

# Localization to the Nucleolus Is a Common Feature of Coronavirus Nucleoproteins, and the Protein May Disrupt Host Cell Division

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**The subcellular localization of transmissible gastroenteritis virus (TGEV) and mouse hepatitis virus (MHV) (group I and group II coronaviruses, respectively) nucleoproteins (N proteins) were examined by confocal microscopy. The proteins were shown to localize either to the cytoplasm alone or to the cytoplasm and a structure in the nucleus. This feature was confirmed to be the nucleolus by using specific antibodies to nucleolin, a major component of the nucleolus, and by confocal microscopy to image sections through a cell expressing N protein. These findings are consistent with our previous report for infectious bronchitis virus (group III coronavirus) (J. A. Hiscox et al., *J. Virol.* 75:506–512, 2001), indicating that nucleolar localization of the N protein is a common feature of the coronavirus family and is possibly of functional significance. Nucleolar localization signals were identified in the domain III region of the N protein from all three coronavirus groups, and this suggested that transport of N protein to the nucleus might be an active process. In addition, our results suggest that the N protein might function to disrupt cell division. Thus, we observed that approximately 30% of cells transfected with the N protein appeared to be undergoing cell division. The most likely explanation for this is that the N protein induced a cell cycle delay or arrest, most likely in the G<sub>2</sub>/M phase. In a fraction of transfected cells expressing coronavirus N proteins, we observed multinucleate cells and dividing cells with nucleoli (which are only present during interphase). These findings are consistent with the possible inhibition of cytokinesis in these cells.**

Coronaviruses are enveloped RNA viruses with nonsegmented, single-stranded, positive-sense RNA genomes of 27 to 32 kb that are 5' capped and 3' polyadenylated (26). The 5' two-thirds of the coronavirus genome encodes the virus contribution to the replicase-transcription complex, Rep1a and Rep1b, the latter resulting from a –1 frameshift (8). During coronavirus replication, a 3'-coterminal nested set of subgenomic mRNAs, which encode other viral proteins, including nucleoprotein (N protein), are synthesized. In part, based on similar genome replication strategies (17, 61), the coronavirus family, *Coronaviridae*, has been grouped together with the arterivirus family, *Arteriviridae*, into the order *Nidovirales* (11). While gene functions and distributions for the two families are similar, there are some differences that might lead to subtle differences in replication strategies. Recently, we have reported that the coronavirus infectious bronchitis virus (IBV) N protein localizes to the cytoplasm and a structure in the nucleus proposed to be the nucleolus in both IBV-infected cells and cells transfected with a plasmid expressing IBV N protein under the control of a PolIII promoter (23). A similar result was reported with the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV) N protein (54), suggesting that localization of N protein to the nucleolus was probably

common to these two virus families and potentially common to all *Nidovirales*.

Coronavirus replication is generally accepted to occur in the cytoplasm of infected cells (66), although for IBV an intact cell nucleus has been proposed to be necessary for virus replication (20). In addition, proteins normally associated with the nucleus have been implicated in the replication of the murine coronavirus mouse hepatitis virus (MHV) (30). The nucleolus is a structure found within the nucleus and is only present during interphase (1). It is the site where rRNA is synthesized and where biogenesis of ribosomal subunits and polymerase III transcripts occurs (10, 57). The nucleolus also sequesters regulatory complexes and has been implicated in the regulation of the cell cycle (10). The possible involvement of the nucleolus in coronavirus replication is not exclusive to coronaviruses. As a consequence of infection or a deliberate process, a number of viruses, including adenoviruses (37) and poliovirus (65), redistribute nucleolin, a major nucleolar antigen, from the nucleolus to the cytoplasm. The nucleolus is the site of Born disease virus replication and transcription (50). A number of virus proteins have been shown to localize to the nucleolus during virus infection including human immunodeficiency virus type 1 Rev (18) and Tat (59) and adenovirus V protein (38). However, the possible role of the nucleolus in coronavirus replication has not been elucidated, and why the N protein would localize to this structure remains unknown. We hypothesized that the IBV N protein might localize to the nucleolus as part of a virus strategy to control both host cell and virus sub-

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genomic RNA (sgRNA) translation by associating with ribosomal subunits and that localization might be cell cycle dependent (23). Furthermore, localization to the nucleolus might be an intrinsic property of the coronavirus N protein.

Coronavirus N proteins vary from 377 to 455 amino acids in length, are highly basic, and have a high (7 to 11%) serine content; serines are potential targets for phosphorylation (29). Three groups of coronaviruses have been identified to date although sequence conservation of the N proteins within the genus is low. For instance, the N proteins of coronaviruses IBV (group III) and porcine transmissible gastroenteritis virus (TGEV; group I) have only 29% identity with that of bovine coronavirus (BCoV; group II) and, within the group II coronaviruses, the N proteins of MHV and BCoV have only 70% identity (28). Based on amino acid sequence comparisons, three structural domains in the coronavirus N protein have been identified (45); of these, domain II was identified as a potential RNA binding site (36, 41) capable of binding both coronavirus- and non-coronavirus-derived RNA sequences in vitro (36, 63), but it might also bind to viral RNAs with greater efficiency than nonviral RNAs (14). The possible function(s) of domains I and III remains unknown. However, recently we have identified a putative ribosome binding site motif and a putative nucleolar localization signal (NuLS) in domain III of the IBV Beaudette N protein, a feature that was conserved in 10 other strains of IBV (23).

Several functions have been postulated for the coronavirus N protein throughout the virus life cycle (29). Primarily, it complexes with the genomic RNA to form a ribonucleocapsid structure (RNP) (16) and has been observed, together with the M protein, to be a component of the viral core (19, 53). The N protein has been shown to associate with the leader RNA sequence (6, 42), located at the 5' end of the genomic RNA and/or to sequences at the 3' end of the genomic RNA (68). As these regions are believed to be involved in synthesis of coronavirus RNA, the N protein has been postulated to have a role in replication of the genomic RNA (12, 15), in the transcription of coronavirus sgRNAs (6, 63), and in translation from the sgRNAs (64). However, replication and transcription have been shown to occur in the absence of N protein in arterivirus equine arteritis virus (39). Although the N protein might not be essential for coronavirus replication, the possibility that it may still be involved in the efficiency of replication cannot be discounted. The N protein may have multiple functions during virus replication. Given that amino acid sequence conservation within the N protein of the three different coronavirus groups is low, the fact that N proteins from group I and group II coronaviruses also localize to the nucleolus suggests that nucleolar localization may be of functional significance. Accordingly, we investigated whether nucleolar localization is a conserved feature among coronavirus N proteins and determined the consequences of N protein expression on host cell proliferation. Our results show that association with the nucleolus is a common feature of the N proteins from the order *Nidovirales*. In addition, expression of N protein leads to an inhibition of host cell proliferation and concomitant polyploidy in some cells, consistent with an inhibition of cytokinesis.

