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Bovine Coronavirus Nonstructural Protein ns2 Is a Phosphoprotein

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To investigate the nature of the bovine coronavirus (BCV) ns2 protein, the gene encoding this protein was cloned and was expressed as a β-galactosidase fusion protein. Antiserum raised against this protein reacted specifically with BCV-infected fixed cells in indirect immunofluorescence microscopy and precipitated an *in vitro* synthesized product approximately 32-kDa in molecular weight and an equivalent protein from BCV-infected cells. The synthesis of ns2 was found to be similar to the structural proteins of BCV and pulse-chase experiments indicated that ns2 protein was stable and that it accumulated in BCV-infected cells. Synthesis of ns2 in the presence of [^{32}P]orthophosphate revealed that it is a phosphoprotein. Phosphoamino acid analysis confirmed the phosphorylated nature of ns2 and identified serine and threonine as its phosphorylated amino acid residues. This is the first demonstration of a phosphorylated nonstructural protein in coronavirus-infected cells. © 1991 Academic Press, Inc.

The Coronaviridae is a family of enveloped viruses that contain an infectious single-stranded, positive-sense RNA genome approximately 30 kb in length. The 3′ terminal 6–8 kb of the genome contain the genes encoding the major structural proteins of these viruses (*1*). The 5′ terminal two thirds of the genome is believed to encode the RNA-dependent RNA polymerase (*2–5*). Coronaviruses direct the synthesis of a nested set of 6–8 subgenomic mRNAs by a unique leader-primed mechanism of transcription (*6*). While the assignment of coronavirus mRNAs to their respective protein products has been well established for the structural proteins, it has been confirmed for only some at the ns proteins. In addition, the biochemical characterization of many of these coronavirus ns proteins is incomplete. Little is known about the function of the ns proteins, but it is generally held that some are probably involved in the replication processes of the virus.

Previously, we reported the sequence of the S and HE genes of BCV (*7, 8*) and an additional ORF upstream from the HE gene with the potential to encode a 32-kDa protein (*9*). Here we report the preparation of BCV ns2-specific antiserum and its use to characterize this protein in infected cells.

To prepare monospecific antiserum, an 871-bp DNA fragment containing the 32K (ns2) ORF was isolated and *Bam*HI sites were created at both ends by site-directed mutagenesis (*10*). The fragment was subcloned into *Bam*HI-cleaved pUEX2 (Amersham) and used to transform *Escherichia coli* strain JM105. After induction at 42° the plasmid, pXns2, directed the synthesis of a 150-kDa β-galactosidase-ns2 fusion protein which was purified and injected into New Zealand White rabbits. Serological response following booster immunizations was monitored by reactivity to the product of cell-free translation of ns2-specific mRNA. ns2-specific mRNA was transcribed from the T7 promoter of pTZ19R (Pharmacia) by the method of Melton *et al.* (*11*). ns2-specific antibody was affinity purified as described previously (*12*). The ns2-specific antiserum precipitated an *in vitro* product with a molecular weight consistent with that expected for the ns2 ORF (Fig. 1, lane 2). Preimmune serum did not recognize the *in vitro* product (Fig. 1, lane 4) nor was the protein made in the absence of ns2-specific mRNA (Fig. 1, lane 3). Cell-free synthesis of the ns2 protein in the presence of canine pancreatic microsomal membranes did not alter its molecular weight or appearance in gels (data not shown). Furthermore, the *in vitro* product comigrated with the ns2 protein from BCV-infected cells (Fig. 1, lane 1) suggesting that little if any post-translational processing of ns2 had occurred.

To investigate the kinetics of synthesis of the ns2 protein, MDBK cells were infected with BCV at a moi of 5 and starved of methionine with methionine-free media 30–60 min prior to labeling for 1 hr with 100 μCi/ml [^{35}S]methionine. Radiolabeled cells were harvested at 4 to 48 hr pi and their products analyzed by immunoprecipitation with ns2-specific antiserum followed by SDS–PAGE (*13*) and fluorography (*14*) (Fig. 2). For the purpose of comparing the synthesis of the ns2 protein with the structural proteins of BCV, duplicate samples were immunoprecipitated with BCV-specific rabbit polyclonal antiserum (*15*). Fig. 2 is a composite figure derived from photographs of the radiographs of the two sample sets. The ns2 protein, like the structural

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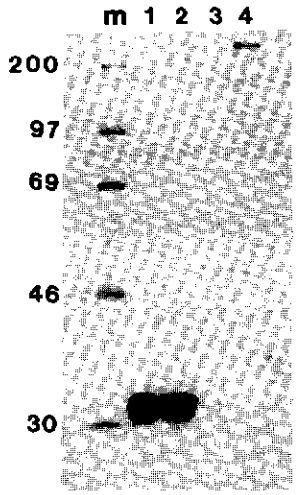

FIG. 1. Antiserum specificity and detection of the *in vitro* and *in vivo* bovine coronavirus ns2. Antiserum raised against βgal-ns2 was used to immunoprecipitate *in vitro* translated ^{35}S-labeled ns2 (lane 2) and a comigrating ^{35}S-labeled protein from BCV-infected cells (lane 1). A precipitable product was not produced *in vitro* in the absence of RNA transcribed from the cloned ns2 gene (lane 3) nor did preimmune serum precipitate the *in vitro* translated product (lane 4). ^{14}C-labeled molecular weight markers (10^{-3}) are indicated (lane m). Samples were separated by 10% SDS–PAGE and processed for fluorography.

