Reverse genetics identifies the product of open reading frame 4 as essential particle assembly factor of Nyamanini virus

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ABSTRACT

We established a reverse genetics system for Nyamanini virus (NYMV) and recovered GFP-expressing virus from full-length cDNA. Using this technology, we assessed the functions of two poorly characterized viral genes. NYMV lacking open reading frame 2 (ORF2) could not be rescued, whereas virus lacking ORF4 was replication-competent. ORF4-deficient NYMV readily established a persisting non-cytolytic infection but failed to produce infectious viral particles, supporting the view that ORF4 represents an essential factor for NYMV particle assembly.

MAIN TEXT

Nyamanini virus (NYMV) is the prototype member of a novel genus in the order Mononegavirales, designated “Nyavirus” (11). Recently, Kuhn and coworkers (7) proposed classifying NYMV as a member of a new mononegaviral family, designated “Nyamiviridae”. NYMV replicates in the nucleus of infected cells (5), and viral budding was proposed to occur at the plasma membrane (11). The NYMV genome contains six major open reading frames (ORF). The viral nucleoprotein N, the glycoprotein G and the polymerase L are encoded by ORF1, ORF5 and ORF6, respectively. We recently showed that ORF3 codes for a polymerase cofactor which is required for NYMV polymerase activity (5). The functions of the proteins encoded by ORF2 and ORF4 remain largely unknown. ORF2 negatively regulates NYMV polymerase activity, presumably through interaction with the ORF3 product (5). ORF2 was further shown to promote production of infectious virus-like particles by an unknown mechanism (5). Formation of virus-like particles required the simultaneous presence of the viral G protein, the product of ORF4 and the product of ORF2, indicating that ORF2 and ORF4 both play a role in NYMV particle assembly (5). It remains unclear, however, whether ORF4 represents the NYMV-specific equivalent of a matrix protein typically found in negative-strand RNA viruses. No substantial homology was detected between ORF4 of NYMV and matrix proteins of other members of the order Mononegavirales (11).

Matrix (M) proteins of negative-strand RNA viruses orchestrate the assembly of viral particles by binding to both, the viral ribonucleoprotein (RNP) complex and cellular membranes (reviewed in (3) and (8)). The importance of M proteins for the assembly and budding of negative-strand RNA viruses was directly demonstrated for rabies virus, measles virus and Sendai virus by employing reverse genetics approaches (2, 6, 10). M-deficient variants of these viruses showed extensive cell-to-cell spreading but failed to produce cell-free infectious particles.
To further characterize the ORF2 and ORF4 genes of NYMV, we set out to establish a reverse genetics system for recovering recombinant virus from cloned cDNA. Using cDNA fragments generated by PCR from RNA of NYMV-infected Vero cells we constructed a rescue plasmid containing the putative full-length NYMV antigenome. As recently described for reverse genetics systems of Borna disease virus and measles virus (9), the full-length NYMV cDNA was inserted into the vector pBR-Pol-II in which expression of the viral antigenomic RNA is controlled by an RNA polymerase II promoter. For correct in vivo processing of the 5’ and 3’ termini of plasmid-derived viral RNA molecules, we inserted sequences encoding a NYMV-specific hammerhead and a hepatitis delta virus ribozyme at the 5’ and 3’ ends of the NYMV antigenome, respectively. The complete sequence of the rescue plasmid pNYMV is provided as supplementary figure 1. For easy discrimination between plasmid-derived recombinant and authentic NYMV we introduced a silent A to G mutation at position 416 (A416G) of the NYMV antigenome, thereby creating a diagnostic XhoI restriction site (Fig. 1A). To recover NYMV from cloned cDNA, we co-transfected 293T cells in 6-well plates with the NYMV antigenome-encoding vector pNYMV (4 µg) and pCA expression plasmids encoding NYMV-N (0.5 µg), polymerase cofactor ORF3 (0.2 µg) and NYMV-L (0.2 µg) (5). Approximately five days post transfection, cytopathic effects were detected in such cultures but not in cultures lacking either of these plasmids (data not shown), indicating productive replication of plasmid-derived NYMV. To confirm successful recovery of recombinant NYMV, we transferred cell-free supernatant from the rescue cell culture onto freshly seeded Vero cells. After a cytopathic effect appeared some three days later, we isolated total RNA from this culture and performed RT-PCR to amplify a fragment of the viral N gene. RNA from authentic NYMV-infected Vero cells served as control. Restriction digestion of the PCR products confirmed the presence of genetically modified viral RNA carrying the diagnostic XhoI restriction site (Fig. 1B), unambiguously demonstrating successful rescue of genetically modified NYMV.