TABLE 1. Sequences of the oligonucleotides used in this study

Oligonucleotide	Sequence (5'-3')	Polarity
MHVJHM5'	<b><i>CTCGAGATGTC</i></b> TTTTGTTCTGGGCA	+
MHVJHM3'	<b><i>GAATTC</i></b> TACACATTAGAGTCATCTT	-
TGEVNXho	<b><i>CTCGAGATGGCCA</i></b> ACCAGGGACAACG	+
TGEVNNot	<b><i>GCGGCCGCTT</i></b> AGTTTCGTTACCTCATCAA	-
IBVNNco	<b><i>AAGATACC</i></b> <u><i>ATGGCAAGCGGTA</i></u> AAGCAGC	+
IBVNΔstop	<b><i>ATATCGAAGCTT</i></b> AAGTTCATTCTCTCTAG	-

<sup>a</sup> Restriction enzyme sites are italicized; noncoronavirus sequences are in boldface; authentic N protein translation start sites and stop sites are underlined.

## MATERIALS AND METHODS

**Cells.** LLC-Pk1 cells were grown in Eagle minimum essential medium supplemented with 10% fetal calf serum (FCS) at 37°C. Vero and L cells were maintained in Dulbecco's modified Eagle's medium with Glutamax-I (Gibco) supplemented with 5% FCS at 37°C. Sf9 cells were cultured in Sf-900II serum-free medium (Gibco BRL) at 28°C.

**Transfection.** Mammalian cells ( $10^5$  per 9.6-cm<sup>2</sup> dish) were grown on glass coverslips and were transfected with 2 μg of plasmid DNA and 16 μg of Lipofectamine in Opti-MEM (Gibco) for 5 h; the medium was replaced with maintenance medium for 24 h prior to fixing with 50% methanol-50% acetone. Coverslips then were incubated for 1 h at 37°C with the appropriate primary antibody (detailed in the text), washed for 10 min in excess phosphate-buffered saline (PBS), reacted with a secondary antibody at a 1:100 dilution (see below), and washed for 10 min in excess PBS. Fluorescent images were viewed with a Leica confocal microscope.

**Oligonucleotides.** The oligonucleotides used in this study were obtained from MWG-Biotech and are listed in Table 1.

**Recombinant DNA techniques.** Procedures for recombinant DNA techniques were either standard (5, 56) or performed according to the manufacturer's instructions.

**PCR.** PCRs were carried out with a mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 3.0 mM MgCl<sub>2</sub>, 10 pmol of each primer, 0.001 μg of template DNA, 0.25 mM deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (Gibco BRL). The reaction was carried out in a total volume of 50 μl. The reaction conditions were 94°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min for 30 cycles. The last (extension) cycle was at 72°C for 6 min.

**Recombinant plasmids.** The MHV N gene was produced by PCR, using *Taq* polymerase, from a plasmid containing an authentic copy of the MHV (JHM strain) N gene (pTR31) (55) using oligonucleotides MHVJHMN5' (corresponding to *Xho*I and the N gene start site) and MHVJHMN3' (corresponding to *Eco*RI and the N gene stop site) and cloned into pCR-2.1 Topo (Invitrogen). The MHV N gene was excised by digestion with *Xho*I and *Eco*RI and cloned into pCi-Neo (Promega) that had been digested with *Xho*I and *Eco*RI, creating pCi-MHV-N, such that transcription of the MHV N gene was under the control of the cytomegalovirus (CMV) promoter; the sequence was confirmed in accordance with standard procedures. The TGEV N gene (strain FS772) was cloned into pCi-Neo in a manner similar to that for the MHV N gene using oligonucleotides TGEVNXho (corresponding to *Xho*I and the N gene start site) and TGEVNNot (corresponding to *Not*I and the N gene stop site), creating pCi-TGEV-N. Additionally, the TGEV N gene (strain FS772) was cloned directly into pCDNA4/HisMax (Invitrogen) using oligonucleotides TGEVNXho and TGEVNNot, such that the TGEV N protein was C-terminal of a His tag, creating pHis-TGEV-N. The sequence and orientation of the insert were confirmed by restriction digestion and sequencing (data not shown).

To synthesize recombinant IBV N protein, the IBV N gene was cloned into pTriEx1.1 (Novagen). Oligonucleotide IBVNNco, corresponding to the 5' end of the gene including the start codon, and an *Nco*I site were introduced at the 5' end of the coding sequence. The stop codon of the IBV N protein was deleted using oligonucleotide IBVNΔstop, which was complementary to the 3' end of the coding sequence, and a *Hind*III site was introduced. The IBV N gene was amplified by PCR from pIBV322, which contains the Beaudette strain N gene (7). pTriExIBVN was created by inserting the PCR product into *Nco*I/*Hind*III-restricted pTriEx1.1 so that, in the resulting construct, the gene would be expressed with a His tag and a herpes simplex virus tag fused to the C terminus.

**Construction of recombinant baculovirus expressing the IBV N protein.** Recombinant virus BacIBVN was generated by homologous recombination after cotransfection of pTriExN together with BacVector-3000 triple-cut virus DNA (Novagen). Baculoviruses were grown in Sf9 cells. Potential recombinant viruses

were plaque purified and amplified, and the N protein was detected by Western blotting (data not shown).

**Transduction of mammalian cells with baculovirus.** Vero cells ( $5 \times 10^5$  cells/well) were plated in 35-mm-diameter dishes and incubated overnight at 37°C in a CO<sub>2</sub> incubator. Baculoviruses were diluted in growth medium (Dulbecco's modified Eagle's medium–10% FCS). Vero cells were incubated with diluted virus at a multiplicity of infection of 100 at 37°C for 2 h in a CO<sub>2</sub> incubator. After transduction, the cells were incubated for a further 22 h in fresh growth medium and then fixed and assayed by indirect immunofluorescence.

**Determination of cell division.** Normal, nontransfected Vero cells and cells transfected with the appropriate construct expressing the N protein were grown on coverslips as described above and fixed with 50% methanol–50% acetone prior to staining with propidium iodide (PI) to visualize nuclear material. The number of cells undergoing cytokinesis or mitosis (cell division) was determined by fluorescence microscopy by counting >100 transfected cells in three different visual fields per slide.

## RESULTS

### Intracellular localization of the MHV and TGEV N proteins.

We previously have shown that the IBV N protein can localize both to the cytoplasm and the nucleolus in both IBV-infected cells and cells transfected with a vector expressing the N protein under the control of a CMV (PolII) promoter (23). To investigate whether localization to both the cytoplasm and nucleolus is a feature common to the N proteins of type I and type II coronaviruses, we cloned TGEV (strain FS772; type I) and MHV (strain JHM; type II) N proteins into eukaryotic expression vector pCi-Neo. In these constructs, transcription of the N genes was placed under the control of a CMV promoter such that translation of the resultant mRNA transcripts would generate authentic coronavirus N proteins. These plasmids were transfected into both species-specific cells (porcine LLC-PK1 cells for TGEV N and mouse L cells for MHV N) and non-species-specific cells (Vero-monkey cells). Cells were incubated at 37°C for 24 h and fixed for analysis by indirect immunofluorescence using mouse monoclonal anti-MHV N protein sera or mouse monoclonal anti-TGEV N sera (DA3) (3), followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibody (Harlan Sera-Lab). Fixed cells were then stained with PI to visualize nuclear DNA and analyzed by confocal microscopy (Fig. 1). The TGEV (Fig. 1A and B) and MHV (Fig. 1C and D) N proteins, expressed from pCi-TGEV-N or pCi-MHV-N, respectively, were distributed throughout the cytoplasm alone or the cytoplasm and a structure in the nucleus identified as the nucleolus. These results were identical to those previously described for the localization of the IBV Beaudette N protein in Vero cells (23). IBV Beaudette has been adapted for growth in Vero cells, and nucleolar localization of the N protein in both species-specific and nonspecific cells indicates that nucleolar localization is a common feature of all coronavirus N proteins.

