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Rescue of Recombinant *Thogoto Virus* from Cloned cDNA

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***Thogoto virus* (THOV) is a tick-transmitted orthomyxovirus with a genome consisting of six negative-stranded RNA segments. To rescue a recombinant THOV, the viral structural proteins were produced from expression plasmids by means of a vaccinia virus expressing the T7 RNA polymerase. Genomic virus RNAs (vRNAs) were generated from plasmids under the control of the RNA polymerase I promoter. Using this system, we could efficiently recover recombinant THOV following transfection of 12 plasmids into 293T cells. To verify the recombinant nature of the rescued virus, specific genetic tags were introduced into two vRNA segments. The availability of this efficient reverse genetics system will allow us to address hitherto-unanswered questions regarding the biology of THOV by manipulating viral genes in the context of infectious virus.**

Thogoto virus (THOV) is the prototype tick-transmitted orthomyxovirus (18). The genome of THOV consists of six single-stranded RNA segments of negative polarity that are encapsidated by the viral nucleoprotein (NP) and associate with the viral RNA polymerase complex to form ribonucleoprotein complexes (vRNPs) (4, 17). Each individual segment codes for a single structural protein: the three subunits of the viral RNA polymerase complex (PB2, PB1, and PA) (11, 25), the viral surface glycoprotein (GP) (12), the NP (26), and the matrix protein (M) (10). Members of the genus *Thogotovirus* are structurally and genetically similar to the influenza viruses but are unique in their ability to infect mammalian as well as tick cells (15). The host change between vertebrates and arthropodes requires specific adaptations to allow the virus to replicate in both cell types. Accordingly, THOV has unique features like the single GP that has no similarities to the influenza virus glycoproteins but has similarity with the surface glycoproteins of baculoviruses (12). In addition, THOV has a unique cap-snatching mechanism, using only the cap structure and one additional nucleotide from cellular mRNAs to initiate viral transcription (2, 26). Moreover, the genome of THOV does not encode additional proteins, like the NS2/NEP or the NS1 of influenza A virus (FLUAV). NS2/NEP is essential for the export of the newly synthesized vRNPs out of the nucleus (13, 16). The nonstructural protein NS1 has been shown to suppress interferon production and the interferon-mediated antiviral response of the infected host cell, most likely by sequestration of double-stranded RNA molecules (7, 23). Since THOV lacks analogous proteins, it depends on the basic set of its six structural proteins to perform nuclear export of the vRNPs and to deal with the interferon-dependent suppression of viral replication. Specific manipulations of the THOV genome should allow to assign such functions to defined viral genes.

We recently succeeded in generating THOV-like particles (24). In this system, synthesis of the six structural THOV proteins together with a model minigenome RNA was sufficient for the formation of functional vRNPs and assembly of infectious virus-like particles. Here, we modified this system by expressing all six genomic vRNA segments from RNA polymerase I-driven expression plasmids instead of the model minigenome. This modification allowed us to rescue infectious recombinant THOV (recTHOV) entirely from cloned cDNAs.

