Betanodavirus B2 Is an RNA Interference Antagonist That Facilitates Intracellular Viral RNA Accumulation

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Betanodaviruses are small positive-sense bipartite RNA viruses that infect a wide variety of fish species and are notorious for causing lethal outbreaks in juvenile fish hatcheries worldwide. The function of a small nonstructural protein, B2, encoded by the subgenomic RNA3 of betanodaviruses, has remained obscure. Greasy grouper nervous necrosis virus, a betanodavirus model, was used to develop a facile DNA-based reverse genetics system that recapitulated the virus infection cycle, and we used this system to show that B2 is a small nonstructural protein that is essential for high level accumulation of viral RNA1 after RNA transfection of fish, mammalian, and avian cells. The defect in RNA1 accumulation in a B2 mutant was partially complemented by supplying B2 RNA in trans. Confocal analysis of the cellular distribution of B2 indicated that B2 is able to enter the nucleus and accumulates there during the late stages of GGNNV infection. Using human HeLa cells as a cellular RNA interference model, we found that B2 could efficiently antagonize RNA interference, which is a property shared by the distantly related alphanodavirus B2 proteins. This function provides an explanation, at least in part, for why B2 mutant RNA1 is severely impaired in its intracellular accumulation.

Greasy grouper nervous necrosis virus (GGNNV) is an RNA virus that elicits nervous necrosis upon infection of a variety of fish species (28). GGNNV belongs to the beta subgroup of the virus family Nodaviridae. The nodavirus genome consists of two single-stranded positive-sense RNA molecules, the 3.1-kb RNA1 and the 1.4-kb RNA2. RNA1 encodes an RNA-dependent RNA polymerase (RdRp or protein A), while RNA2 encodes the coat protein, α (25). In addition, a subgenomic RNA transcribed from the 3′ end of RNA1, termed RNA3 (10, 26), encodes a protein of unknown function called B2.

We have previously investigated the subcellular localization of protein A and found that it targets mitochondrial membranes via an N-terminal stretch of approximately 40 amino acids (10), which is also the case with other nodaviruses (23, 24). Furthermore, we have characterized the nucleolus localization and apoptotic inducer domains within the α coat protein of the GGNNV (11, 12). However, the function and properties of the remaining GGNNV protein, B2, have remained a mystery.

The betanodaviruses of fish studied to date all possess putative B2 proteins with very high sequence homology. The betanodavirus B2 proteins consist of only 75 amino acids and do not possess any characteristic sequence motifs or domains. Several of the alphanodavirus B2 proteins, however, have been characterized and appear to be involved in the suppression of cellular RNA interference, which is now known to act as an intracellular poison against virus infection (7). The B2 protein of Nodamura virus (NoV), which infects both insects and rodents, is required for maximal autonomous replication of RNA1 and RNA3 after transfection of a wide variety of cell lines with RNA1 transcripts synthesized in vivo (17). Recent data indicate that the B2 proteins of both NoV and another alphanodavirus, Flock House virus (FHV), are able to block host-mediated RNA interference (RNAi) in eukaryotes by preventing Dicer-mediated RNA cleavage (20–22, 27). These data suggest that the alphanodavirus B2 proteins function to prevent destruction of viral double-stranded RNA (dsRNA) replicating intermediates that would otherwise trigger RNAi in the host cell.

Despite these remarkable findings in the alphanodaviruses, the B2 proteins of betanodaviruses share little in common with their alphanodavirus counterparts (8). Betanodavirus B2s range in size from 90 to 137 amino acids and share little significant sequence homology with one another, with the exception of those from the Boolarra and Black Beetle viruses (8, 18), which share 77.4% sequence identity. Although an alignment of the B2 protein of GGNNV to those of NoV and FHV shows several small stretches of conserved amino acids (Fig. 1), the identities (10.2 and 7.1% for NoV and FHV, respectively) are only marginally higher than would be expected to occur by chance. The possible biological roles and properties of the B2 proteins from betanodaviruses cannot therefore be easily deduced by direct sequence comparisons with their alphanodavirus counterparts.

In the present study we have developed a DNA-based reverse genetics system for GGNNV and show, by using both reverse genetics and real-time reverse transcription-PCR (RT-PCR) detection of intracellular RNA1 accumulation, that B2 is essential for maximal RNA1 accumulation in a diverse range of cell types. We go on to use a replicon-based RNAi system in HeLa cells to show that B2, even in the presence of high levels of a shRNA expression vector, is able to effectively block the host RNAi response, revealing a role for B2 as an RNAi antagonist.
Materials and Methods

Cells and viruses. Asian sea bass (SB) fibroblast cell culture and infection by GGNNV was performed as described previously (13), while BSR T7/5 (5), HeLa, and DF-1 (14) cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS). Unless otherwise indicated, SB cells were grown at 28°C, BSR T7/5 and HeLa cells were grown at 37°C in a 5% CO2 humidified chamber and DF-1 cells were grown at 37°C in a 5% CO2 humidified chamber.