To determine whether recovery of a GFP-expressing NYMV would likewise be possible, we generated the rescue plasmid pNYMV-GFP (Fig. 1A). For this, we modified pNYMV by inserting an expression cassette for humanized GFP (hrGFP) between the viral ORF3 and ORF4 genes. To ensure proper expression of the foreign gene, the hrGFP cassette was flanked by copies of the transcriptional start signal from the viral G gene (S\(_G\)) and the transcriptional termination signal from the ORF3 gene (T\(_{ORF3}\)) (Fig. 1A). The complete sequence of the rescue plasmid pNYMV-GFP is provided as supplementary figure 2. To reconstitute virus, pNYMV-GFP was transfected into 293T cells together with expression plasmids coding for
the NYMV proteins N, ORF3 and L. Four days later, GFP-positive cells first were detectable, and by day 8 post transfection the GFP-expressing virus had successfully spread through almost the entire cell culture (Fig. 1C).

To determine whether the various recombinant NYMV variants might exhibit reduced fitness, we infected Vero cells either with authentic NYMV, recombinant NYMV-(A416G) or recombinant NYMV-GFP at an MOI of 0.01. To measure both, cell-associated as well as cell-released virus, the cells were scraped into the culture supernatant every 24 hours and the virus was liberated from its host cells by three repeated freeze-thaw cycles. Titration on Vero cells using the 50% tissue culture infective dose (TCID$_{50}$) assay revealed indistinguishable growth properties of authentic NYMV and recombinant NYMV-(A416G), whereas the GFP-expressing NYMV seemed to multiply at slightly reduced rate compared to authentic or recombinant NYMV lacking the foreign gene cassette (Fig. 1D).

We next used the NYMV rescue system to assess the function of two hitherto poorly characterized NYMV proteins. To this end we tried to recover recombinant viruses in which either ORF2 (NYMV-$\Delta$ORF2/GFP) or ORF4 (NYMV-$\Delta$ORF4/GFP) was replaced by hrGFP. Complete sequences of the corresponding rescue plasmids are provided in the supplementary figures 3 and 4. Unlike pNYMV-GFP (Fig. 1C), vector pNYMV-$\Delta$ORF2/GFP failed to support virus recovery in repeated attempts. Although individual cells displayed strong GFP signals by day 7 post transfection, pNYMV-$\Delta$ORF2/GFP did not spread through the culture. Instead, green cells were frequently observed to round up, and GFP-positive cells had almost completely disappeared by day 14 post transfection (data not shown). The fact that fluorescent cells did appear at early times post transfection reveals that the rescue plasmid was functional, indicating that the product of ORF2 is essential for NYMV propagation.

In striking contrast, NYMV-$\Delta$ORF4/GFP was successfully recovered under similar experimental conditions. However, propagation of NYMV-$\Delta$ORF4/GFP was strongly attenuated, and nearly complete infection of the culture was only achieved by day 28 post plasmid transfection (Fig. 2A). Interestingly, NYMV-$\Delta$ORF4/GFP established a persisting infection in the host cells without inducing any detectable cytopathic effects. Furthermore, NYMV-$\Delta$ORF4/GFP seemed to spread only between neighboring cells, indicating impaired ability of the virus to form infectious particles. In line with this hypothesis and in contrast to findings with NYMV-GFP, we were unable to detect infectious virus in the supernatant of the cell culture throughout the course of the rescue experiment (Fig. 2B, left panel, and data not shown). Notably, if the ORF4 product was provided in trans by transiently transfecting plasmid pCA-ORF4 into persistently infected rescue cells, NYMV-$\Delta$ORF4/GFP was
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successfully recovered as evident from the appearance of infectious virus in the culture supernatant (Fig. 2B, right panel). These results strongly suggested that ORF4 of NYMV plays a key role in viral particle assembly.

To support this hypothesis, we performed comparative ultrathin section electron microscopy of 293T cells infected with either NYMV-GFP or NYMV-ΔORF4/GFP and screened for the presence of viral particles. Numerous vesicles seemingly containing enveloped viral particles were present in cells infected with NYMV-GFP for 96 h (Fig. 3A, left panels). The size of these enveloped particles was approximately 70-100 nm in diameter which is in good agreement with the NYMV size estimate from a recent study (11). Vesicular structures with such content were not detected in uninfected 293T cells (data not shown). Importantly, 293T cells persistently infected with mutant NYMV-ΔORF4/GFP also did not show such particle-loaded vesicles (Fig. 3A, right panel), although they contained high levels of viral antigen (Fig. 3B). Taken together, these observations demonstrate that the ORF4 product is essential for the formation of NYMV particles.

