic *C. elegans*-based reverse genetics system to generate recombinant mutants of Orsay virus. This capability will open new frontiers of viral research in *C. elegans*. In theory, it should now be possible to create fluorescent versions Orsay virus, for example, by fusion of green fluorescent protein (GFP) or introduction of a fluorescent RNA aptamer (34) that could be used to visualize and follow infection in live animals. Such a virus would also be ideal as a reporter for high-throughput functional screens of host genes that affect Orsay virus infectivity and replication. Of course, as with all reverse genetics systems, there are likely to be constraints on the size of the insertion that can be tolerated at both the genome and protein levels. Structural studies of the Orsay capsid and capsid-delta fusion would be helpful in providing insight into sites that may be more amenable to manipulation. Other applications of this system in the future could include efforts to make viruses that cannot express the capsid-delta fusion protein to facilitate efforts to define the function of this mysterious protein. Furthermore, by choosing tissue-specific promoters for initial expression constructs, it may also be possible to direct Orsay virus expression to specific tissues, as has recently been described for *Drosophila* with modified Sindbis virus (28). The successful development of Orsay virus reverse genetics also provides the possibility of generating similar systems for the other two *Caenorhabditis* nematode-infecting viruses, Le Blanc and Santeuil viruses (2). As both of those viruses have the opposite host specificity of Orsay virus (i.e., they can infect *Caenorhabditis briggsae* but not *C. elegans*), the availability of reverse genetics would enable the creation of reassortant or chimeric viruses that could then be used to explore viral and host factors that govern infection specificity. To conclude, the Orsay virus reverse genetics system developed in this work will greatly facilitate studies of Orsay virus and its interactions with its host, *C. elegans*.

ACKNOWLEDGMENTS

The *C. elegans* N2 and *rde-1* (WM27) strains used in this work were provided by the Caenorhabditis Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (grant P40 OD010440). We thank Shouwei Ding for sharing plasmid vector pPD49.83 and Michael Crowder for sharing plasmid pRF4. We also thank Xianrong Mao and Qun Zheng for their technical help with the establishment of microinjection.

D.W. holds an Investigator in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund.

REFERENCES

1. **Félix MA, Ashe A, Piffaretti J, Wu G, Nuez I, Belicard T, Jiang Y, Zhao G, Franz CJ, Goldstein LD, Sanroman M, Miska EA, Wang D.** 2011. Natural and experimental infection of Caenorhabditis nematodes by novel viruses related to nodaviruses. PLoS Biol. **9:**e1000586. http://dx.doi.org/10.1371/journal.pbio.1000586.
2. **Franz CJ, Zhao G, Felix MA, Wang D.** 2012. Complete genome sequence of Le Blanc virus, a third Caenorhabditis nematode-infecting virus. J. Virol. **86:**11940. http://dx.doi.org/10.1128/JVI.02025-12.
3. **Jiang H, Franz CJ, Wu G, Renshaw H, Zhao G, Firth AE, Wang D.** 2014. Orsay virus utilizes ribosomal frameshifting to express a novel protein that is incorporated into virions. Virology **450-451:**213–221. http://dx.doi.org/10.1016/j.virol.2013.12.016.
4. **Lu R, Maduro M, Li F, Li HW, Broitman-Maduro G, Li WX, Ding SW.** 2005. Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature **436:**1040–1043. http://dx.doi.org/10.1038/nature03870.
5. **Schott DH, Cureton DK, Whelan SP, Hunter CP.** 2005. An antiviral role for the RNA interference machinery in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U. S. A. **102:**18420–18424. http://dx.doi.org/10.1073/pnas.0507123102.
6. **Wilkins C, Dishongh R, Moore SC, Whitt MA, Chow M, Machaca K.** 2005. RNA interference is an antiviral defence mechanism in Caenorhabditis elegans. Nature **436:**1044–1047. http://dx.doi.org/10.1038/nature03957.
7. **Liu WH, Lin YL, Wang JP, Liou W, Hou RF, Wu YC, Liao CL.** 2006. Restriction of vaccinia virus replication by a ced-3 and ced-4-dependent pathway in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U. S. A. **103:**4174–4179. http://dx.doi.org/10.1073/pnas.0506442103.
8. **Guo X, Zhang R, Wang J, Ding SW, Lu R.** 2013. Homologous RIG-I-like helicase proteins direct RNAi-mediated antiviral immunity in C. elegans by distinct mechanisms. Proc. Natl. Acad. Sci. U. S. A. **110:**16085–16090. http://dx.doi.org/10.1073/pnas.1307453110.
9. **Ashe A, Belicard T, Le Pen J, Sarkies P, Frezal L, Lehrbach NJ, Felix MA, Miska EA.** 2013. A deletion polymorphism in the Caenorhabditis elegans RIG-I homolog disables viral RNA dicing and antiviral immunity. eLife **2:**e00994. http://dx.doi.org/10.7554/eLife.00994.
10. **Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y.** 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. U. S. A. **96:**9345–9350. http://dx.doi.org/10.1073/pnas.96.16.9345.
11. **Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A.** 1999. Rescue of influenza A virus from recombinant DNA. J. Virol. **73:**9679–9682.
12. **Conzelmann KK, Schnell M.** 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. J. Virol. **68:**713–719.
13. **Kobayashi T, Antar AA, Boehme KW, Danthi P, Eby EA, Guglielmi**

KM, Holm GH, Johnson EM, Maginnis MS, Naik S, Skelton WB, Wetzel JD, Wilson GJ, Chappell JD, Dermody TS. 2007. A plasmid-based reverse genetics system for animal double-stranded RNA viruses. Cell Host Microbe **1:**147–157. http://dx.doi.org/10.1016/j.chom.2007.03.003.