**Nucleolar localization of the coronavirus N protein is not an artifact of the plasmid-based expression system.** The expression of the MHV and TGEV N proteins and that previously described for IBV (23) relied on the transfection of mammalian cells with a plasmid that expressed the N gene under the control of a CMV promoter. In addition, we have previously shown that the IBV N protein localizes to the nucleolus in infected cells (23). While the plasmid-based expression system has several advantages for studying subtle phenotypes of the N protein, which may be masked in the context of a virus-infected cell, we wanted to eliminate the possibility that nucleolar lo-

calization was an artifact of the expression system. Therefore we studied the localization of the N protein in a non-plasmid-based system. The IBV N protein was cloned into the *Nco*I and *Hind*III sites of pTriEx1.1 (Novagen), creating pTriExIBVN, such that expression of the IBV N protein was under the control of the chicken  $\beta$ -actin promoter. This vector contained baculovirus sequences flanking the cloning site, which permitted the generation of a recombinant baculovirus using the BacVector-3000 vector (Novagen), creating BacIBVN. Vero cells were transduced with BacIBVN and incubated at 37°C for 24 h prior to fixing and staining with polyclonal sera against IBV as described previously (23). The IBV N protein expressed from BacIBVN localized to both the cytoplasm and the nucleolus (Fig. 1E), identical to the pattern observed in cells either infected with IBV or transfected with a plasmid expressing the IBV N protein (23). Thus localization of the coronavirus N protein to the nucleolus could not be attributed to either transfection or the plasmid-based expression system and therefore was an intrinsic property of the N protein itself.

**Identification of the nucleolus.** The eukaryotic nucleus contains a number of domains or subcompartments, which include nucleoli, nuclear Cajal bodies (CBs), nuclear speckles, transcription and replication foci, and chromosome territories (27). The subnuclear structure identified in Fig. 1 and in our previous study (23) is consistent with the nucleolus, in that localization of the IBV N protein was observed only in cells that were in interphase (when nucleoli are present) (1). However, to confirm that this structure was located in the nucleus rather than being an artifact located in the cytoplasm, Vero cells were transfected with pCi-TGEV-N and incubated at 37°C for 24 h and fixed for analysis by indirect immunofluorescence using mouse monoclonal DA3, specific to the TGEV N protein, followed by a FITC-labeled goat anti-mouse antibody (Harlan Sera-Lab). The fixed cells were stained with PI and analyzed by confocal microscopy by taking nine 0.1- $\mu$ m-thick sections through a cell where the TGEV N protein localized to both the cytoplasm and nucleolus. The sectioned cell was identified as being in the G<sub>1</sub> phase of the cell cycle as two nucleoli were present in one nucleus (1) (Fig. 2A). Analysis of the various sections (Fig. 2B) indicated that the structures preliminarily identified as nucleoli were only present in the middle sections of the cell, in the same focal plane as the nucleus, and thus are most likely within the nucleus, rather than being an artifact in the cytoplasm or associated with the cell (a similar result was observed in sections of Vero cells expressing the MHV N protein [data not shown]). To confirm conclusively that this structure was the nucleolus, HeLa cells were transfected with pCi-MHV-N, incubated at 37°C for 24 h, fixed, and labeled with rabbit anti-MHV polyclonal sera followed by antirabbit Alexa Fluor 564 (Molecular Probes) to visualize the MHV N protein and with mouse antinucleolin monoclonal sera (Leinco Laboratory) followed by a FITC-labeled goat anti-mouse antibody (Harlan Sera-Lab) to visualize the nucleolus. Nucleolin is a major nucleolus-specific protein (22, 58). Confocal microscopy indicated that the MHV N protein localized to the structure in the nucleus that was recognized by the antinucleolin antibody, which is consistent with the structure being the nucleolus (Fig. 3).

**Nucleolar localization of the N protein is not concentration dependent.** Soluble proteins of less than approximately 60 kDa

