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Recombinant Sindbis virus expressing functional GFP in the nonstructural protein nsP3

Research Article

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Abbreviations: fetal bovine serum, FBS; green fluorescent protein, (GFP); multiplicity of infection, (MOI); polyacrylamide gel electrophoresis, (PAGE); Sindbis virus, (SINV); sodium dodecyl sulfate, (SDS)

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Summary

Sindbis virus vectors usually express foreign genes cloned in the structural region of the viral genome. In this report, we tested the possibility of expressing genes in the nonstructural region. We made a recombinant virus with a GFP gene inserted in the nsP3 sequence. The resulting Toto1101/GFP virus was infectious and grew to the same high titer as the parental virus. The nsP3-GFP fusion protein, like nsP3 itself, was phosphorylated. With confocal fluorescence microscopy, we found that nsP3-GFP, as a component of viral RNA replication complex, was on the plasma membrane early in infection (2 h post-infection), but quickly moved to punctate intracellular structures and remained there throughout the course of infection. The observed fluorescence indicates that GFP is functionally expressed in this nonstructural region and other marker or therapeutic genes should be able to be expressed in the same fashion, thus improving the utility of these viral vectors.

I. Introduction

Sindbis virus (SINV) is an enveloped plus-strand RNA virus belonging to the *Alphavirus* genus of the family *Togaviridae* (Strauss and Strauss, 1994). The SINV RNA genome contains 11,703 nucleotides and is completely sequenced (Strauss et al, 1984). With increasing understanding of the viral genome organization and replication strategy, SINV has been developed into one of the most efficient expression vectors for gene transfer in cultured cell lines (Frolov et al, 1996; Piper et al, 1994; Xiong et al, 1989) and animal models (Altman-Hamamdzic et al, 1997; Jeromin et al, 2003; van Marle et al, 2003). SINV vectors have been used to deliver genes to antigen-presenting cells to trigger specific immune responses to pathogens (Hahn et al, 1992; Pugachev et al, 1995; Tsuji et al, 1998; Kamrud et al, 1999) and cancers (Cheng et al, 2002; Tseng et al, 2004a; Chikkanna-Gowda et al, 2005; Wollmann et al, 2005). Furthermore, recent studies show that SINV vectors are targeted to and exhibit oncolytic activity towards a variety of tumors *in vivo* (Tseng et al, 2004b), making them promising gene therapy vehicles against cancer (Lundstrom, 2003; Hay, 2004; Yamanaka, 2004; Atkins et al, 2005).

There are generally two types of SINV vectors: replicons and infectious recombinant viruses (Frolov et al, 1996; Lundstrom, 2001). The replicons contain only viral nonstructural genes whose products (nsP1-4) are responsible for viral RNA replication/amplification, while viral structural genes (capsid and envelope glycoproteins) are replaced by foreign genes to be expressed. Because of the lack of structural proteins, the replicons are not packaged into infectious virions and thus are self-contained in the transfected cells. However, they can form infectious virions if the structural proteins are provided *in trans*. The more recently developed DNA vectors can perhaps be viewed as a form of replicons (Dubensky et al, 1996; Berglund et al, 1998; Yamanaka and Xanthopoulos, 2004). The recombinant viruses, on the other hand, contain all SINV genetic information with foreign genes inserted in certain structural regions of the viral genome under the control of a SINV subgenomic promoter (Hahn et al, 1992). These are live viruses that undergo normal viral replication cycle and in the process express the cloned foreign genes.