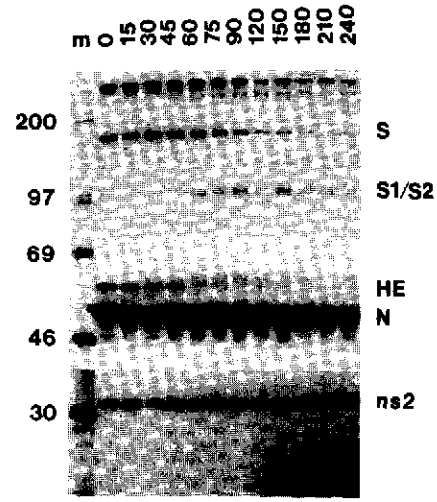

FIG. 3. Pulse-chase labeling of BCV-infected cells. At 18 hr pi, BCV-infected cells were ^{35}S-labeled for 15 min and chased from times 0 to 240 min post-pulse. Cells were harvested at the times indicated, and lysates were prepared and immunoprecipitated in two sets with either whole virus-specific antiserum (top) or ns2-specific antiserum (bottom). Each sample set was separated by SDS–PAGE, fluorographed, and a composite figure, as for Fig. 2, was assembled. Virus-specific proteins are indicated (S, S1/S2, HE, N, and ns2) as are the ^{14}C-labeled molecular weight markers (10^{-3}).

proteins of BCV, is synthesized early, detectable at 6 hr pi upon long exposure of the gel (data not shown), and throughout the infectious cycle, as late as 72 hr pi (data not shown). The peak of ns2 synthesis occurred, as for the structural proteins, at about 24 hr pi (Fig. 2). Not unlike most nonstructural proteins, BCV ns2 is synthesized in limited amounts when compared to the virion structural polypeptides. Similar but less protracted kinetics have been described for the ns2 of MHV-A59 (*16, 17*).

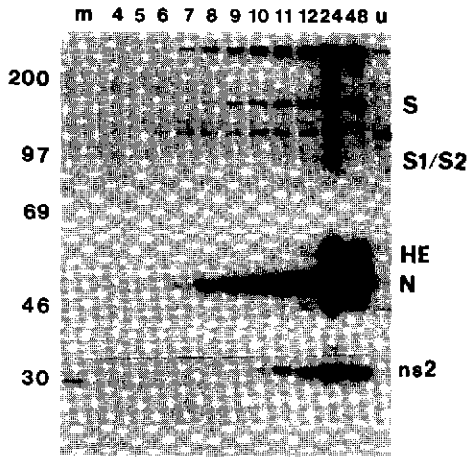

FIG. 2. Kinetics of BCV protein synthesis. ^{35}S-labeled BCV-infected cells were harvested hourly at times 4 through 12, 24, and 48 hr pi. Lysates prepared from these cells were divided into two sets and immunoprecipitated with either whole virus-specific antiserum (top) or ns2-specific antiserum (bottom). Each sample set was separated by SDS–PAGE, fluorographed, and a composite photograph was assembled from two photographs to produce Fig. 2. The uninfected sample is indicated (lane u) as are the virus-specific proteins (S, S1/S2, HE, N, and ns2) and the ^{14}C-labeled molecular weight markers (10^{-3}).

The intracellular turnover rate and processing of the ns2 protein was investigated by pulse-chase experiments. The radiolabeling of cells, sample preparation, and analysis were as described above except infected cells were labeled for 15 min at 18 hr pi and chased with media containing 10× the normal concentration of cold methionine. Contrary to a previous characterization of the analogous ns2 protein in MHV-infected cells (*16*), the ns2 of BCV was observed to be stable and it accumulated in BCV-infected cells with little or no detectable turnover during a 4-hr chase period (Fig. 3). The reason for this difference is not clear; however, it may have some functional significance or could simply involve some intrinsic property of the MHV ns2 protein as well as the cells used to grow the virus. The apparently unaltered molecular weight of ns2 supports the *in vitro* data described above that suggested an absence of ns2 processing. While similar stability and a lack of processing was observed for the N protein, other structural proteins such as the S and HE are observed to be processed further by cleavage and/or glycosylation over the same period of time (Fig. 3).