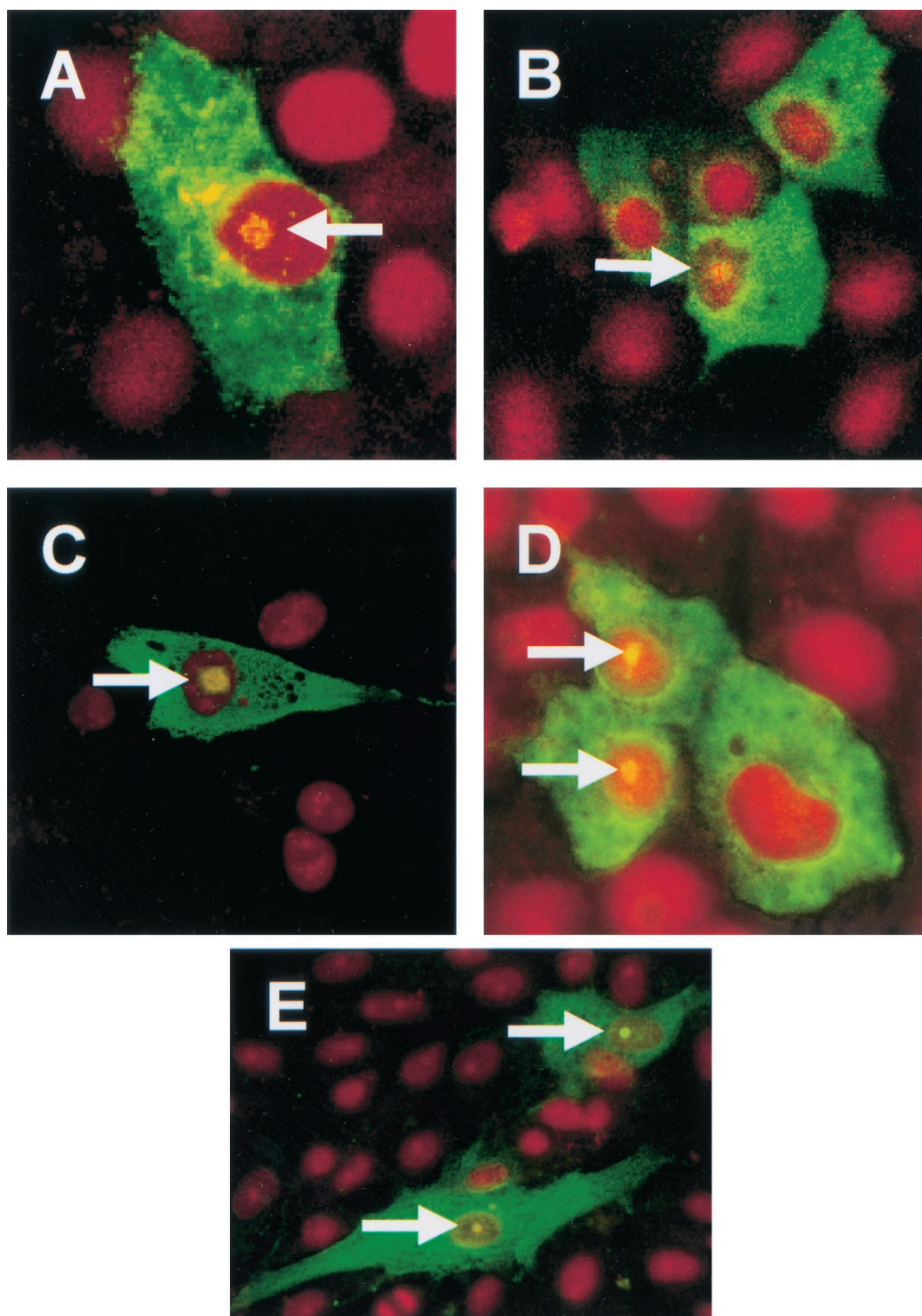


FIG. 1. Indirect detection of TGEV (A and B) and MHV (C and D) N proteins in transfected cells and the IBV (E) N protein in transduced cells by immunofluorescence. LLC-PK1 cells (A), L cells (C), and Vero cells (B and D) were transfected with either pCi-TGEV-N or pCi-MHV-N or were transduced with BacIBVN (E). They were incubated for 24 h, fixed, and analyzed by indirect immunofluorescence using appropriate antibodies (green) (see text). Additionally, cells were stained with PI to directly visualize nuclear DNA (red). Differentially fluorescing images were gathered separately from the same 0.5- $\mu$ m-thick optical section by using a confocal microscope and the appropriate filter. The two images were digitally superimposed to depict the distribution of the appropriate coronavirus N protein and nuclear DNA. Arrow, position of a nucleolus. Magnifications,  $\times 62$  (A to D) and  $\times 16$  (2.73 zoom) (E).

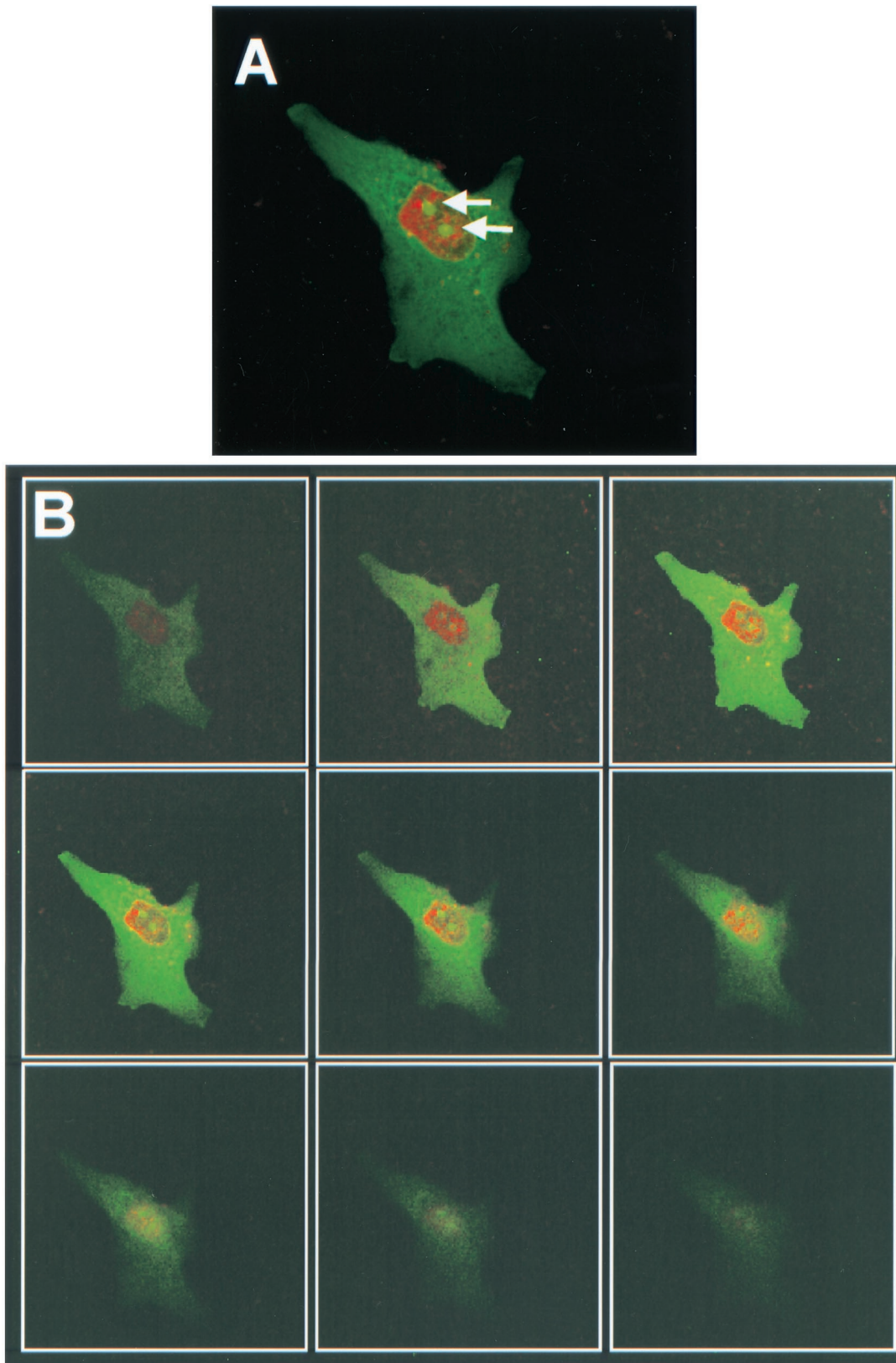


FIG. 2. Detection of TGEV N proteins by indirect immunofluorescence in transfected cells. Vero cells were transfected with pCi-TGEV-N, incubated for 24 h, fixed, and analyzed by indirect immunofluorescence using appropriate antibodies (green) (see text). Additionally, cells were stained with PI to visualize nuclear DNA (red). Differentially fluorescing images were gathered separately from the same 0.5- $\mu\text{m}$ -thick optical sections by using a confocal microscope and the appropriate filter. The two images were digitally superimposed to depict the distribution of TGEV N protein and nuclear DNA (A). Arrow, position of a nucleolus. (B) The confocal microscope was used to take 0.1- $\mu\text{m}$ -thick sections of the cell shown in panel A. The section on the top left is nearest the coverslip, and the section on the bottom right is nearest the media. Magnification,  $\times 62$  (and  $\times 2$  zoom).