Construction of RNA1 and RNA2 cDNA transcription plasmids. Infected SB culture supernatants were collected at 3 to 4 days postinfection and centrifuged for 10 min at 10,000× g to remove cells and debris. GGNNV was pelleted from 10 ml of the supernatant by centrifugation at 220,000× g for 3 h at 4°C, and RNA was isolated by using TRIzol reagent (Invitrogen). The extracted RNA was subjected to a two-step RT-PCR to amplify RNA1 and incorporate terminal BbsI restriction sites for cloning. RT was performed in 20-μl reactions with StrataScript reverse transcriptase (Stratagene) using 800 ng of total RNA and the RNA1-BbsI-REV1 primer (5'-GCGGAAGACATACCCCGCCGAAGCGATA CGACGACTAAAG-3'). The PCR was performed in a 20-μl volume with 2 μl of the RT reaction described above, the RNA1-BbsI-FWD1 (5'-GCCGGAAGACATACCCCGCCGAGTTGAGAAGCGATC-3'), and RNA1-BbsI-REV1 primers, and Pfu Turbo DNA polymerase (Stratagene). The PCR thermocycle consisted of an initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min with a final extension at 72°C for 10 min.

Products of the RT-PCR were separated on a 1% agarose-TAE gel, and the DNA band corresponding in size to full-length RNA1 (3.1 kb) was excised and purified by using a QiAquick gel purification kit (QIAGEN). This DNA was digested with BbsI and ligated to TTVTR(0.0) transcription vector (19). Ligation mixtures were used to transform Escherichia coli DH5α, and plasmid DNA from six transformants was verified by DNA sequencing. One plasmid, designated pGGNNV1(0,0), was found to be free of cDNA mutations compared to the published GGNNV RNA1 sequence (28) and was used in further studies. Variants of pGGNNV1(0,0) containing 5' or 3' nonviral residues were created in the same way using appropriate modified primers. RNA2 cDNA transcription vectors were created in the same way using the primers RNA2-BbsI-FWD1 (5'-GCCGGAAGACATACCCCGCCGAGTTGAGAAGCGATC-3') and RNA2-BbsI-REV1 (5'-GCCGGAAGACATACCCCGCCGAGTTGAGAAGCGATC-3'). For clarity, the naming system for the plasmids used here is based on the presence of 5' and 3' nonviral nucleotides following transcription and ribozyme cleavage. Thus, the designation (0,0) indicates that the resulting RNA has no nonviral nucleotides, whereas (1,0) and (2,0) indicate the presence of one or two nonviral residues at the 5' and 3' termini, respectively.

In vitro site-directed mutagenesis. Mutagenesis of the B2 start codon was performed in a 20-μl volume containing 30 ng of pGGNNV1(0,0), 0.1 μM concentrations of each mutagenesis primer (B2-MUT-FWD, 5'-CAAAACTAGTAGACGACCACCAAAATCTACAAG-3'; B2-MUT-REV, 5'-TGTGTTGAGTTGCTTCGGTCTGTGATTTGTT-3'), 0.2 μM concentrations of each deoxynucleoside triphosphate, 2 μl of Pfu Turbo 10X reaction buffer, and 1 U of Pfu Turbo DNA polymerase (Stratagene). Mutagenesis primers were 5' phosphorylated with T4 polynucleotide kinase (New England Biolabs) prior to use. The PCR thermocycle consisted of an initial denaturation at 95°C for 2 min, followed by six cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 12 min, with a final extension at 72°C for 10 min. The products of this reaction were treated with 20 U of DpnI (New England Biolabs) for 1 h to remove methylated parental DNA prior to transformation of E. coli DH5α. Three mutant plasmids were sequenced to confirm incorporation of the desired mutation and one of these, designated pGGNNV1B2(0,0), was used in subsequent work.

In vitro transcription. Transcription reactions were performed in 50-μl reaction volumes containing 5 μg of plasmid DNA, ribo-m-G cap analog, and T7 RNA polymerase (Promega) as recommended by the manufacturer. Template DNA was removed by digestion with 5 U of RQ1 RNase-free DNase (Promega) for 30 min, and RNA was purified by using TRizol reagent (Invitrogen). RNA was quantified by using a NanoDrop ND1000 spectrophotometer and analyzed by denaturing formaldehyde-agarose gel electrophoresis.

Transient transfections. For RNA transfections, cells were grown to 70 to 90% confluence, treated with trypsin, and washed once in fresh medium without FBS for BSR T7/5, HeLa, and DF-1 cells or in medium containing 1% FBS for SB cells. Cells were then resuspended in the washing medium at a density of 1 × 10^6 to 5 × 10^6 cells/ml. Cell suspension volumes of 0.1 to 0.2 ml were mixed with 16 μg of electroporated 2-μm cuvettes using a Bio-Rad Gene Pulser X-cell apparatus. The electroporation protocols for each cell type were as follows: SB cells, 250-μs square pulse at 450 V; BSR T7/5 cells, 25-ms square pulse at 140 V; HeLa cells, exponential pulse at 160 V, 500 μF, infinitive resistance; and DF-1 cells, 250-μs square pulse at 900 V. After electroporation the cells were transferred to 24-well culture plates containing complete medium and incubated at 28°C. DNA transfections were performed using Lipofectamine and PLUS reagent (Invitrogen). RNA was quantified by using a NanoDrop ND1000 spectrophotometer and analyzed by denaturing formaldehyde-agarose gel electrophoresis.