All SINV vectors so far keep the nonstructural region intact, because it encodes the nonstructural proteins (nsP1-4) for the amplification of viral RNA, which in turn is

important for the expression of foreign genes. Several point mutations in the nsP2 gene are found to reduce the viral RNA replication to a relatively low level that no longer causes cytopathic effect and readily establishes persistent infection (Frolov et al, 1999; Perri et al, 2000). These mutations have been incorporated into a new class of non-cytopathic SINV vectors (Agapov et al, 1998). In the current study, we explore the possibility of direct cloning and expressing foreign genes in the nonstructural region. To this end, we have generated a recombinant SINV that contains an in-frame insertion of the entire cDNA of GFP (green fluorescent protein) in nsP3, which is known to tolerate some in-frame insertions and deletions in its C-terminal variable domain (Davis et al, 1989; Lastarza et al, 1994). This recombinant SINV (termed Toto1101/GFP) remains infectious and grows to the same high titer as the parental virus, demonstrating the plasticity of nsP3 and the feasibility of expressing a functional fusion protein in this region. Incorporation of this type of modification into SINV vectors should improve their utility in terms of simultaneous expression of multiple genes in both structural and nonstructural regions.

II. Materials and Methods

A. Cells

The BHK-21 (baby hamster kidney) cell line was used in this study. Cell monolayers were grown in 35-mm tissue culture dishes or 6-well plates in α-MEM (invitrogen) containing 5% fetal bovine serum (FBS) (Invitrogen) and incubated at 37ºC in a cell culture incubator with 5% CO_2.

B. Generation of the Toto1101/GFP virus

The GFP cDNA was amplified by PCR with pEGFP-C1 (BD Biosciences) as template and oligonucleotide primers containing *Spe*I site. After digestion with *Spe*I, the GFP cDNA was directly inserted into the unique *Spe*I site of Toto1101, a full-length cDNA clone of Sindbis virus. In the resulting Toto1101/GFP plasmid, the GFP reading frame was the same as that of nsP3 and the translation product was expected to be a nsP3-GFP fusion protein with GFP sandwiched between amino acids 388 and 389 of nsP3. The GFP cDNA sequence was confirmed by direct DNA sequencing. The Toto1101/GFP plasmid was then used as a template for *in vitro* transcription by SP6 RNA polymerase as previously described and the resulting RNA transcript was used for transfection of BHK-21 cell monolayers via lipofectin-mediated procedure (Invitrogen). Briefly, 1 μg of the RNA transcript was mixed with 200 μl of PBS (phosphate-buffered saline) containing 8 μg of lipofectin and incubated on ice for 10 min. The transfection mixture was then added to the cell monolayers and incubated at room temperature for 10 min, before replacing the transfection mixture with 3 ml of growth medium (α-MEM containing 5% FBS). The cells were incubated at 37ºC in an incubator with 5% CO_2. The medium containing the released viruses was harvested 48 h later and frozen at -80ºC as virus stocks.

C. Titration of virus stocks by plaque assay

Virus stocks were serially diluted in PBS containing 1% FBS and 200 μl of each dilution were added to each well of BHK-21 cell monolayers. The cells were incubated at room temperature for 30 min, followed by direct overlay of each well with 3 ml of 1% agarose in α-MEM. The cells were then incubated at 37°C in an incubator with 5% CO_2. After 3 days, the cells were fixed by adding 1 ml of 7% formaldehyde to each well and incubating at room temperature for 30 min. The agarose overlay was then carefully removed with a spatula and cell monolayers were stained with crystal violet to visualize and count the number of viral plaques.

D. One-step growth curve

BHK-21 cell monolayers in 35-mm culture dishes were infected with Toto1101/GFP and the parental Toto1101 viruses, diluted in 200 μl of PBS containing 1% FBS, at a multiplicity of infection (MOI) of 20 PFU/cell. After virus absorption at room temperature for 1 h, the infection mix was aspirated and the cells were rinsed with PBS, followed by addition of 3 ml α-MEM containing 5% FBS to each dish and incubation in a 37°C incubator with 5% CO_2. At 1 h post-infection, the medium was replaced with 3 ml of fresh pre-warmed (37°C) α-MEM containing 5% FBS, followed by continued incubation at 37°C. A small amount of medium (10 μl) was collected from each dish at the following time points: 4 h, 6 h, 8 h, 12 h and 24 h post-infection and the virus titer in the medium was determined by the plaque assay as described above.