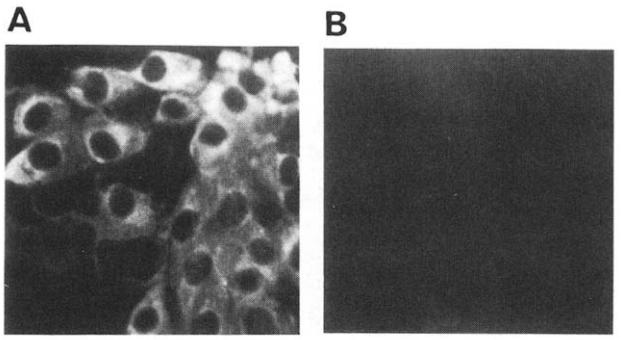

FIG. 4. Indirect immunofluorescent staining of BCV-infected cells. At 18 hr pi, cells were fixed and stained for fluorescent microscopy using FITC-conjugated goat anti-rabbit IgG as secondary antibody. (A) Infected cells stained with ns2-specific antiserum. (B) Uninfected cells stained with ns2-specific antiserum.

The intracellular location of the ns2 protein was determined by immunofluorescence microscopy. MDBK cells were grown on glass chamber slides (Lab-Tek) and infected with BCV at a moi of 5. At 18 hr pi, the cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100. Cells were incubated first with rabbit anti-ns2 antibody, then with FITC-conjugated goat anti-rabbit antibody (Southern Biotechnology Associates, Inc.) and photographed under uv light. The ns2 protein was localized to the cytosol of infected cells (Fig. 4A). This would be expected as the deduced amino acid sequence does not contain within it the characteristic hydrophobic, potentially membrane-spanning, domains associated with membrane proteins. In support, surface staining for ns2 in unfixed cells was not observed (data not shown) nor was staining evident in uninfected cells (Fig. 4B). BCV ns2 was not detected in radiolabeled virus preparations and is, therefore, considered to be a nonstructural protein (data not shown). The cellular location of the MHV homologue was also determined by immunofluorescence of infected cells and, as well, by the immune precipitation of the protein from subcellular fractions prepared from infected cells (*17*). As for BCV ns2, the deduced amino acid sequence of MHV ns2 protein is devoid of hydrophobic domains (*18, 19*). The MHV homologue is also only ever detected in infected cells and is, therefore, considered to be a nonstructural protein (*16*).

As the state of phosphorylation governs the role some viral proteins have in replication, we sought to determine if the ns2 protein was phosphorylated. When BCV infected cells were radiolabeled with [^{32}P]orthophosphate, the rabbit anti-ns2 serum precipitated a single band which comigrated with the ^{35}S-labeled ns2 (data not shown). To confirm the phosphorylated nature of ns2 and determine which amino acids contained phosphate groups, phosphoamino acid analyses were performed. The products of acid hydrolysis of immune precipitated, gel-isolated ns2 were mixed with phosphoamino acid markers and separated by thin layer electrophoresis (*20*). The radiolabeled phosphoserine and phosphothreonine residues detected in the ns2 protein are indicated (Fig. 5). The figure also indicates that phosphoserine was the more abundant of the two phosphoamino acids. Similar experiments for MHV have not been reported, so whether the MHV ns2 is phosphorylated is not presently known, however, considering the similarity between the ns2 of BCV and MHV, it is likely that the MHV homologue is also phosphorylated. The origin of the kinase(s) responsible for the phosphorylation of BCV ns2 is not known. It may be phosphorylated by a kinase activity similar to that demonstrated in purified MHV preparations (*21*) or, alternatively, could be phosphorylated by cellular kinases. Because of this uncertainty it is difficult to speculate on the significance of the phosphorylated nature of the ns2; however, phosphorylated ns proteins from other positive strand viruses have been demonstrated to function in viral replication (*22, 23*). Support for this possible function is provided by the homology between the ns2 of BCV and MHV. While the overall homology between the BCV ns2 and the MHV ns2 protein is only about 45%, the purported nu-

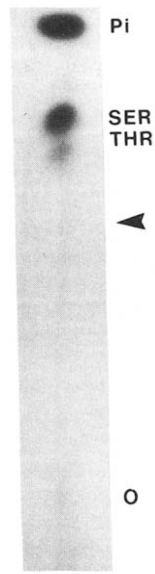

FIG. 5. Phosphoamino acid analysis. ^{32}P-labeled ns2 protein isolated from gels was subjected to acid hydrolysis, mixed with phosphorylated markers, and separated by thin layer electrophoresis (TLE). TLE plates were dried, sprayed with ninhydrin to detect the phosphorylated amino acid markers, and radiographed overnight. Radiolabeled phosphoserine and phosphothreonine are indicated. No radiolabeled phosphotyrosine was detected (arrow). O, origin. Pi, [^{32}P]orthophosphate.

cleotide binding domain reported in the MHV ns2 (*18*) is retained almost completely in BCV ns2 (15 out of 23 amino acids including conservative substitutions). While perhaps indicative of a preserved function one must approach this sort of relationship with caution until similar functions are demonstrated in each virus.

The ability to determine the *in vitro* function of BCV ns2 may not be possible if it is nonessential for virus growth *in vitro* as demonstrated for MHV-JHM ns2 (*24*). If, however, ns2 is essential for BCV replication *in vitro* then not only would study of its function by conventional methods be possible but it would provide a unique opportunity to study coronavirus evolution. To this end, we have undertaken to determine the essential/nonessential nature of BCV ns2.

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