Quantitative real-time RT-PCR. GGNNV RNA1 accumulation was monitored by a real-time RT-PCR assay specific for RNA1 and normalized against cellular 18S rRNA. Total RNA was prepared from GGNNV-infected SB cells or transfected cells in 24-well culture plates using TRIzol reagent (Invitrogen). Extracted RNA was treated with RQ1 RNase-free DNase (Promega), re-extracted with TRIzol reagent, and then quantitated by using a NanoDrop spectrophotometer. RT reactions were performed with avian myeloblastosis virus reverse transcriptase (Promega) using 100 ng of RNA and the relevant primers as recommended by the manufacturer. The absence of contaminating DNA and the presence of a single specific PCR product was confirmed by endpoint PCR and agarose gel electrophoresis. Reactions were performed in 20-μl volumes containing cDNA template, 0.2 μM concentrations of each primer, 0.2 μM concentrations of each deoxynucleoside triphosphate, 1.5 mM MgCl2, and 250 ng of Plasmid DNA polymerase and reaction buffer. The PCR thermocycle consisted of an initial denaturation at 94°C for 5 min, followed by either 35 cycles (for quality tests) or 25 cycles (for standard curve tests) of 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s, with a final elongation at 72°C for 2 min. Products were separated on a 1.2% agarose TAE gel stained with ethidium bromide and visualized by UV illumination.

Real-time RT-PCR assays for RNA1 used the RNA1-FWD1 (5'-CCCTCACGCCTTCCGGTGAGTCCGCTTAGAA3') and RNA1-REV1 (5'-CAATGGAACCTAAGGATGGAAGGT3') primer pair, which target a 148-nucleotide (nt) region of RNA1, and were normalized with universal 18S rRNA primers (18S-FWD, 5'-CCGGCTACACATCCGAAAGGA3'; 18S-REV, 5'-CGCTGGAATTCGCCGCTC3') in separate reactions. Reactions were performed using the RNA Master SYBR Green I system and LightCycler (Roche) as recommended by the supplier. The PCR thermocycle consisted of an initial denaturation at 94°C for 5 min, followed by a variable number of cycles of 95°C for 5 s, 55°C for 5 s and 72°C for 25 s. Subsequent melting curve analysis and C_T value determinations were performed using Roche LightCycler software version 3.5. PCR target copy numbers in test reactions were determined by comparison with known amounts of RNA1 and 18S rRNA transcripts generated in vitro using T7 RNA polymerase and pBluescript-SK(+) or pBluescript-SK(−) containing the relevant PCR amplicons ligated at the Smal site. RNA levels are expressed as normalized units, calculated by dividing the calculated copy number of RNA1 target by the calculated copy number of 18S rRNA and multiplying the result by one million to obtain easily interpretable figures.
Real-time RT-PCR assays for enhanced green fluorescent protein (EGFP) transcript levels in transfected HeLa cells were performed as described above with primers EGFP-FWD (5'-ACGTCTATATCAGGGCGG-3') and EGFP-REV (5'-TGATGACGCTTTC-3') and normalized by using the 18S rRNA primers as described above. EGFP transcript levels were calculated by comparison of C_\text{t} values with a standard curve generated from serial dilutions of EGFP RNA as generated from the EGFP ampiclon cloned at the SalI site of pCDNA3.1(+). The resulting copy numbers were normalized to 18S RNA copy numbers prior to comparison.

Immunoblotting. For generation of polyclonal anti-B2 antibodies, the B2 open reading frame (ORF) was first amplified from GGNNV(0,0), inserted into the pQE30 expression vector (QIAGEN) to create an N-terminal His_6 affinity tag fusion, overexpressed in E. coli M13(pREP4) expression host, and purified by using Ni_2+NiNTA nickel chelate affinity resin as recommended by the manufacturer (QIAGEN). Antibodies to the His_6-B2 protein were raised in guinea pig as described previously (10) and validated by immunoblotting with the purified His_6-B2 protein. The generation of polyclonal antibodies to protein A and α coat protein has been described elsewhere (10, 11). Total cell proteins (30 to 50 μg per well) prepared from infected SB or transfected BSR T7/5 cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% (for protein A) or 15% (for B2) gels and subjected to immunoblotting (10) with 1:1,000 and 1:2,000 dilutions of anti-protein A and anti-B2 antibodies, respectively. Signals from horseradish peroxidase-conjugated rabbit anti-guinea pig secondary antibodies (Dako) at a 1:1,000 dilution were developed by using Super Signal West Femto chemiluminescent substrate (Pierce) and visualized by autoradiography.