E. Pulse-chase labeling and immunoprecipitation of nsP3

BHK-21 cell monolayers in 35-mm culture dishes were infected with Toto1101/GFP and the parental Toto1101 viruses as described above. At 3 h post-infection, the medium was removed and the cells were pulse-labeled for 15 min in methionine-free α-MEM containing 50 Ci/ml ^{35}S-methionine (MP Biomedicals), followed by different times of chase as indicated in normal growth medium (α-MEM with 5% FBS). The cells were then rinsed once with ice-cold PBS and lysed with 200 μl of 1% sodium dodecyl sulfate (SDS). The lysates were boiled for 3 min and either stored at -70°C or used directly for immunoprecipitation with the rabbit antiserum monospecific for nsP3 as previously described (Li and Rice, 1989). The immunoprecipitated proteins on γBind G-Sepharose beads (Amersham Biosciences) were resuspended in 30 μl of Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol, 12.5% glycerol, 0.01% bromophenol blue), boiled for 3 min and centrifuged for 1 min. Proteins released in the supernatants were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The gels were treated with 1 M sodium salicylate (in 10% methanol) for 30 min, dried and exposed to Kodak BioMax films for autoradiography.

F. Confocal fluorescence microscopy

BHK-21 cells were grown on cover slips and infected with the Toto1101/GFP virus as described above. At the indicated times, cells were rinsed once with PBS and fixed in 4% paraformaldehyde (in PBS) (Electron Microscopy Sciences) for 30 min. The cover slips were mounted in PBS on glass slides (Fisher Scientific) and the fluorescence was observed and documented via a Leica confocal laser scanning microscope (Liang and Li, 2000).

III. Results and Discussion

A. Toto1101/GFP virus is viable and grows to high titer

The Toto1101/GFP RNA transcript produced the same high titer of infectious virions (2 x 10^9 PFU/ml) as the parental Toto1101 transcript, indicating that nsP3 remains functional, despite the GFP insertion. We then

determined the single-step growth curve of the viruses at 37°C. The Toto1101/GFP and Toto1101 virus stocks derived from the transfection were used to infect fresh BHK cell monolayers at 20 PFU/cell and media containing released viruses were collected at the indicated times after infection, followed by titration of the virus samples. The Toto1101/GFP virus replication showed a small but reproducible delay early in infection with lower virus yields (2-4 fold) than the parental Toto1101 virus at 4 h and 6 h post-infection (**Figure 1**). However, Toto1101/GFP quickly caught up and the virus yield was essentially the same as that of Toto1101 at 8 h post-infection and throughout later time points (**Figure 1**).

We next examined directly the infectivity of the RNA transcript of Toto1101/GFP in comparison to that of parental Toto1101 by plaque assays upon transfection. Consistent with the delay in the growth curve (**Figure 1**), Toto1101/GFP RNA transcript produced somewhat less plaques (5 x 10^5 PFU/μg) than the parental Toto1101 transcript (1.5 x 10^6 PFU/μg), reflecting some negative effect on nsP3, which is known to function in viral RNA synthesis (Lemm et al, 1994; Shirako and Strauss, 1994; Wang et al, 1994). However, the plaque morphology and virus yields are the same for both viruses.

B. The nsP3-GFP fusion protein is expressed properly and post-translationally processed

We did pulse-chase labeling experiments with [^{35}S]methionine (ICN Translabel) (Li et al, 1990) in Toto1101/GFP and Toto1101 virus-infected cells, followed by immunoprecipitation with a nsP3-specific rabbit antiserum (Li et al, 1990), SDS-PAGE and autoradiography. It was previously reported that after a short labeling (10-15 min), newly made nsP3 appeared as a 70 kDa protein. However, it was gradually chased into multiple phosphorylated forms with the largest, predominant form migrating at approximately 100 kDa (Li et al, 1990). We confirmed this observation with the parental Toto1101 virus, which served as a control (**Figure 2**). In this case, immediately after the 15-min pulse-labeling, we observed polyprotein precursors P123 and P34 in addition to the processed nsP3 (**Figure 2**, indicated on the left). Most of P123 was chased into nsP3 within 30 min, but P34 was stable throughout the chase (as long as 90 min). The mature nsP3 product was chased into higher molecular weight phosphorylated forms ranging from 72-100 kDa (**Figure 2**) (Li et al, 1990). In Toto1101/GFP-infected cells, on the other hand, the 15-min pulse-labeling produced the corresponding precursors P123-GFP and P34-GFP as well as the processed mature