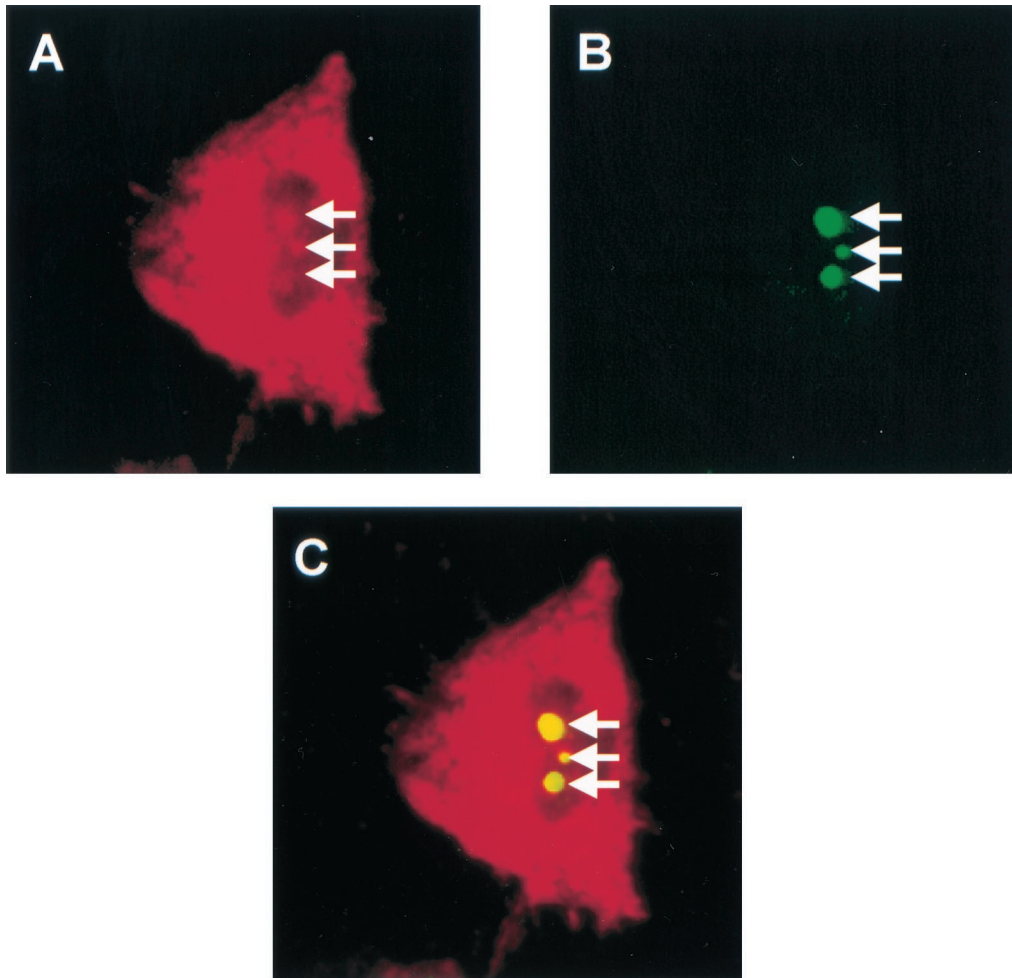


FIG. 3. Detection of MHV N protein by indirect immunofluorescence in transfected cells. HeLa cells were transfected with pCi-MHV-N, incubated for 24 h, and analyzed by indirect immunofluorescence using appropriate antibodies to detect the N protein (red) and nucleolin (green) (see text). Differentially fluorescing images were gathered separately from the same 0.5- $\mu$ m-thick optical section by using a confocal microscope and the appropriate filter. The two images (A and B) were digitally superimposed to depict the distribution of the MHV N protein and nucleolin (C). Yellow indicates colocalization. Magnification,  $\times 61$ .

can diffuse passively into the nucleoplasm through the nuclear pore complex and could in principle diffuse in and out of the nucleolar compartment (52). However, nonsoluble nuclear components are expected to become restricted to the nucleolus depending on their binding sites (10). If nucleolar localization of the N protein occurred randomly in a concentration-dependent manner, then we would predict that transfected cells expressing more N protein would have a greater number of nucleoli containing the N protein. To investigate this, Vero cells were transfected with pCi-MHV-N, incubated at 37°C for 24 h, and fixed for analysis by indirect immunofluorescence using mouse monoclonal anti-MHV N protein sera followed by a FITC-labeled goat anti-mouse antibody (Harlan Sera-Lab). The fixed cells were stained with PI to visualize nuclear DNA and analyzed by confocal microscopy (Fig. 4). Assuming that the amount of fluorescence from FITC is proportional to the amount of N protein in a cell, then variations in N protein concentrations among cells can be compared. Comparison of cells with the N protein in the cytoplasm with cells with the N protein in both the cytoplasm and nucleolus indicated that

nucleolar localization was not directly proportional to the amount of N protein within the cell (Fig. 4). A similar result was observed in cells expressing either the IBV N protein (Fig. 1E) or the TGEV N protein (data not shown).

**MHV and TGEV N proteins contain putative nucleolar localization signals and may associate with rRNA.** Several nuclear localization signals (NLS) have been identified; these include the pat4 motif, which consists of a continuous stretch of four basic amino acids (arginine or lysine), and the pat7 sequence, which starts with a proline and which is followed within three residues by a segment containing three basic residues out of four (43). NuLSs are less well understood but usually incorporate an NLS. Using these criteria, amino acid sequence analysis indicated that TGEV N protein (amino acids 331 to 350; RPSEVAKEQRKRKRSRSKSAE) has a potential pat4 motif (underlined) and that the MHV N protein (amino acids 381 to 400; QDGGADVVSPPKQPKRGTK) has a potential pat7 motif (underlined), both located in the third domain of the N protein. A putative pat7 motif also was located in the domain III region of the IBV Beaudette strain N protein