Immunolocalization and confocal microscopy. Indirect immunofluorescence assays (IFA) were performed with infected or transfected cells in either four-well chamber slides or 24-well tissue culture plates. Cells were fixed for 30 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed thrice in PBS-Tween 20 (0.05% [vol/vol]) and then treated with a 1:100 dilution of polyclonal antibody for 2 h at 37°C. Cells were washed again with PBS-Tween 20 and treated with a 1:100 dilution of fluorescein isothiocyanate-conjugated rabbit anti-guinea pig polyclonal antibodies (Dako) at a 1:1,000 dilution were developed by using Super Signal West Femto chemiluminescent substrate (Pierce) and visualized by autoradiography.

RESULTS

B2 is a small nonstructural protein expressed during GGNNV infection. Several betanodavirus research groups have predicted the existence of a B2 protein based on sequence analysis of the RNA3 subgenomic RNA, although this has not yet been confirmed experimentally. To resolve this issue, we prepared polyclonal antibodies against a His_6-tagged B2 protein and used them to probe cellular proteins of SB cells infected with GGNNV by immunoblotting. As expected, we observed a small band of approximately 8 kDa expressed within 3 h of infection, which remained during the 24-h sampling period (Fig. 2). The size of the protein was measured as approximately 8 kDa, which corresponds reasonably well with the predicted molecular mass of B2, which is 8.5 kDa. As expected, the viral replicase protein A was also detectable and accumulated during the sampling period (Fig. 2). These results clearly indicate that B2 is a small nonstructural protein expressed during GGNNV infection of SB cells.

Establishment of a DNA-based GGNNV reverse genetics system. Investigations of nodavirus biology have been greatly expedited by the development of reverse genetics systems that function to provide a suitable host cell with nodavirus RNA1 and RNA2. Together these RNAs are sufficient to support infection of the host cell. The approach we took toward GGNNV reverse genetics was the creation of a DNA-based nodavirus replicon vector that can be directly introduced into T7 RNA polymerase-expressing BSR T7/5 cells (5) to liberate infectious nodavirus. Thus, as a first step toward investigating the involvement of protein B2 in GGNNV infection, we constructed a series of plasmids using the T7 promoter-based transcription vector TVT7R(0,0) with the aim of establishing a reverse genetics system for GGNNV. The TVT7R(0,0) vector facilitates the generation of RNA transcripts containing defined 5' and 3' termini through placement of DNA inserted between the 5' T7 promoter and 3' HDV antigenic ribozyme/T7 terminator elements (Fig. 3A). Three versions of RNA1 cDNA were synthesized, including one with the native GGNNV RNA1 sequence and two versions with additional nonviral residues. Of the three cDNAs, two were ligated to TVT7R(0,0) to yield pGGNNV1(0,0), pGGNNV1(1,0), and pGGNNV1(2,0), respectively. These additional nonviral residues were added as a means of improving transcription initiation by T7 RNA polymerase, as has been done for other nodavirus transcription plasmids (9, 15, 19). In vitro transcription with the 5' G or GG RNA1 versions as templates liberated substantially more of the 3.1-kb RNA1 transcript (Fig. 4A), although native RNA1 template still generated significant quantities of RNA1. The positive influence of the 5' G residues on transcription of RNA1 also occurred in vivo, since transfection of BSR T7/5 cells with pGGNNV1(1,0) and pGGNNV1(2,0) yielded 15 and 20 times more RNA1 transcript, respectively, than pGGNNV1(0,0) (Fig. 4B). Given

![Image](http://jvi.asm.org/Downloaded from http://jvi.asm.org)
that pGGNNV1(0,0) yielded usable quantities of RNA1 in vitro, we used this construct to generate RNA1 transcripts in vitro for RNA transfection but used pGGNNV1(2,0) for DNA transfection experiments involving the T7 RNA polymerase-expressing BSR T7/5 cells. RNA2 vectors, designated pGGNNV2(0,0), pGGNNV2(1,0), and pGGNNV2(2,0) were created by using the same approach (Fig. 3B). With these vectors, however, we found that the addition of two 5′G residues resulted in virtually complete apoptotic destruction of all cotransfected BSR T7/5 cells within 12 h (data not shown), presumably due to overexpression of the potent apoptotic inducer B2 encoded by RNA2. In spite of this, we found that the native RNA2 vector pGGNNV2(0,0) was sufficient for production of infectious GGNNV in BSR T7/5 cells (shown below), which is in agreement with previous observations for alphaherpesvirus infectious clones (19).