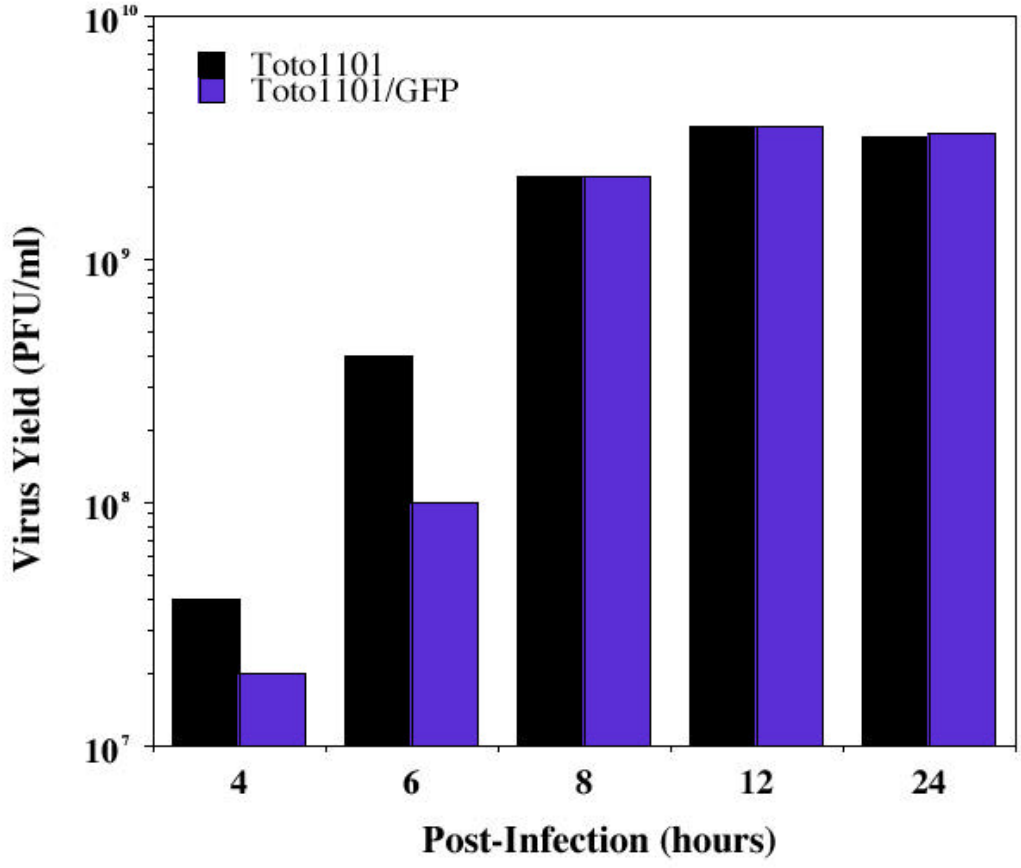

Figure 1. Single-step growth curves of Toto1101/GFP and Toto1101 viruses. Virus samples were collected at the indicated times and titered by plaque assays. The bar graph shows the virus accumulation over a 24-hour period. The data represent the mean of duplicate samples and were reproducible in two independent experiments.