MATERIALS AND METHODS

Plasmid constructs. The structural proteins of THOV were produced from the expression vectors pG7-PB2, pBS-PB1, pBS-PA, pBS-GP, pG7-NP, and pBS-M, all under the control of the T7 RNA polymerase promoter, as described previously (24, 27). These cDNA plasmids were used as templates to generate RNA polymerase I constructs for the expression of the full-length genomic segments of THOV. The cDNAs were amplified by PCR using primers containing *Bsm*BI restriction sites and sequences corresponding to the 3′ and 5′ noncoding sequences of the genomic segments (all accession numbers are from GenBank): segment 1, nucleotides (nt) 1 to 14 and 2325 to 2375 (accession no. Y17873); segment 2, nt 1 to 25 and 2159 to 2212 (accession no. AF004985); segment 3, nt 1 to 20 and 1890 to 1927 (accession no. AF006073); segment 4, nt 1 to 15 and 1555 to 1574 (accession no. M77280); segment 5, nt 1 to 20 and 1386 to 1418 (accession no. X96872); and segment 6, nt 1 to 20 and 937 to 956 (accession no. AF236794). The sequences of the primers will be provided on request. The PCR products were digested with *Bsm*BI and inserted into the *Bsm*BI site of pHH21 between the human RNA polymerase I promoter and terminator regions (kindly provided by Gerd Hobom, Justus Liebig University, Giessen, Germany) (14), yielding pHH21-vPB2, pHH21-vPB1, pHH21-vPA, pHH21-vGP, pHH21-vNP, and pHH21-vM. To introduce silent mutations into the cDNA of pHH21-vGP and pHH21-vNP, we amplified two overlapping cDNA fragments with a common *Kpn*I or *Nsi*I site, respectively. For the PCR, we combined primer S4/PstI (H45; nt 1273 to 1310) (5′ CTCTGGTACCCTTCTGCAGCCGAAGTCGATTTTAG GGG 3′) and the reverse-sense counterpart (H46; nt 1263 to 1286) with the primers coding for the noncoding regions of segment 4 and primer S5/ClaI (H41; nt 631 to 660) (5′ GGAAATCGATCGTCGGGCACCTCAAGCGCC 3′) and the reverse-sense counterpart (H42; nt 611 to 645) with the primers coding for the noncoding regions of segment 5. These internal primers introduced a unique *Pst*I restriction site into the segment 4 cDNA at position 1290 and a second *Cla*I restriction site into segment 5 cDNA at position 637. The PCR products were digested with *Bsm*BI/*Kpn*I for segment 4 and *Bsm*BI/*Nsi*I for segment 5 and inserted into pHH21 in a three-molecule ligation reaction. The sequences of all PCR-derived cDNA constructs were confirmed by sequencing.

Cells, viruses, and antibodies. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. We used 293T human embryonic kidney cells for transfection and African

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green monkey kidney (Vero) cells and BHK-21 cells for the cultivation of the viruses.

Thogoto virus strain SiAr126 (wild-type THOV) (1) was used as a control. The recombinant MVA-T7 vaccinia virus expressing the T7 RNA polymerase was kindly provided by Gerd Sutter (GSF, Neuherberg, Germany) (22).

A polyclonal hyperimmunized guinea pig antiserum directed against THOV proteins (kindly provided by P. A. Nuttall, NERC Institute of Virology and Environmental Microbiology, Oxford, United Kingdom) (9) was used for the neutralization experiments.

Generation of recTHOV. A monolayer of 293T cells (10^6 cells in 35-mm-diameter dishes) was infected with 10 PFU of MVA-T7 per cell for 1 h at 37°C. Then, the cells were transfected with the T7 expression plasmids and the RNA polymerase I expression plasmids with LipofectAMINE 2000 (Gibco BRL). The 12 plasmids were used in the following quantities: 500 ng of pG7-PB2, 500 ng of pBS-PB1, 500 ng of pBS-PA, 500 ng of pBS-GP, 2.5 μg of pG7-NP, 250 ng of pBS-M, and 500 ng of each pHH21 expression plasmid. After 5 h, the transfection solution was replaced with 1 ml of DMEM with 5% fetal calf serum and 20 mM HEPES (pH 7.3), and the cells were further incubated at 37°C for 4 to 5 days. The supernatant was then collected, cleared from cell debris, and passaged onto a monolayer of Vero cells (3×10^6 in 60-mm-diameter dishes). After 1 h of virus attachment, the inoculum was exchanged for 5 ml of DMEM with 5% fetal calf serum and 20 mM HEPES (pH 7.3), and the cells were further incubated for 5 days or until a cytopathic effect was visible. recTHOV present in the supernatant was subjected to plaque purification on Vero cells.

Genetic analysis of recTHOV. To detect the silent mutations introduced into segments 4 and 5, the supernatants of Vero cells (10^6 cells in a 35-mm-diameter dish) infected with wild-type THOV or recTHOV were used to isolate vRNAs from polyethylene glycol-precipitated virus particles. A total of 1.25 ml of supernatant was mixed with 250 μl of polyethylene glycol 8000 (40% in 2.5 M NaCl). The mixture was incubated for 30 min on ice and then spun down in a microcentrifuge at 15,000 × *g* for 20 min. The pellets were resuspended in 200 μl of a solution containing 10 mM Tris (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, and 0.3% sodium dodecyl sulfate. A total of 200 μl phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the samples were incubated at 56°C for 10 min with occasional mixing. The RNA was precipitated from the aqueous phase with ethanol. vRNAs were reverse transcribed using primer H66 (nt 481 to 500) for segment 4 and primer H71 (nt 467 and 486) for segment 5, and the cDNAs were then amplified by PCR with primers specific for segment 4 (nt 481 to 500 and 1536 to 1574) and segment 5 (nt 467 to 486 and 1373 to 1418). The reverse transcriptase (RT)-PCR products were analyzed for the presence of the novel restriction sites by digestion of the PCR products of segment 4 with *Pst*I and the PCR products of segment 5 with *Cla*I.