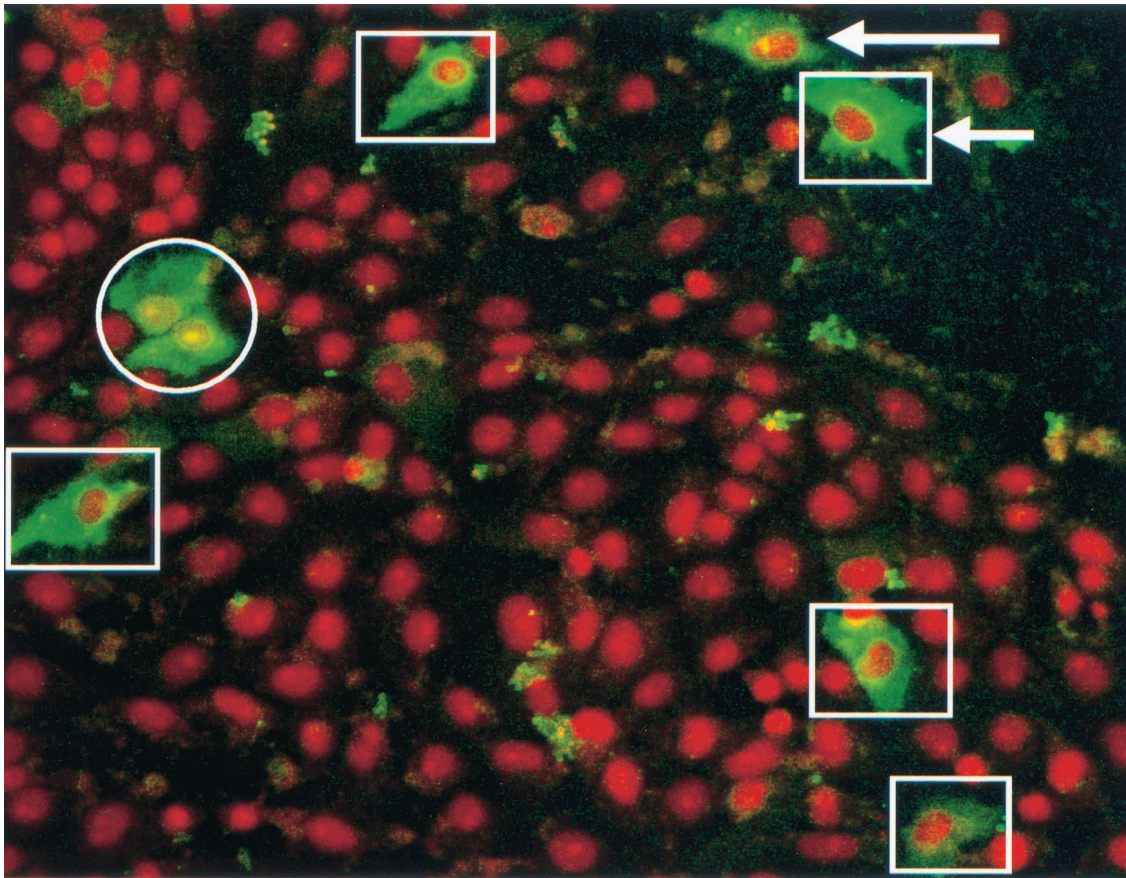


FIG. 4. Detection of MHV N proteins by indirect immunofluorescence in transfected cells. Vero cells were transfected with pCi-MHV-N, incubated for 24 h, fixed, and analyzed by indirect immunofluorescence using appropriate antibodies (green) (see text). Additionally, cells were stained with PI to visualize nuclear DNA (red). Differentially fluorescing images were gathered separately from the same 0.5- $\mu$ m-thick optical sections by using a confocal microscope and the appropriate filter. The two images were digitally superimposed to depict the distribution of the MHV N protein and nuclear DNA. Squares, cells in which the MHV N protein localized to the cytoplasm; circles, cells where the MHV N protein localized to both the cytoplasm and the nucleolus. Magnification,  $\times 16$ .

(23). Thus, the conservation of a putative NuLS in domain III of N proteins representative of the three different coronavirus groups suggests that it may be of functional significance.

**The coronavirus N protein might affect cell division.** During the course of this study, we observed that approximately 25 to 30% of transfected Vero cells expressing the TGEV N protein at various time points posttransfection were apparently undergoing cell division (Fig. 5A and B), unlike what is found in mock-transfected cells (Table 2). Assuming that most mammalian cells, on average, take approximately 24 h to go through the cell cycle, with mitosis and cytokinesis taking approximately 1 h, then in any 24-h period 4% of cells would be expected to be dividing. This figure is in agreement with the number of cells undergoing division in the mock-transfected cells (Fig. 5C; Table 2). Apart from an intrinsic property of the N protein, a number of possibilities could account for this observation. First, the backbone vector, pCi-Neo, contains a neomycin resistance gene under the control of an simian virus 40 early promoter (Promega), and expression of neomycin could result in the observation of a greater proportion of cells undergoing division. Second, expression of a foreign gene from the CMV promoter could lead to a similar result. Third, the

coronavirus N protein is an RNA binding protein and binding of cellular RNAs could disrupt normal cell division.

To test the above hypotheses, we transfected Vero cells with pEGFP (which expressed enhanced green fluorescent protein (EGFP) under the control of a CMV promoter), pCDNA3-NP (which expressed influenza B virus nucleoprotein [NP] under the control of a CMV promoter), and pHis-TGEV-N, which expressed the TGEV N protein with an N-terminal His tag under the control of a CMV promoter and which contained the zeocin rather than the neomycin resistance gene. Two of the vectors, pEGFP and pCDNA3-NP, had the neomycin resistance gene in common with pCi-TGEV-N, so if expression of neomycin led to an increased number of cells undergoing cell division, then this might be discriminated by using pHis-TGEV-N. Expression of EGFP also tested the hypothesis that expression of a foreign gene from a CMV promoter led to increased cell division. Influenza B virus N protein is a known RNA binding protein (2, 25) that localizes to the nucleus (62). Expression of this protein tested the hypothesis that RNA binding disrupted the cell cycle.

Vero cells transfected with pEGFP and pCDNA3-NP were fixed after 24 h; EGFP expression was analyzed directly, and