We showed here that the genetic manipulation of NYMV is feasible if standard reverse genetics technology is employed that has successfully been used for other negative-strand RNA viruses in the past. We found that NYMV can tolerate the insertion of a foreign gene which will greatly facilitate future experimental work with this virus for which no good antibodies are available at present. We began to use the newly developed reverse genetics technology to investigate the roles of two viral proteins with unclear functions. We found that NYMV lacking the ability to synthesize ORF2 could not be recovered, whereas NYMV lacking ORF4 remained replication-competent and could establish a persisting infection. However, ORF4-deficient NYMV failed to produce infectious particles, as demonstrated by the absence of virus-like structures and infectious virus in persistently infected cells or culture supernatant, respectively. These results are in good agreement with our previously formulated hypothesis (5) that ORF4 of NYMV may represent the functional equivalent of matrix proteins of other negative-strand RNA viruses. In fact, our ORF4-deficient NYMV and matrix-deficient rabies virus (10), measles virus (2) and Sendai virus (6) mutants show a similar phenotype in that they lose the ability to produce cell-free infectious viral particles and acquire the ability to persist in their host cells. Our results are further in good agreement with the view (5) that ORF2 of NYMV codes for a protein with functional similarities to the X protein of Borna disease virus (12, 13) and VP24 of filoviruses (1, 4, 14) which both serve essential viral functions.
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REFERENCES

**FIGURE LEGENDS**

**Figure 1:** A reverse genetics system for the recovery of recombinant NYMV. (A) Schematic representation of the NYMV antigenome-encoding plasmids used for rescuing recombinant NYMV. A diagnostic *XhoI* restriction site was introduced at position 416 (A416G) of the NYMV antigenome in order to distinguish between authentic and recombinant NYMV. For expression of GFP, a cassette encoding hrGFP was introduced between the ORF3 and ORF4 genes. H: hammerhead ribozyme, δ: hepatitis delta virus ribozyme, S_G: G-gene transcriptional start sequence, T_ORF3: ORF3-gene transcriptional termination sequence. (B) Successful recovery of recombinant wild-type NYMV. 293T cells were co-transfected with a plasmid coding for the NYMV antigenome harboring a diagnostic *XhoI* restriction site (pNYMV) and expression vectors for the NYMV polymerase components N, ORF3 and L. Three to five days post transfection, cytopathic effects (CPE) became detectable in the rescue cells. The cell-free supernatant was then transferred to freshly seeded Vero cells. After re-appearance of CPE, total RNA was isolated from these cells or from Vero cells infected with authentic NYMV. RT-PCR and digestion of the PCR products with *XhoI* confirmed the presence of genetically modified genomic viral RNA in the rescue cells. Primers used for amplification of cDNA are depicted as grey arrows in panel A. (C) Spread of recombinant NYMV expressing GFP in the rescue cells. 293T cells were co-transfected with plasmid pNYMV-GFP encoding the NYMV antigenome containing the hrGFP gene (see panel A) and expression vectors for the NYMV polymerase components. Spread of the GFP signal was visualized by fluorescence microscopy at the indicated time points after starting the rescue experiment. (D) Growth properties of authentic NYMV, recombinant NYMV-(A416G) and NYMV-GFP in Vero cells. Vero cells were infected with the indicated viruses using an MOI of 0.01. At the indicated time points post infection, the cells were scraped into the culture supernatant and the virus was liberated from the cells by 3 cycles of freeze-thawing. Viral titers were determined using the TCID₅₀ assay.

**Figure 2:** ORF4 is required for NYMV particle assembly. (A) Dissemination of NYMV lacking ORF4 occurs only between neighboring cells. 293T cells were co-transfected with a plasmid encoding the NYMV antigenome in which ORF4 was replaced by hrGFP (pNYMV-ΔORF4/GFP) and expression vectors for the NYMV polymerase components. Spread of the GFP signal in the rescue cells was visualized by fluorescence microscopy at the indicated time points after starting the rescue experiment. (B) Providing the ORF4 protein *in trans* results in accumulation of infectious NYMV in the culture supernatant. 293T cells
persistently infected with NYMV-ΔORF4/GFP were transfected with an expression vector encoding the ORF4 protein (pCA-ORF4) or with an empty vector as control (pCA-Ø). Three days later, viral titers in cell-free supernatants were determined by analyzing the ability of inducing fluorescent foci in indicator cells. Titers are expressed as fluorescent foci-forming units (FFU) per ml. Bars represent the average values of three independent experiments. Standard deviations are shown. ND = not detectable.

**Figure 3**: Ultrathin section electron microscopy analysis reveals absence of virus-like structures in cells infected with NYMV-ΔORF4/GFP. (A) Electron microscopy analysis of 293T cells either infected with NYMV-GFP for 96 h (left panels) or persistently infected with NYMV-ΔORF4/GFP (right panel). Structures reminiscent of enveloped viruses of ~70-100 nm diameter were abundantly present in cytoplasmic vesicles in cells infected with NYMV-GFP but not in cells persistently infected with NYMV-ΔORF4/GFP. (B) Western blot analysis of extracts from uninfected 293T cells, 293T cells infected with NYMV-GFP for 96 h or 293T cells persistently infected with NYMV-ΔORF4/GFP. The blot was probed with antiserum detecting NYMV proteins N and ORF3 (5) and a primary antibody against beta-tubulin.