Growth properties and virus plaque assay. To determine the growth characteristics of recTHOV, the viruses of two independent transfection experiments were plaque purified and used to prepare virus stocks on Vero cells. In parallel, virus stocks of wild-type THOV were prepared from plaque-purified viruses. BHK cells in 25-cm^2 flasks were infected with these plaque-purified wild-type and recTHOV isolates at a multiplicity of infection of 0.01 PFU per cell and incubated at 37°C. At different time points, the supernatants were assayed for infectious virus by titration on Vero cells. The virus titers were calculated as reciprocals of the 50% tissue culture infective dose per ml. For plaque assays, monolayers of Vero cells in six-well macroplates (35 mm) were infected with about 100 PFU of THOV. For plaque reduction assays, the viruses were incubated on ice with a partially neutralizing solution of guinea pig antiserum for 60 min prior to infection. After incubation at 37°C for 1 h, the virus inoculum was removed, and medium containing 2% fetal calf serum, 20 mM HEPES (pH 7.3), 0.4% Noble agar, and 0.002% DEAE-dextran was added. After incubation at 37°C for 4 days, the agar overlay was removed, and the cells were stained with a solution of 1% crystal violet, 3.6% formaldehyde, 1% methanol, and 20% ethanol.

RESULTS AND DISCUSSION

Rescue of infectious recTHOV. To generate recTHOV, we cotransfected the full set of the six RNA polymerase I constructs encoding the six individual viral genomic RNA segments in negative-sense orientation together with the six T7 expression plasmids encoding the THOV structural proteins into 293T cells which were infected with MVA-T7 (Fig. 1). Infection with the attenuated recombinant vaccinia virus MVA-T7 (22) provided the bacteriophage T7 RNA polymerase necessary to synthesize the structural proteins of THOV. MVA-T7 infection did not cause cytopathic effects, and no progeny vaccinia viruses were produced. After 4 days, the supernatants of the transfected cells were passaged onto Vero cells known to be highly permissive for THOV (6). Plaque formation on Vero cells revealed that we were able to rescue recTHOV in most transfection experiments.

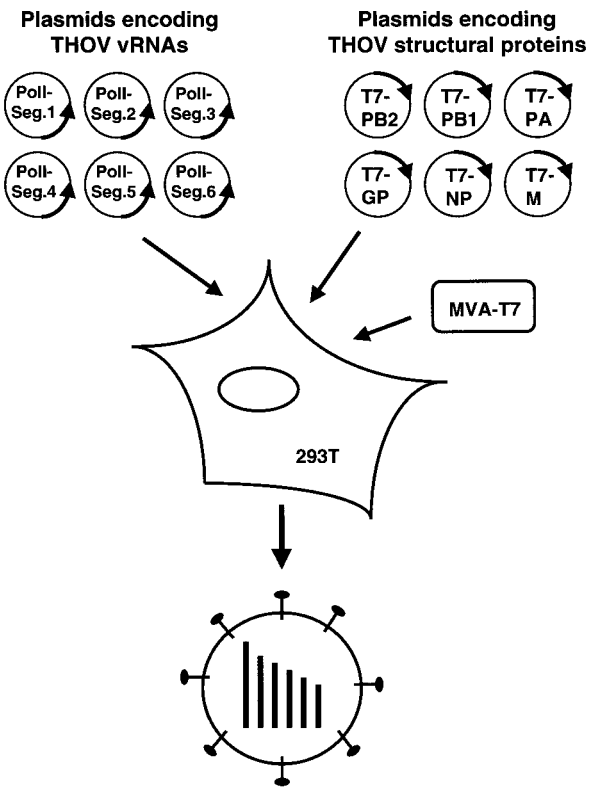

FIG. 1. Reverse genetics system for the generation of recTHOV. Twelve plasmids were transfected into MVA-T7-infected 293T cells. The six genomic negative-sense RNA segments were produced from expression plasmids containing the human RNA polymerase I promoter (Poll-Seg. 1 to 6). The six structural proteins of THOV were synthesized from expression plasmids under the control of the T7 promoter; the T7 RNA polymerase was provided by a recombinant vaccinia virus, MVA-T7. Infectious recTHOV was generated and released into the cell supernatant.