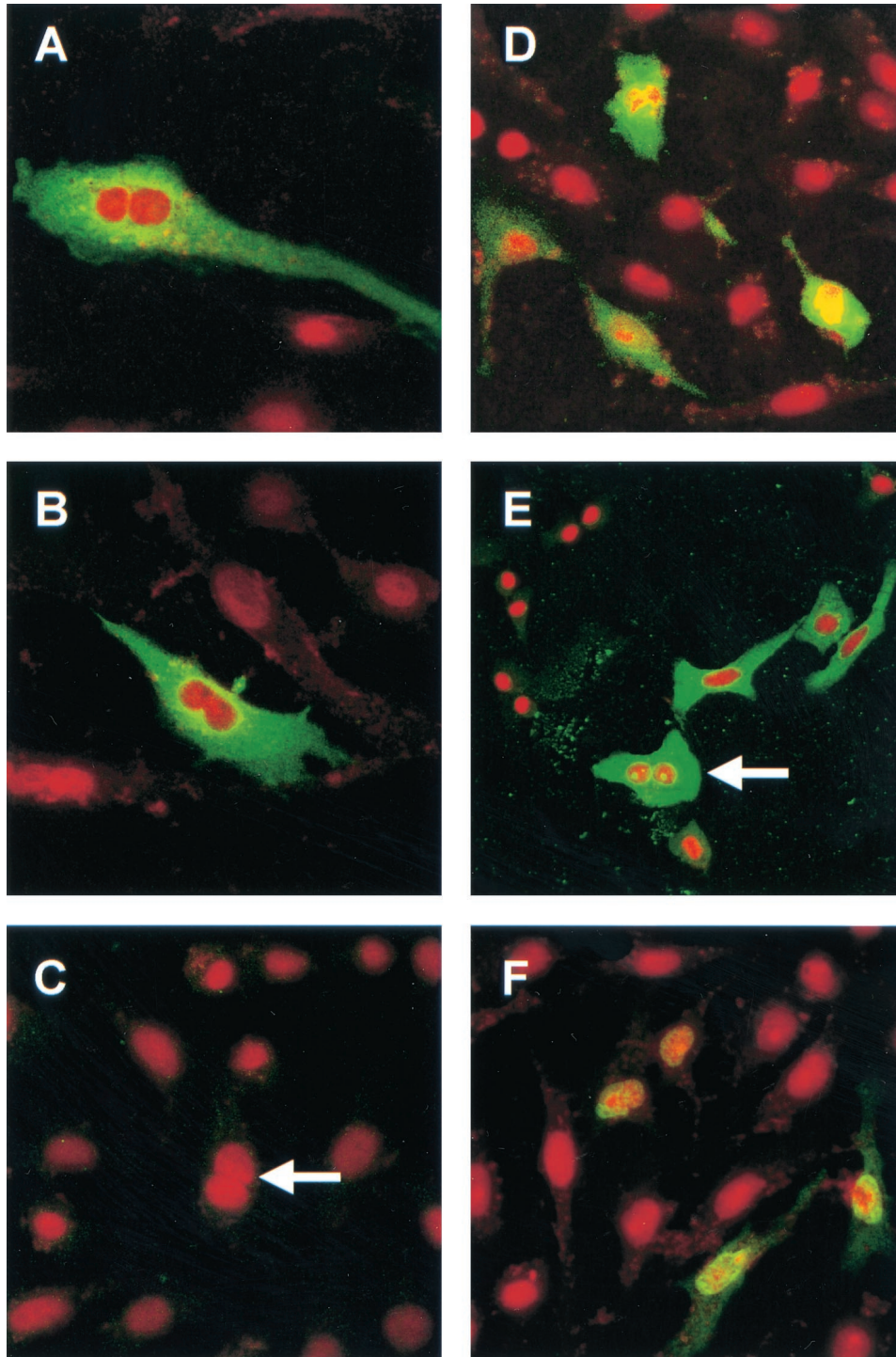


FIG. 5. Detection of TGEV N proteins (A, B, and E) and influenza B virus NP (F) by indirect immunofluorescence and detection of EGFP (D) (all green) and cell nuclei (A to F) (red) by direct immunofluorescence. Vero cells were transfected with either pCi-TGEV-N, pCDNA3-NP, or pEGFP, incubated for 24 h, fixed, and analyzed by indirect immunofluorescence using appropriate techniques (see text). Additionally, cells were stained with PI to directly visualize nuclear DNA. Differentially fluorescing images were gathered separately from the same 0.5- $\mu$ m-thick optical sections by using a confocal microscope and the appropriate filter. The two images were digitally superimposed to depict the distribution of the appropriate protein and nuclear DNA. Magnification,  $\times 62$ .

influenza virus NP was analyzed by indirect immunofluorescence with a mouse anti-B virus NP monoclonal antibody (MAS774b; Harlan Sera-Lab) and an anti-mouse immunoglobulin G-FITC conjugate. Both samples were mounted in PI

to visualize nuclear DNA. Both EGFP and influenza B virus NP were distributed evenly between the cytoplasm and nucleus (Fig. 5D and F, respectively). An average of 3 cells out of 100 were observed to be undergoing cell division in cells expressing



TABLE 2. Cell division in cells transfected with pCi-TGEV-N<sup>a</sup>

Time post-transfection (h)	No. of transfected cells		No. of mock-transfected cells	
	Counted	Undergoing cell division (%)	Counted	Undergoing cell division (%)
24	101	31 (31)	1,524	47 (3)
36	101	24 (24)	930	38 (4)
48	101	29 (29)	1,782	82 (5)
60	100	16 (16)	1,018	37 (4)
72	105	28 (27)	1,120	19 (2)

<sup>a</sup> Percentages of Vero cells transfected with pCi-TGEV-N and mock-transfected cells undergoing telophase or cytokinesis (cell division) were determined by fluorescence microscopy by counting ~100 transfected cells and 930 mock-transfected cells in three different visual fields per slide.

EGFP, whereas an average of 5 cells out of 100 in cells expressing influenza B virus NP were undergoing division. The background level of cell division in Vero cells was established to be between 2 and 5% (Table 2), which was in accordance with the predicted number. From these results we conclude that neither expression of neomycin nor expression of a foreign gene (EGFP gene) nor expression of an RNA binding protein (NP) affected cell division. This suggested that an intrinsic property of the TGEV N protein was responsible for disrupting cell division in transfected cells.

To confirm that the observation was not an artifact of expression of a protein from the pCi-Neo vector, the TGEV N protein was expressed in Vero cells from a vector with a backbone different from pCi-TGEV-N, pHis-TGEV-N. Transfected cells were fixed at 24, 36, and 48 h posttransfection for analysis by indirect immunofluorescence using mouse monoclonal anti-TGEV N sera (DA3), followed by a FITC-labeled goat anti-mouse antibody (Harlan Sera-Lab) (Fig. 5E). Fixed cells were stained with PI and analyzed by confocal microscopy. At all time points analyzed, over 25% of the cells transfected with pHis-TGEV-N were observed to be undergoing mitosis (Table 3); this is a result similar to that observed in cells transfected with pCi-TGEV-N.

## DISCUSSION

Although the *Arteriviridae* and *Coronaviridae* have considerable differences in virion architecture and genetic complexity, they are very similar in replication strategy and genome organization (17). The N proteins of the coronaviruses and arteriviruses are different in size (50 and 14 kDa, respectively) and in amino acid sequence; however, both are thought to play a major role in the formation of the virus core. Any other similarities between the N proteins, such as in intracellular localization, could suggest an important function of this protein that has been conserved between the two virus families. Rowland et al. (54) found that the N protein of PRRSV, an arterivirus, localized to both the cytoplasm and nucleolus in a subpopulation of cells infected with PRRSV and in cells transfected with vectors expressing the PRRSV N protein. Recently, we described a similar observation with the IBV (group III) N protein (23), and taken together with this study, where the N proteins of both TGEV (group I) and MHV (group II) coronaviruses localize to both the cytoplasm and nucleolus (Fig. 1) in both species-specific and nonspecific cells, these data sug-

gest that localization of the N protein to the nucleolus may be of functional significance in the order *Nidovirales*.