nsP3-GFP, which is larger (about 100 kDa) than nsP3 itself reflecting GFP insertion (**Figure 2**, indicated on the right). Both the expected size and the recognition by the nsP3-specific antibody indicated that this 100 kDa protein is the nsP3-GFP fusion protein. Like their Toto1101 counterparts, P123-GFP was mostly chased into nsP3-GFP within 30 min with a slightly slower kinetics, while P34-GFP was stable throughout the 90-min chase (**Figure 2**, indicated on the right). Likewise, nsP3-GFP was chased into higher molecular weight forms, suggesting proper post-translational processing (phosphorylation) of the protein (**Figure 2**). We noticed a new nsP3-related fragment, which was termed nsP3x (**Figure 2**), appeared only in Toto1101/GFP-infected cells. It was smaller than nsP3, but was recognized by nsP3 antibody and was chased into a higher molecular weight form (**Figure 2**). It was likely a degradation product of nsP3-GFP.

C. Localization of nsP3-GFP to the plasma membrane and intracellular structures

We next determined if the GFP portion is properly folded and functional by examining the green fluorescence of GFP via confocal fluorescence microscopy (Liang and Li, 2000) during the time course of Toto1101/GFP virus infection of BHK cells. This also allowed us to determine the intracellular localization of nsP3-GFP. The GFP fluorescence was detected as early as 2 h post-infection (**Figure 3**). At this time, nsP3-GFP was found at the plasma membrane (**Figure 3**, indicated by arrows) as well as in punctate intracellular structures. Interestingly, the plasma membrane-localized nsP3-GFP tended to be at the junction region between neighboring cells rather than at the free rim of the cell (**Figure 3B**). By 3 h post-infection, however, all nsP3-GFP moved to the punctate intracellular structures (**Figure 4A**) and remained there throughout virus infection. These nsP3-GFP containing intracellular structures moved progressively towards the center of the cell during the course of infection and eventually clustered at the perinuclear region at late infection (10 h post-infection) (**Figure 4B**). The punctate pattern of the nsP3-GFP structures is similar to the nsP3-positive late endosome/lysosome-like vesicles in SINV-infected cells described previously (Froshauer et al, 1988).

The early targeting of nsP3-GFP, probably in the form of polyproteins P1234 and P123 (Salonen et al, 2003), to the plasma membrane suggests that functional viral RNA replication/transcription complexes (Lemm et al, 1994; Shirako and Strauss, 1994) may form and initiate the minus-strand RNA synthesis at the plasma membrane before moving inward to the cytoplasm, possibly via endocytosis. Further studies may provide insight into the functional differences between plasma membrane-localized and late endosome/lysosome-localized viral RNA replication/transcription complexes and our Toto1101/GFP virus should be useful in monitoring the formation and dynamics of the replication/transcription complexes in live cells. In addition, functional expression of GFP in nsP3 suggests that other reporter or therapeutic genes may also be expressed in this nonstructural region, thus improving the utility of SINV expression vectors. Because the nonstructural genes are usually expressed at a lower level than the structural genes, foreign genes cloned in this region are likely to express at a lower level as well.

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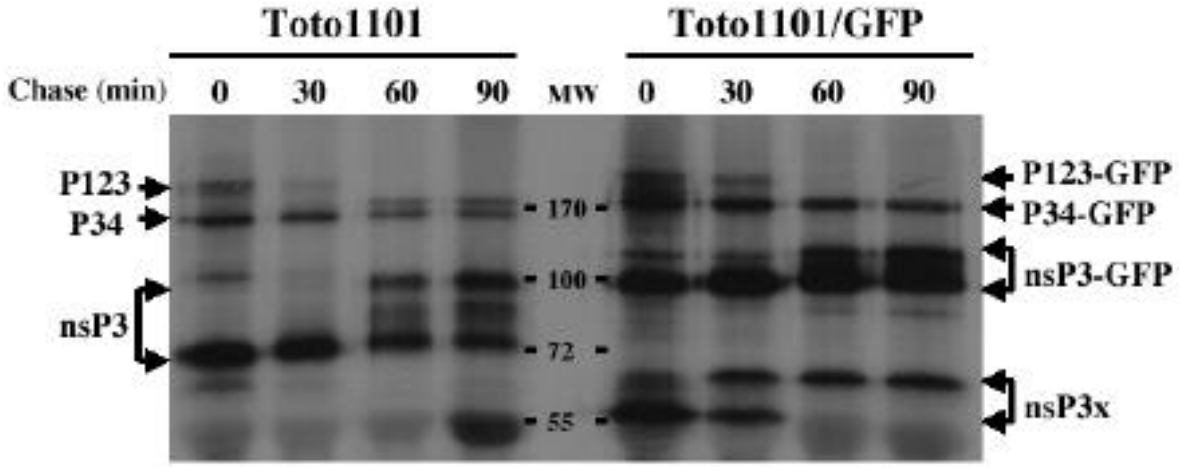