To monitor the time course of recTHOV production, aliquots of the supernatants of the transfected cells were removed every 24 h and titrated for infectivity. Table 1 summarizes the results of these experiments. Transfection of the full set of 12 plasmids into 10^6 cells yielded recTHOV between 48 and 96 h (Table 1, experiments 1 and 3). In most experiments, the initial titers of recombinant virus increased up to 10^7 PFU/ml, probably due to amplification of newly formed recombinant viruses in the 293T cell culture; whereas in some experiments, no infectious virus could be detected (Table 1, experiment 2).

In principle, synthesis of the three subunits of the vRNA polymerase and the nucleoprotein together with the genomic vRNAs should be sufficient to generate recombinant viruses, as has been demonstrated for FLUAV (5, 14). This would allow the omission of the T7 expression constructs coding for GP and M from the set of transfected plasmids. We attempted to rescue recTHOV by omitting the T7 expression plasmid coding

TABLE 1. Generation of recTHOV, following plasmid transfection of 293T cells

Hours after plasmid transfection	Virus titers in culture supernatant (PFU/ml) in expt[a]:				
	1 (complete)	2 (complete)	3 (complete)	4 (−T7/M)	5 (−T7/M)
24	0	0	0	0	0
48	10	0	0	0	0
72	1.1×10^4	0	0	0	0
96	1×10^6	0	90	0	1
120	1.5×10^7	0	2.4×10^3	1	2.4×10^3
144	7.5×10^7	0	4.4×10^4	3×10^3	8.7×10^4
172	5×10^7	0	1.5×10^6	4.6×10^4	2×10^7

[a] 293T cells were transfected with the full set of 12 expression plasmids (complete) or with all plasmids except the M-encoding helper plasmid (−T7/M). At different time points, the virus titer of the culture supernatants was determined.

for M. This also led to the formation of progeny (Table 1, experiments 4 and 5). Similarly, we were able to recover recTHOV by using only the four expression plasmids coding for the three polymerase subunits and NP (data not shown).

In the rescue system described here, two established protocols for the rescue of negative-strand RNA viruses were combined. The T7 RNA polymerase was used to produce the required viral proteins in high quantities. The cellular RNA polymerase I expression system was used to provide vRNA molecules with the correct 3′ and 5′ ends of the authentic viral genome segments, as described for the rescue of FLUAV (5, 14). It should be noted that recTHOV was rescued from RNA polymerase I expression plasmids producing negative-sense vRNAs. This is in contrast to earlier studies describing the recovery of recombinant negative-strand RNA viruses using positive-sense, antigenomic RNAs (3, 19, 20, 21). In these studies, expression of positive-sense antigenomic RNAs was chosen to avoid any risks of hybridization with the mRNA transcripts coding for the support proteins. In our rescue system, formation of such double-stranded RNA species was presumably prevented by physically separating the synthesis of mRNA transcripts from that of vRNA transcripts within the cytoplasmic and nuclear compartments. Recently published systems to generate recombinant FLUAV used nuclear RNA polymerase I to express the vRNA segments and nuclear RNA polymerase II to produce mRNAs (5, 8, 14), suggesting that, at least for FLUAV, the simultaneous expression of positive-sense mRNA and negative-sense vRNA species in the same cellular compartment was not a problem for the rescue of recombinant viruses.

Identification of the genetically tagged recTHOV. To prove that the rescued virus was derived from the transfected cDNAs and did not represent a laboratory contamination, we introduced silent mutations into the cDNAs encoding segments 4 and 5. The altered nucleotide sequences resulted in a new *Pst*I restriction site in segment 4 and a second *Cla*I restriction site in segment 5. In both cases, the amino acid sequence of the encoded viral proteins was not altered. To identify the silent mutations, recTHOV obtained after transfection of the 12 plasmids was plaque purified and propagated on Vero cells.