14. **Huang Q, Deng X, Yan Z, Cheng F, Luo Y, Shen W, Lei-Butters DC, Chen AY, Li Y, Tang L, Soderlund-Venermo M, Engelhardt JF, Qiu J.** 2012. Establishment of a reverse genetics system for studying human bocavirus in human airway epithelia. PLoS Pathog. **8:**e1002899. http://dx.doi.org/10.1371/journal.ppat.1002899.
15. **Racaniello VR, Baltimore D.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214:**916–919. http://dx.doi.org/10.1126/science.6272391.
16. **Johnson KN, Ball LA.** 2001. Recovery of infectious pariacoto virus from cDNA clones and identification of susceptible cell lines. J. Virol. **75:**12220–12227. http://dx.doi.org/10.1128/JVI.75.24.12220-12227.2001.
17. **Iwamoto T, Mise K, Mori K, Arimoto M, Nakai T, Okuno T.** 2001. Establishment of an infectious RNA transcription system for Striped jack nervous necrosis virus, the type species of the betanodaviruses. J. Gen. Virol. **82:**2653–2662.
18. **Ball LA, Johnson KL.** 1999. Reverse genetics of nodaviruses. Adv. Virus Res. **53:**229–244. http://dx.doi.org/10.1016/S0065-3527(08)60350-4.
19. **Ball LA.** 1992. Cellular expression of a functional nodavirus RNA replicon from vaccinia virus vectors. J. Virol. **66:**2335–2345.
20. **Biacchesi S.** 2011. The reverse genetics applied to fish RNA viruses. Vet. Res. **42:**12. http://dx.doi.org/10.1186/1297-9716-42-12.
21. **Mello C, Fire A.** 1995. DNA transformation. Methods Cell Biol. **48:**451–482. http://dx.doi.org/10.1016/S0091-679X(08)61399-0.
22. **Tabara H, Grishok A, Mello CC.** 1998. RNAi in C. elegans: soaking in the genome sequence. Science **282:**430–431. http://dx.doi.org/10.1126/science.282.5388.430.
23. **Mello CC, Kramer JM, Stinchcomb D, Ambros V.** 1991. Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J. **10:**3959–3970.
24. **Stinchcomb DT, Shaw JE, Carr SH, Hirsh D.** 1985. Extrachromosomal DNA transformation of Caenorhabditis elegans. Mol. Cell. Biol. **5:**3484–3496.
25. **Lu R, Yigit E, Li WX, Ding SW.** 2009. An RIG-I-Like RNA helicase mediates antiviral RNAi downstream of viral siRNA biogenesis in Caenorhabditis elegans. PLoS Pathog. **5:**e1000286. http://dx.doi.org/10.1371/journal.ppat.1000286.
26. **Rechavi O, Minevich G, Hobert O.** 2011. Transgenerational inheritance of an acquired small RNA-based antiviral response in C. elegans. Cell **147:**1248–1256. http://dx.doi.org/10.1016/j.cell.2011.10.042.
27. **Guo X, Lu R.** 2013. Characterization of virus-encoded RNA interference suppressors in Caenorhabditis elegans. J. Virol. 87:5414–5423. http://dx.doi.org/10.1128/JVI.00148-13.
28. **Wernet MF, Klovstad M, Clandinin TR.** 2014. Generation of infectious virus particles from inducible transgenic genomes. Curr. Biol. **24:**R107–R108. http://dx.doi.org/10.1016/j.cub.2013.12.009.
29. **Brenner S.** 1974. The genetics of Caenorhabditis elegans. Genetics **77:**71–94.
30. **Stringham EG, Dixon DK, Jones D, Candido EP.** 1992. Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic Caenorhabditis elegans. Mol. Biol. Cell **3:**221–233. http://dx.doi.org/10.1091/mbc.3.2.221.
31. **Perrotta AT, Been MD.** 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature **350:**434–436. http://dx.doi.org/10.1038/350434a0.
32. **Kramer JM, French RP, Park EC, Johnson JJ.** 1990. The Caenorhabditis elegans rol-6 gene, which interacts with the sqt-1 collagen gene to determine organismal morphology, encodes a collagen. Mol. Cell. Biol. **10:**2081–2089.
33. **Franz CJ, Renshaw H, Frezal L, Jiang Y, Felix MA, Wang D.** 2014. Orsay, Santeuil and Le Blanc viruses primarily infect intestinal cells in Caenorhabditis nematodes. Virology **448:**255–264. http://dx.doi.org/10.1016/j.virol.2013.09.024.
34. **Paige JS, Wu KY, Jaffrey SR.** 2011. RNA mimics of green fluorescent protein. Science **333:**642–646. http://dx.doi.org/10.1126/science.1207339.