The two plasmids pGGNNV2(2,0) and pGGNNV2(0,0) were used to transfect BSR T7/5 cells and, following a 48-h incubation at 28°C, the cells were lysed and the supernatant used to inoculate subconfluent Asian sea bass (SB) fibroblasts, which are permissive for GGNNV infection (12). An obvious cytopathic effect typical of GGNNV infection was evident in the inoculated cells after 48 h (Fig. 5A), whereas the presence of the GGNNV a coat protein was detected at 24 h by an IFA using an anti-a antibody (Fig. 5B). We also confirmed that the recombinant virus, rGGNNV, exhibited replication kinetics similar to the wild-type virus using a real-time RT-PCR assay that is specific for RNA1 and normalized against cellular 18s rRNA, a reliable indicator of cell number (3). As shown in Fig. 5C, RNA1 synthesis commenced within 3 h postinfection in both GGNNV- and rGGNNV-infected cells, with RNA1 continuing to accumulate over the 44-h sampling period. The total amplification of RNA1 by GGNNV and rGGNNV during this period was 36- and 78-fold, respectively, although the kinetics of replication for both viruses is essentially identical. Taken together, these data indicate that a plasmid cotransfection system using BSR T7/5 cells provides a relatively simple method
for recapitulating the GGNNV infection cycle from plasmid replicons.

A RNA1 B2 mutant is impaired in RNA1 accumulation. To elucidate the role, if any, of protein B2 in GGNNV infection, we created a variant of pGGNNV1(2,0) by site-directed mutagenesis to eliminate the B2 start codon. The resulting mutant, pGGNNV1ΔB2(2,0), contains a single T-to-C point mutation that changes the ATG start codon of B2 to ACG and does not alter the amino acid sequence of the overlapping protein A ORF (Fig. 3A). The effect of this mutation was confirmed by immunoblotting transfected BSR T7/5 cell extracts with protein A and B2 antisera, using GGNNV-infected SB cell extracts as a positive control. The results of this experiment are shown in Fig. 3C, where a 110-kDa band corresponding to the protein A protein was detected in all extracts, while the B2 band was only present in BSR T7/5 cells transfected with pGGNNV1(2,0) or in infected SB cells. Thus, the T-to-C point mutation of the B2 start codon appeared to be sufficient to ablate B2 translation but not interfere with protein A translation.

To investigate the role of B2 in GGNNV infection, we co-transfected BSR T7/5 cells with the pGGNNV1ΔB2(2,0) and pGGNNV2(0,0) plasmids as described above and used the resulting lysates to infect SB cells. To our surprise, no IFN-positive cells were detected in the first passage (data not shown), although after five consecutive supernatant passages we were able to obtain an abundance of IFN signals resembling those obtained from the wild-type clones. However, after extracting total RNA from these cells and subjecting it to a RNA1-specific RT-PCR, DNA cloning, and sequencing, we found that, of 10 independent clones analyzed, all had a C-to-T reversion at the B2 start codon (data not shown). These data suggested that the B2 protein was important in GGNNV infection and/or replication, although its role remained unclear.

As an alternative approach to investigating the role of B2 in GGNNV infection, we asked the question of how B2 might contribute to RNA1 accumulation in the absence of a full virus replication cycle. Alphanodavirus RNA1s are capable of autonomous replication, and this property has recently been exploited to show that NoV B2 is required for maximal RNA1 accumulation in vivo. Thus, we generated capped RNA1 in vitro using pGGNNV1(0,0) or pGGNNV1ΔB2(0,0) as the template and used this RNA to transfect SB cells. We then extracted cellular RNA at several time points after transfection up to 9 h, at which time wild-type RNA1 levels were found to be more than 95% of the maximal level observed at up to 24 h posttransfection (data not shown). Real-time RT-PCR analysis revealed that RNA1 accumulation in the B2 mutant was significantly reduced in transfected SB cells (Fig. 6A), with the total amount at 9 h posttransfection being eight times lower than the wild-type level (29.9 versus 246 U). The RNA1 level in the mutant at 9 h posttransfection were, however, substantially more than those observed after 3 h (1.2 versus 29.9 U; Fig. 6A), indicating that RNA1ΔB2 was still capable of replication. To confirm that the B2 start codon mutation was retained throughout the transfection/repllication period, the B2 region of RNA1 was amplified from transfected SB cell RNA extracts by using RT-PCR and cloned into the E. coli cloning vector pUC18. DNA sequencing of plasmids derived from 10 of the resulting E. coli transformants indicated that in all cases the B2 start codon mutation was preserved.

Johnson et al. (17) previously made the fascinating observation that autonomous replication of NoV RNA1ΔB2 was drastically reduced compared to wild-type RNA1 in some cell types, such as HeLa, but not in others, such as BSR T7/5. We were thus interested to determine whether a similar phenomenon occurred with GGNNV RNA1ΔB2. To this end, both
to about 6 h for the other cell types. Importantly, RNA1 accumulation in the B2 mutant was impaired in each of these cell types to various degrees (Fig. 6B, C, and D), with the total amount of RNA1 observed at the 9 h posttransfection (or 7.5 h for BSR T7/5) being 3.6-, 17-, and 7.2-fold lower in the B2 mutant than the wild type for BSR T7/5, DF-1, and HeLa cells, respectively. From these results it seemed clear that B2 was important for RNA1 accumulation in both permissive and non-permissive cell types.