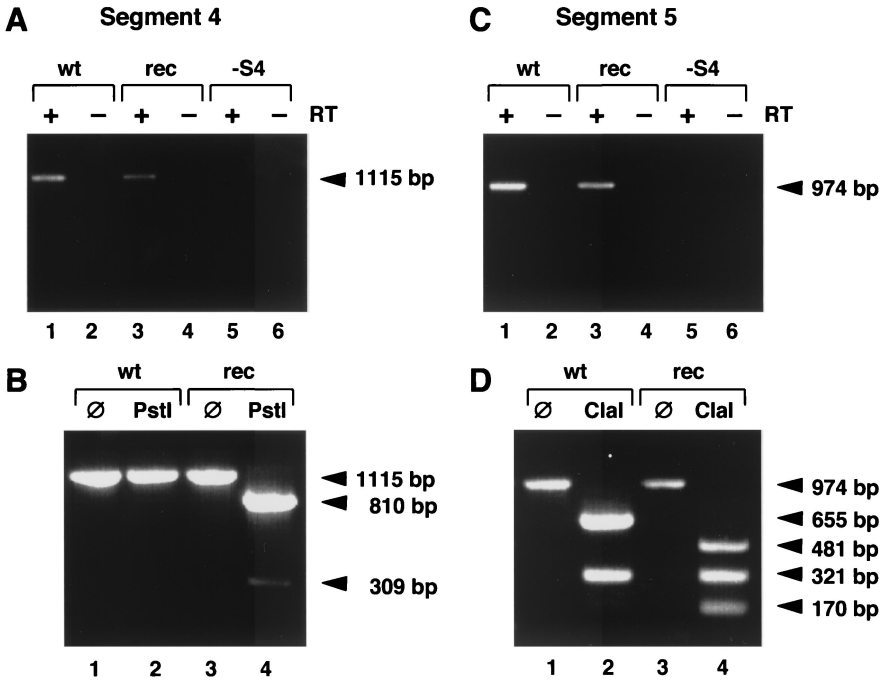

FIG. 2. recTHOV carries genetic markers. vRNA was isolated from virus particles obtained from supernatants of recTHOV-infected (rec) or wild-type THOV-infected (wt) cells. As a control, supernatants of Vero cells treated with the supernatants of 293T cells which had been transfected with plasmids for all vRNA segments except segment 4 (-S4) were used for RNA isolation. Segment 4 (A and B) and segment 5 (C and D) genomic vRNAs were detected by RT-PCR with primers that allow the amplification of a 1,115-bp segment 4 fragment (position 481 to 1574) and a 974-bp segment 5 fragment (position 467 to 1418). RT-PCRs without RT enzyme (−RT) were used as controls. The presence of the newly created *Pst*I site in the cDNA of segment 4 (B) and of the additional *Cla*I restriction site in the cDNA of segment 5 (D) of the recTHOV was determined by restriction analysis of the RT-PCR products. The products were analyzed by agarose gel electrophoresis in the presence of ethidium bromide. The molecular sizes of the fragments are indicated at the right.

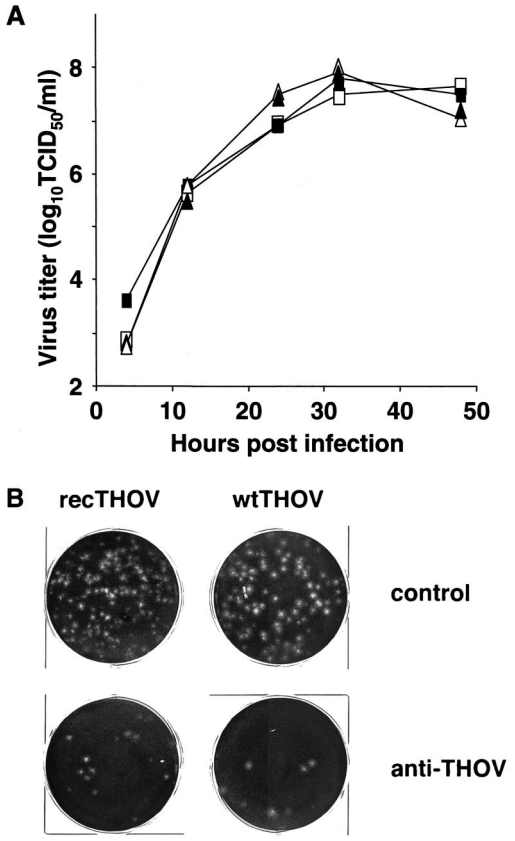

FIG. 3. Growth characteristics of recTHOV. (A) Growth curves of recTHOV and wild-type THOV. BHK cells were infected with plaque-purified isolates of THOV at a multiplicity of infection of 0.01 PFU per cell. At the indicated times after infection, the virus titer in the supernatant was determined. □, wild-type isolate 1; ■, wild-type isolate 2; △, recTHOV isolate 1; ▲, recTHOV isolate 2. (B) Plaque formation of recTHOV. Vero cell monolayers were infected with about 100 PFU of recTHOV or wild-type THOV and incubated under soft agar. For neutralization, 100 PFU of wild-type THOV or recTHOV was preincubated with a guinea pig antiserum directed against THOV for 60 min before infection of Vero cells. After 4 days, cells were fixed and stained with crystal violet.