Figure 2. Post-translational processing of nsP3-GFP. BHK cells infected with either the Toto1101/GFP virus (indicated) or the parental Toto1101 virus (indicated) were pulse-labeled with [^{35}S]methionine for 15 min, followed by different times of chase as indicated in normal growth medium. Cells were lysed in 1% SDS and the lysates were immunoprecipitated with a nsP3-specific antibody as previously described (Li and Rice, 1989), followed by SDS-PAGE and autoradiography. Note the mobility shift of both nsP3 and nsP3-GFP after the chase, which is indicative of post-translational phosphorylation. The nsP3 and its precursors are indicated on the left, while the nsP3-GFP and its precursors are indicated on the right. A possible degradation product of nsP3-GFP, referred to as nsP3x, is also indicated on the right. Molecular weight standards (in kDa) are indicated in the middle.

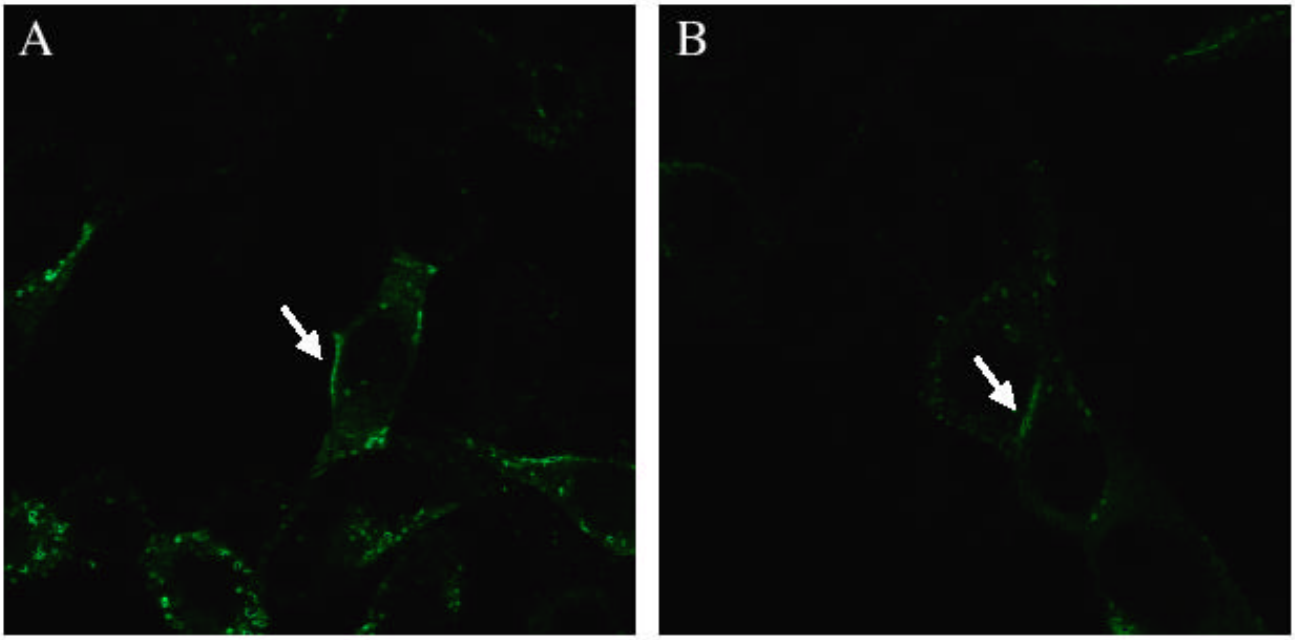