The mechanism by which the N protein localizes to the nucleolus was not determined in this study. However, nuclear pore complexes allow the passive transport in both directions between the cytoplasm and nucleoplasm of ions, small molecules, and proteins with molecular masses up to 40 to 60 kDa (46). The transport of larger proteins through the pore is an active process requiring ATP, and such proteins must contain suitable NLS (43, 52). Coronavirus N proteins from all three groups meet both criteria in that they are less than 50 kDa in size and contain putative NLS (pat4 and pat7 motifs) and might therefore be expected to enter the nucleus via both passive and active routes. Our previously published work suggests that transport of N proteins into the nucleus (and subsequently to the nucleolus) might, in part, be an active process, as an IBV N-green fluorescent protein fusion protein with a mass of 74 kDa (which would, therefore, not be expected to diffuse into the nucleoplasm unless it contained an NLS) localized to both the cytoplasm and nucleolus (23). Data suggest that domains I and III of the N protein are dispensable for RNA binding (36, 41). The putative NuLS identified within the domain III region of the N protein in this and a previous study (23) might be responsible for the active transport of the N protein into the nucleus. It is feasible that the N protein might then associate with rRNA in the nucleolus via the RNA binding domain (domain II). The BCoV N protein has been shown to associate with rRNA (14).

NuLSs are not restricted to pat4 and pat7 motifs, and arginine or lysine clusters may be sufficient or, conversely, may not be required at all. For example, nucleolar localization of the Marek's disease virus bZIP oncoprotein, MEQ, is mediated by an arginine-rich region (34), whereas putative NuLSs could not be identified in the adenovirus IVa2 gene product, which localizes to the nucleolus (35). Therefore, an alternative possibility is that the coronavirus N protein associates with cellular proteins that are targeted to the nucleolus, such as fibrillarin (4) and nucleolin (22, 58). Indeed, nucleolar components are involved in ribosome biogenesis (13), and the N protein might associate with preribosomal proteins synthesized in the cytoplasm and might be imported into the nucleolus. Fibrillarin is associated with CBs (60), and these move to and from nucleoli (47). Adenovirus infection results in the redistribution of fibrillarin, with a possibly effect on viral and host cell translation (49). Similar to polio- or adenovirus infection (65, 37) coronavirus infection might result in the redistribution of nucleolar antigens.

The function of N protein in the nucleolus is unknown, and

TABLE 3. Cell division in cells transfected with pHis-TGEV-N<sup>a</sup>

Time post-transfection (h)	No. of cells	
	Counted	Undergoing cell division (%)
24	251	92 (37)
36	253	85 (34)
48	303	80 (26)

<sup>a</sup> Percentages of Vero cells transfected with pHis-TGEV-N undergoing telophase or cytokinesis (cell division) were determined by fluorescence microscopy by counting ~250 cells in three different visual fields per slide.

our observations could be of an artifact that occurs as a consequence of the N protein diffusing into the nucleus and associating with rRNA. Alternatively, the N protein might play a role in the regulation of the cell cycle, as proteins that associate with the nucleolus previously have been implicated in cell cycle regulation (10). Interestingly, CDK2 and cyclin E localize to CBs in a cell cycle-dependent manner (33). A further alternative is that nucleolar localization of the N protein could be part of a general virus strategy to sequester ribosomal subunits for preferential translation of virus sgRNAs. Several other possibilities also exist (23).

The coronavirus N protein probably has multiple functions during the virus life cycle (29). Evidence presented here suggests that the coronavirus N protein also might function to inhibit cell division. There are two possibilities to account for our observed data that approximately 25% of cells transfected with the TGEV N protein appear to be undergoing cell division: the N protein could cause increased cellular proliferation and hence speed up mitosis or it could induce cell cycle delay or arrest. Of these two possibilities, we favor the latter scenario since a delay in mitosis and/or inhibition of cytokinesis could lead to an accumulation of cells in the M phase of the cell cycle and hence the appearance that a significant number of cells are undergoing cell division. In a fraction of transfected cells expressing coronavirus N proteins we observed multinucleate cells (data not shown), consistent with the fact that cytokinesis has been inhibited in these cells. This is not an unprecedented finding for mammalian cells since terminally differentiated adult cardiac myocytes, which lose the ability to divide as the heart develops, also are multinucleated (see reference 31 for a review). In addition, the "dividing" cell expressing the TGEV N protein in Fig. 5E has a nucleolus in each nucleus (nuclei are separated by a cleavage furrow), indicating that this cell, with respect to the nucleoli, is in interphase (nucleoli are absent in dividing cells [1]). The mechanism by which cytokinesis is inhibited in these cells is unknown at this time although it may involve a down-regulation in the activity and expression of the CDC2-cyclin B complex as is the case in mature cardiac myocytes (9). It is feasible that such a mechanism is also responsible for the effects of the N protein. Taken together, these data suggest that the N protein causes aberrant cell division, although a possible link between nucleolar localization of the N protein and cell cycle control is not established in this study.

It is not unusual for viruses and viral proteins to interact with the cell cycle machinery to promote virus replication; this is a common feature of DNA viruses that replicate in the nucleus (44). Retroviruses also disrupt the cell cycle; thus, human immunodeficiency virus type 1 Vpr arrests cells in the G<sub>2</sub> phase (24), resulting in an increase in virus production (51). Altering the host cell cycle appears to be less common in RNA viruses and has not been extensively described in the literature. For the negative-strand RNA viruses, there are several examples of control of the cell cycle. The reovirus nonstructural protein  $\sigma$ 1s arrests cells in G<sub>2</sub>/M during infection (48). Measles virus infection results in a G<sub>0</sub> block (40), and the paramyxovirus and simian virus (SV) V protein prolong the cell cycle by delaying cells in G<sub>1</sub> and G<sub>2</sub> (32). For positive-strand RNA viruses, the phenomenon of interacting with the cell cycle machinery is less common. The hepatitis C virus (HCV) NS5a protein has been reported to promote cell growth by repressing transcription of

the cyclin-dependent kinase inhibitor gene encoding p21<sup>WAF1</sup> (21). In addition, the HCV core protein interacts with this protein (67).

Lin and Lamb (32) postulated that enveloped viruses might delay the cell cycle in order to promote suitable conditions for virus assembly, especially if the virus life cycle is longer than the cell cycle. Intercellular sites of assembly for enveloped viruses include the Golgi and the endoplasmic reticulum, which are disrupted during cell division. The N protein might, therefore, have a function similar to that of the SV V protein, although coronavirus replication is quicker than that of SV and shorter than the cell cycle. However, it might be advantageous for a virus that by chance infected a cell in the latter stages of the cell cycle to delay the onset of mitosis for as long as possible. Alternatively, coronaviruses could also be controlling the cell cycle in order to create an environment favorable for the translation of virus sgRNAs. Translation of host proteins is approximately 25% in cells arrested in the G<sub>2</sub>/M phase or mitosis compared to that in interphase cells, because the cap-binding protein loses the ability to bind to the cap structure. Some cellular mRNAs whose encoded proteins are necessary during mitosis contain internal ribosome entry site elements, presumably to overcome the block in cap-dependent translation (55). Coronavirus sgRNAs and genomic RNA are capped and polyadenylated, and cap-dependent translation is believed to be responsible for translation of all of the coronavirus proteins with the exception of the envelope (E) protein, whose translation is possibly cap independent. The N protein might therefore have the dual function of delaying the cell cycle to promote intracellular conditions for virus assembly and also sequestering ribosomes for translation of viral proteins.

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