**A B2 mutant RNA1 can be partially complemented.** Taken together, the results presented above suggested that the B2 protein was required for maximal accumulation of RNA1 but did not exclude the possibility that the start codon mutation we created to prevent B2 translation was deleterious in and of itself in terms of RNA1 stability or replication. Indeed, RNA viruses are widely known to be extremely sensitive to mutation and can be considered as existing on the threshold of error catastrophe (6). With this in mind, we sought to complement the B2 lesion by generating capped in vitro transcripts of either the B2 ORF by itself (nt 2753 to 2980 of RNA1) or ΔB2 transcripts containing the B2 start codon point mutation and using them to cotransfect the four cell types along with RNA1ΔB2. For this experiment the B2 ORF transcripts were transfected at a 55-fold (for SB cells) or 11-fold (for BSR T7/5, DF-1, and HeLa cells) molar excess relative to RNA1ΔB2. Excess B2 transcript was used for SB cells to compensate for RNA1 accumulation in these cells with those in cells transfected with RNA1 or RNA1ΔB2 alone. It is important to note that the real-time RT-PCR assay used to detect RNA1 accumulation targeted a region well outside of the B2 ORF in RNA1 (see Fig. 3A), and was therefore not compromised by the addition of B2 ORF transcripts. Cotransfection with B2 ORF had a modest but significant impact on RNA1ΔB2 accumulation (Table 1), with RNA1ΔB2 accumulation being complemented to an efficiency of more than 20% in DF-1 and HeLa cells. In addition, there was no significant difference in RNA1 accumulation between RNA1ΔB2 and the RNA1ΔB2+ΔB2 transfections (Table 1), indicating that the point mutated ΔB2 ORF did not influence RNA1 accumula-

**FIG. 6.** B2 is required for maximal accumulation of RNA1 in a variety of cell types. RNA1 (●) or RNA1ΔB2 (■) transcripts were transfected into SB (A), BSR T7/5 (B), DF-1 (C), or HeLa (D) cells, and RNA1 accumulation was monitored over time by real-time RT-PCR, normalized against cellular 18S rRNA levels. Values shown are the means of three independent determinations, performed in duplicate. Error bars indicate the standard error of the mean.

**TABLE 1.** B2 RNA transcripts supplied in trans can partially complement the RNA1 accumulation defect of the RNA1ΔB2 mutant

<table>
<thead>
<tr>
<th>Cell type</th>
<th>RNA1 level in transfected cells*</th>
<th>Complementation efficiency by B2 ORF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB</td>
<td>RNA1</td>
<td>RNA1ΔB2</td>
</tr>
<tr>
<td>BSR T7/5</td>
<td>246</td>
<td>29.9</td>
</tr>
<tr>
<td>DF-1</td>
<td>326</td>
<td>90.6</td>
</tr>
<tr>
<td>HeLa</td>
<td>314</td>
<td>11.8</td>
</tr>
</tbody>
</table>

* RNA1 levels, determined by the real-time RT-PCR assay, were measured using RNA prepared from RNA-transfected cells at either 9 (SB, DF-1, and HeLa cells) or 7.5 (BSR T7/5 cells) h posttransfection. Values are the mean of three independent determinations, with the standard error being less than 12% of the mean for each test.

b Capped RNA transcripts generated by T7 RNA polymerase runoff transcription using linearized pCDNA3.1(+) containing the GGNNV B2 ORF or the ΔB2 derivative of the B2 ORF were used to cotransfect cells with RNA1ΔB2. B2 or ΔB2 RNA was added at a level of 500 ng (SB cells) or 100 ng (BSR T7/5, DF-1, and HeLa cells) per transfection, with RNA1 or RNA1ΔB2 at 100 ng per reaction. Given the calculated size difference between the B2 and RNA1ΔB2 transcripts, these represent 55- or 11-fold molar excesses of B2 transcript. Total SB RNA was added to all transfections to bring the final amount of RNA to 1 ng per transfection.

c Complementation efficiency is the difference between RNA1 levels observed in the RNA1ΔB2+ΔB2 and RNA1ΔB2+ΔB2 experiments expressed as a percentage of the wild-type level.
A recombinant His6-B2 protein was expressed in E. coli, and next looked at its subcellular localization. For the present study the GGNNV B2 facilitates viral RNA accumulation.

Visualization of B2 in infected and transfected cells. To gain a better insight into the biological properties of the B2 protein, we next looked at its subcellular localization. For the present study a recombinant His6-B2 protein was expressed in E. coli, purified, and used to raise polyclonal antibodies in guinea pig serum. The results of this experiment were thought significant enough for us to conclude that the negative effect of the B2 start codon mutation on RNA1 accumulation was due to a lack of B2 protein rather than a decrease in RNA1 stability or replication competence.

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Visualization of GGNNV B2 in infected and transfected cells. SB cells either infected with GGNNV (A and C to E), mock infected (B), or transfected with pcDNA-B2 (F), pEGFP (G), pcDNA-B2, or pEGFP-B2 (H) were fixed at the indicated times postinfection or posttransfection using paraformaldehyde, subjected to IFA with anti-B2 polyclonal antibody where appropriate, and their nuclei were counterstained with DAPI (shown in red false coloring). Overlap between DAPI and EGFP is visible as yellow coloring. Fluorescence was visualized by using a Zeiss LSM510 META laser scanning microscope. Images are representative of the protein localization observed in the majority (>70% of at least 50 cells) of cells visualized. Bars in the lower right corners of the images represent 10 μm.