Figure 3. Localization of nsP3-GFP during early infection. BHK cells grown on cover slips were infected with the Toto1101/GFP virus and incubated at 37°C. At 2 h post-infection, the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences), mounted on glass slides (Fishser Scientific) and viewed via a Leica confocal fluorescence microscope. Shown are two confocal images of different fields. Panels A shows free single cells, while panel B shows two or more cell clusters. Arrows indicate plasma membrane localized nsP3-GFP.

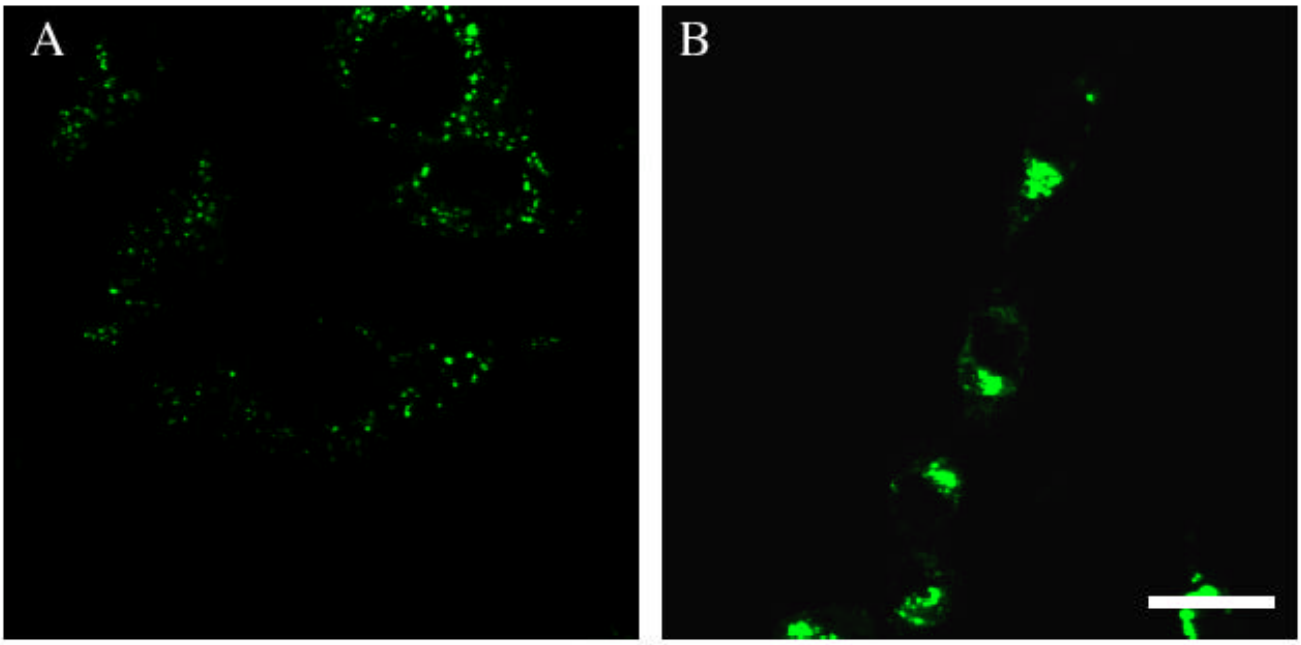

Figure 4. Localization of nsP3-GFP during late infection. The experimental procedure was identical to the Figure 3 legend, except that the cells were fixed and processed for microscopy at later times. Panels A and B show confocal images taken at 3 and 10 h post-infection. Bar=20 μm.

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