The progeny virus was harvested from the cell supernatant, and genomic RNA was extracted. The vRNA preparation was used to amplify short cDNA fragments by RT-PCR using primers specific for segments 4 and 5. In parallel, the same protocol was applied to wild-type virus. Analysis of the RT-PCR products by agarose gel electrophoresis revealed cDNA fragments of the expected sizes of 1,115 and 974 bp for segments 4 and 5, respectively (Fig. 2A and C, lanes 1 and 3). Amplification of the same vRNA samples without the RT step failed to produce positive signals (Fig. 2A and C, lanes 2 and 4), excluding the presence of cDNA contaminations in the vRNA preparations. As a further control, cells were transfected with the full set of expression plasmids except that for vRNA segment 4. The supernatant of this transfection experiment was passaged onto Vero cells, and the resulting supernatant was treated exactly as described for the isolation of vRNA from virus-infected cells. Analysis of this RNA preparation did not show any signal in the RT-PCR (Fig. 2A and C, lanes 5 and 6), as expected. Next, the RT-PCR products were incubated with the appropriate restriction enzymes. Treatment of the PCR product derived from segment 4 of recTHOV with *Pst*I resulted in two fragments, whereas only one band, corresponding to the uncleaved PCR product, was detected in the case of wild-type THOV (Fig. 2B). Similarly, digestion of the RT-PCR products of segment 5 with *Cla*I revealed the presence of the extra *Cla*I restriction site in the cDNA derived from recTHOV but not that from wild-type THOV (Fig. 2D). These results demonstrate that the rescued virus was a true recombinant virus derived from the transfected cDNAs.

Characterization of recTHOV. We compared the growth properties of the rescued virus with that of wild-type THOV in BHK cells (Fig. 3A). Wild-type THOV and recTHOV from two independent transfection experiments were plaque purified. Stocks derived from these purified viruses were used to infect BHK cells. Yields of progeny viruses in the culture supernatants were determined at different time points postinfection. The recTHOV isolates and the wild-type viruses produced titers of about 4×10^7 to 8×10^7 50% tissue culture infective doses per ml of the culture supernatant at 32 h postinfection. Clearly, recTHOV did not differ appreciably from the wild-type virus in either growth rate or the maximal titer reached.

To further characterize the rescued virus, we performed plaque assays on Vero cell monolayers. As shown in Fig. 3B, the plaque size of wild-type and recTHOV was approximately equal, again indicating that the two viruses had comparable growth characteristics. We further compared the neutralizing capacity of a polyclonal antiserum directed against THOV by preincubating about 100 PFU of recTHOV or wild-type THOV with the antibody before testing for infectivity by plaque assay. The antiserum was used at a dilution to allow some breakthrough of the viruses. Growth of both viruses was reduced to a similar degree, indicating that recTHOV and wild-type THOV are antigenically identical (Fig. 3B).

In summary, we have established an efficient system for the rescue of recTHOV entirely from cDNA without the need of a homologous helper virus. Our system combines the strong T7-driven synthesis of the viral structural proteins with the RNA polymerase I-dependent expression of the six genomic RNA segments. The recovered recTHOV showed properties similar to that of authentic wild-type THOV. Therefore, THOV is the second orthomyxovirus for which a reverse genetics system is now available. This system will allow the study of THOV-specific aspects of the orthomyxovirus life cycle by observing the effects of specific mutations in the viral genome. It can be used to study open questions of the biology of THOV: the importance of the baculovirus-like GP of THOVs for the host change between mammals and ticks or the influenza C virus-like coding strategy of the THOV M (10). In addition, questions about virally encoded activities like the NS2/NEP-dependent nuclear export pathway or the M2 ion channel activity, which are essential for FLUAV multiplication but seem to be dispensable for THOV, can now be studied in the context of recTHOV.

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