FIG. 7. Visualization of GGNNV B2 in infected and transfected cells. SB cells either infected with GGNNV (A and C to E), mock infected (B), or transfected with pcDNA-B2 (F), pEGFP (G), pcDNA-B2, or pEGFP-B2 (H) were fixed at the indicated times postinfection or posttransfection using paraformaldehyde, subjected to IFA with anti-B2 polyclonal antibody where appropriate, and their nuclei were counterstained with DAPI (shown in red false coloring). Overlap between DAPI and EGFP is visible as yellow coloring. Fluorescence was visualized by using a Zeiss LSM510 META laser scanning microscope. Images are representative of the protein localization observed in the majority (>70% of at least 50 cells) of cells visualized. Bars in the lower right corners of the images represent 10 μm.

The functional similarity of the cellular RNAi response among different species and the comparable behaviors of the RNA1ΔB2 mutant among the four cell types used above gave us reason to believe that an effect on RNAi by B2 in HeLa cells should also be biologically relevant to other cell types. In our previous work (10, 11) involving the remaining two alphanodavirus B2 proteins, protein A and α, did not reveal a significant degree of specific colocalization, suggesting that B2 does not specifically interact with these proteins during infection.

Cells transfected with pcDNA-B2 displayed an essentially homogeneous distribution of B2 (Fig. 7F) at 24 h posttransfection. In the case of the EGFP fusions, both EGFP-B2 and B2-EGFP localized throughout the cell but occurred predominantly in the nucleus (Fig. 7H and I), similar to the distribution of unmodified EGFP (Fig. 7G). We can thus conclude from these data that GGNNV B2 is indeed able to enter the nucleus and accumulates there during the late stages of infection. In addition, both unmodified B2 and its EGFP fusions localize throughout the cell and, in the case of the EGFP fusions, accumulate in the nucleus.

GGNNV B2 antagonizes RNAi. Recent studies reporting on the function of B2 in alphanodaviruses have indicated that their B2 proteins function as RNAi antagonists that can block activity of the Dicer RNase both in vivo and in vitro (20, 21, 27), thus preventing the formation of siRNAs from longer dsRNA precursors. The blocking of RNAi by B2 presumably facilitates the accumulation of dsRNA viral replication intermediates. Given the similar effect that GGNNV B2 had on RNA1 accumulation (see Fig. 6) compared to its NoV B2 counterpart, we reasoned that GGNNV B2 might also be able to antagonize RNAi. To test this hypothesis, we devised a simple RNAi assay using HeLa cells, whose RNAi response was shown to be highly efficient in a previous study of NoV B2 (17). The functional similarity of the cellular RNAi response among different species and the comparable behaviors of the RNA1ΔB2 mutant among the four cell types used above gave us reason to believe that an effect on RNAi by B2 in HeLa cells should also be biologically relevant to other cell types. In our assay pEGFP (the RNAi target), pcDNA, or pcDNA-B2 and a U6 promoter-based shRNA vector expressing either a specific anti-EGFP shRNA or scrambled shRNA were used to transiently transfect HeLa cells.

After cotransfection of HeLa cells with the shRNA and GFP vectors, we visualized EGFP expression by microscopy and noticed a marked decrease in EGFP expression in cells transfected with the specific anti-EGFP shRNA vector in the absence of B2 (Fig. 8A, compare the first and third images). However, this response was virtually abolished in cells cotransfected with a B2-expressing plasmid (Fig. 8A, compare the second and fourth images), suggesting that B2 was blocking RNAi. We then quantitated the level in EGFP mRNA in the transfected cells by an EGFP-specific real-time RT-PCR assay, normalized to cellular 18s rRNA levels (Fig. 8B). We found that a molar ratio of 10:1 shRNA plasmid to EGFP target specifically silenced EGFP expression by 86%, whereas a 1:1 ratio resulted in only 48% silencing compared to the nonspecific control (Fig. 8C). In the presence of a B2-expressing plasmid, however, even at a 10:1 ratio a silencing of only 26% was measured, while at 5:1 and 1:1 ratios there was no detectable decrease in EGFP mRNA levels compared to the nonspecific control (Fig. 8C). Virtually identical results were ob-
tained when either B2-EGFP or EGFP-B2 proteins were used in place of the native B2 protein and EGFP RNAi target (data not shown), indicating that the B2 moiety of these fusion proteins fully retains the ability to block RNAi. Based on these results we conclude that B2 can efficiently antagonize the RNAi response of HeLa cells.

**DISCUSSION**

Our current work has uncovered a number of intriguing properties of the GGNNV B2 protein, not least of which is its identity as a bona fide protein expressed during betanodavirus infection. Past work has predicted the presence of B2 based on RNA3 sequence analysis (26, 28), and we have now confirmed that, at least for GGNNV, B2 is a small nonstructural protein that is expressed during infection in SB cells and also after transfection of BSR T7/5 cells with an RNA1 replicon vector. Moreover, we have now demonstrated that B2 is essential for maximal intracellular accumulation of GGNNV RNA1 and that B2, like its alphanodavirus counterparts, can act as a potent antagonist of RNAi.

Our initial failure to obtain an infectious GGNNV B2 mutant using plasmid-based reverse genetics makes it difficult to ascertain exactly how B2 functions during a native infection, where RNA2 would also be present. Recent work with the FHV alphanodavirus has revealed that in vivo replication of RNA2 by protein A requires RNA3 but not the B2 protein product (9), suggesting that a complex interplay exists between the three RNAs of nodaviruses and also the protein A and B2...
nonstructural proteins, which facilitate their replication and accumulation. It is clear from our results that B2, while not absolutely essential for replication in fish or mammalian cells, is important for high-level accumulation of RNA1. As a point of clarity, the term accumulation is used rather than replication because there is currently no evidence to suggest that B2 communicates either directly or indirectly with protein A during RNA1 replication.

The reduction of RNA1 accumulation observed in the B2 mutant RNA1 correlates well with the observed RNA1 antagonist function of B2. One can envisage a situation where RNA1 commences its intracellular replication cycle, which would naturally involve the formation of a long dsRNA replication intermediate, and the cellular RNAi response is invoked to destroy the viral RNA, as is the case with FHV (21). Wild-type RNA1 would combat this response by blocking RNAi through Dicer cleavage (27), although we do not yet know whether GGNNV B2 also targets Dicer cleavage. In a B2 mutant the host RNAi response would be expected to drastically reduce RNA1 accumulation without hindrance, although the ability of RNA1ΔB2 to accumulate at low levels (see Fig. 6) suggests that the RNAi response of the four cell types tested here is not sufficient to completely block RNA1 accumulation even in the absence of B2.

A noteworthy difference between our results and those of Johnson et al. (17), who reported on the influence of NoV B2 on RNA1 accumulation, was the ability of NoV RNA1ΔB2 to accumulate to wild-type levels in BSR T7/5 cells, whereas we found an obvious decrease in RNA1 accumulation with GGNNV RNA1ΔB2. Clearly, at this stage we cannot rule out the possibility of mechanistic differences between GGNNV and NoV B2 proteins, although it is still possible that NoV RNA1ΔB2 is impaired in accumulation at the early stages of replication, which were not monitored in their study (17). This said, there is still relatively good agreement with the two sets of results in that of the cell types tested here, RNA1ΔB2 was least impaired in BSR T7/5 cells and accumulated to higher levels in these cells than in HeLa cells, which Johnson et al. (17) attributed in part to differences in the RNAi response between the cell types. It will be interesting to see if GGNNV B2 and the B2 proteins of alphanodaviruses are functionally interchangeable and, given the similarities in the behavior of the RNA1ΔB2 mutants and the B2 proteins as RNAi antagonists, it seems probable that they would be able to complement one another.

Both cytoplasmic and nuclear localization of the B2 protein was confirmed by immunolocalization of infected SB cells, where it was shown that B2 is predominantly cytoplasmic and accumulates at the early stages of infection but enters the nucleus and accumulates there during the late stage of infection. The data obtained with cymogevalovirus-expressed native B2 and EGFP fusions appeared to mimic the nuclear accumulation pattern of localization observed in infected cells. Although B2 does not possess any typical nuclear localization signals, it seems likely that its small size enables it to freely enter the nucleus. Recent work with Nodamura virus B2 (27) indicated that EGFP-B2(NoV) was cytoplasmic (4), although in that study the localization of native NoV B2 was not investigated and thus it remains to be seen whether this protein is also cytoplasmic. That GGNNV B2 might behave differently to NoV B2 is still not surprising given the sequence and size difference between the two proteins, although it is clear from our immunofluorescence data that GGNNV B2 can enter and accumulate in the nucleus during the late stages of infection.

It is an exciting time for Nodavirus biology. A degree of biological function can for the first time be assigned to both the nonstructural and structural proteins of alpha- and betanodaviruses, and the development of facile reverse genetics systems for both virus subgroups will no doubt make further exploration of the infection process of this remarkable family of viruses even more expedient. Particularly in the case of betanodaviruses, where virus outbreaks in fish hatcheries are still rampant worldwide (1), such developments should facilitate the development of defined attenuated betanodavirus vaccines. With respect to the B2 protein, we are now in the process of more fully characterizing its cellular function. New data from our lab indicate that GGNNV B2, like its alphanodavirus counterparts, is a sequence-nonspecific dsRNA binding protein that suppresses not only RNAi but also other dsRNA-dependent cellular antiviral responses (B. J. Fenner et al., manuscript in preparation). The prospect of B2 being able to block such ubiquitous and important biochemical pathways is in our eyes simply astonishing.

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